

Tissue distribution of the dystrophin-related gene product and expression in the *mdx* and *dy* mouse

(dystrophin-related protein/chromosome 6)

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Communicated by David J. Weatherall, January 11, 1991 (received for review November 5, 1990)

ABSTRACT We have previously reported a dystrophin-related locus (*DMDL* for Duchenne muscular dystrophy-like) on human chromosome 6 that maps close to the *dy* mutation on mouse chromosome 10. Here we show that this gene is expressed in a wide range of tissues at varying levels. The transcript is particularly abundant in several human fetal tissues, including heart, placenta, and intestine. Studies with antisera raised against a *DMDL* fusion protein identify a 400,000 *M_r* protein in all mouse tissues tested, including those of *mdx* and *dy* mice. Unlike the dystrophin gene, the *DMDL* gene transcript is not differentially spliced at the 3' end in either fetal muscle or brain.

Dystrophin has been identified as the protein product defective in Duchenne muscular dystrophy (DMD); however, very little is known about its precise function (1–3). Sequence comparisons have revealed features in common with cytoskeletal proteins. The amino-terminal region of dystrophin shows homology to the actin binding region of α -actinin (4, 5) and the central rod domain shows structural similarities to the triple helical configuration of spectrin (6). In contrast, the carboxyl-terminal domain of 420 amino acids does not show homology to any previously characterized proteins. This latter domain is thought to be important in the integration of dystrophin into a glycoprotein complex localized at the muscle membrane surface (7, 8). The carboxyl-terminal region of the gene is differentially spliced in muscle and brain, which suggests the production of isoforms with differing interactions with membrane proteins (9). In addition, this region of dystrophin is important for the correct functioning of the molecule *in vivo* as deletions covering this domain result in DMD rather than the milder Becker muscular dystrophy (10–13).

Recently, we demonstrated the existence of an mRNA in human fetal muscle that shares a high degree of sequence homology with the carboxyl-terminal region of dystrophin (14). The gene encoding this transcript is localized to human chromosome 6 and the locus has been designated *DMDL* for DMD-like in Human Gene Mapping 10 (15). The *DMDL* gene shares structural similarities with dystrophin: the transcript is large, 13 kilobases (kb), and is multiexonic with a similar distribution of 3' exons and introns.

Although no human disease has yet been attributed to mutations at the *DMDL* locus, the mouse homologue of this gene, designated *Dmdl*, has been shown to be syntenic with the *dy* (dystrophin muscularis) locus on mouse chromosome 10 (16). The *dy* mutation is recessive and results in a severe

neuromuscular disease in the mouse in which skeletal muscle shows degenerative changes (17). These observations suggest that the *Dmdl* gene may be a candidate for the *dy* mutation.

In this paper, we present the tissue distribution of the *DMDL* transcript in humans and compare this distribution with the presence of a 400,000 *M_r* protein in mouse tissues. We also investigate the occurrence of the *DMDL* polypeptide in liver and muscle of mice with mutations in the *dy* locus.

EXPERIMENTAL METHODS

RNA Preparation and Northern and Slot Blot Analyses. RNA was isolated from homogenized tissues by precipitation in 3 M LiCl/6 M urea at 4°C and stored in ethanol at –70°C. Northern blots were prepared by denaturing RNA in formamide/formaldehyde at 65°C prior to electrophoresis in a 1% agarose gel in 20 mM Mops buffer (pH 7.0) and 2.2 M formaldehyde and transfer to nylon membranes (Pall Bio-dyne; ref. 18). The blots were hybridized with ³²P-labeled DNA fragments and then washed three times for 20 min each in 50 mM sodium phosphate, pH 7.5/1% SDS at 65°C. The *DMDL* probes were not found to cross-hybridize with dystrophin mRNA under these conditions. Slot blot analysis involved denaturation of RNA in glyoxal (19) and direct transfer to a membrane using a slot blot apparatus (Schleicher & Schuell) as described (20). Following hybridization, membranes were washed twice for 15 min each in 0.2× standard saline citrate (SSC)/0.1% SDS at room temperature and at 50°C and finally in 0.1× SSC/0.1% SDS at 50°C for 15 min. mRNA levels were quantified by densitometric scanning of autoradiographs using a Sigma FTR20 densitometer linked to a Shimadzu CR3A chromatopac peak integrator (Oriental Scientific, Dyson Instruments, Tyne and Wear, U.K.).

Differential RNA Splicing Analysis. Two-microgram samples of total RNAs were annealed to 50 ng of hexadeoxynucleotide primers (Pharmacia) in 10 μ l and converted to single-stranded cDNA in 25 μ l using avian myeloblastosis virus reverse transcriptase (Northumbria Biologicals, Northumberland, U.K.) and a cDNA synthesis kit (Promega). Reaction mixtures were incubated at 42°C for 2 hr and 2.5- μ l samples were stored at –20°C. Polymerase chain reaction (PCR) amplifications used 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.1% Nonidet P-40, MgCl₂ at 1.5 mM or 2 mM, 0.2 mM dNTPs, 0.5 unit of Taq DNA polymerase (ampli Taq; Perkin-Elmer/Cetus), and each oligonucleotide at 0.5 μ M in a final volume of 25 μ l in the presence of 2.5- μ l samples of cDNA or 2.5 μ l of H₂O as negative control. Mixes were

routinely UV-irradiated for 5 min (21) prior to the addition of enzyme and exogenous cDNA. Amplification involved denaturation at 94°C for 1 min, primer annealing at 65°C for 30 sec, and extension at 72°C for 2 min, repeated 45 times using a Perkin-Elmer/Cetus thermal cycler; the reaction mixtures were stored at -20°C. Half of each reaction mixture was electrophoresed in a 3% Nusieve (FMC) plus 1% agarose (BRL) gel and then stained with ethidium bromide.

DNA Analysis. High molecular weight DNA from various animal species was prepared as previously described (22) or purchased from IBI. DNAs were digested with restriction enzymes, electrophoresed in 0.8% agarose gels, and transferred to nylon membranes (Hybond N; Amersham). The blots were hybridized in a 50% formamide mix at 42°C (22) and finally washed in 3× SSC/0.1% SDS at 65°C for 30–60 min prior to exposure to Fuji x-ray film at -70°C for 1–7 days. In the case of hybridization to nematode DNA (see Fig. 4C), the blot was incubated at 24°C rather than 42°C and was washed twice for 15 min each in 3.4× SSC/0.1% SDS at 60°C. Oligonucleotide hybridization was performed in 6× SSC, 10× Denhardt's solution, and 50 µg of denatured, sonicated salmon sperm DNA per ml at 63°C for 2 hr. The blot was then washed in 6× SSC/0.1% SDS at 63°C for 10 min prior to exposure to x-ray film.

DNA probes were prepared using commercially supplied random-primed labeling kits (Amersham and Boehringer Mannheim) following the manufacturers' instructions. Oligonucleotides were 5' end-labeled following standard procedures (18).

Antibodies and Western Blotting. A 1.05-kb fragment of the cDNA Bfm3 (start position, nucleotide 510; ref. 14) was

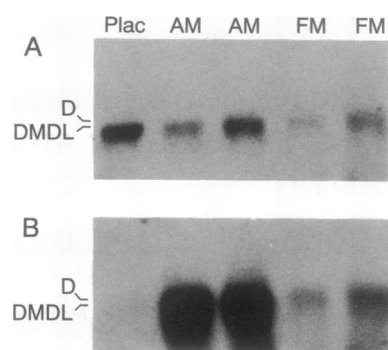


FIG. 1. Northern analysis of DMDL and dystrophin transcripts. RNAs were loaded, from left to right: placenta (Plac, 12 µg of total RNA); adult muscle [AM, 9 µg and 10 µg of poly(A)⁺]; fetal muscle [FM, 8 µg and 10 µg of poly(A)⁺ from 24-week fetus]. The Northern blot was probed sequentially with the 1.9-kb *EcoRI*-*HindIII* fragment of Bfm3 (ref. 14; A) and the 1.2-kb *HindIII* fragment of the 3' dystrophin cDNA, Cf115 (refs. 14 and 24; B); bound probe was removed between hybridizations. Locations of DMDL and dystrophin (D) transcripts are indicated.

cloned into the *Sma* I site of the expression vector pEX2 and introduced into *Escherichia coli* POP2136 cells. Induction and purification of the β-galactosidase fusion protein and immunization of BALB/c mice were performed as described (23). Tissue samples were homogenized in boiling extraction buffer (4 ml/g) containing 10% SDS and the extracts were subjected to SDS/PAGE followed by Western transfer (23). The blots were incubated with mouse antiserum or control

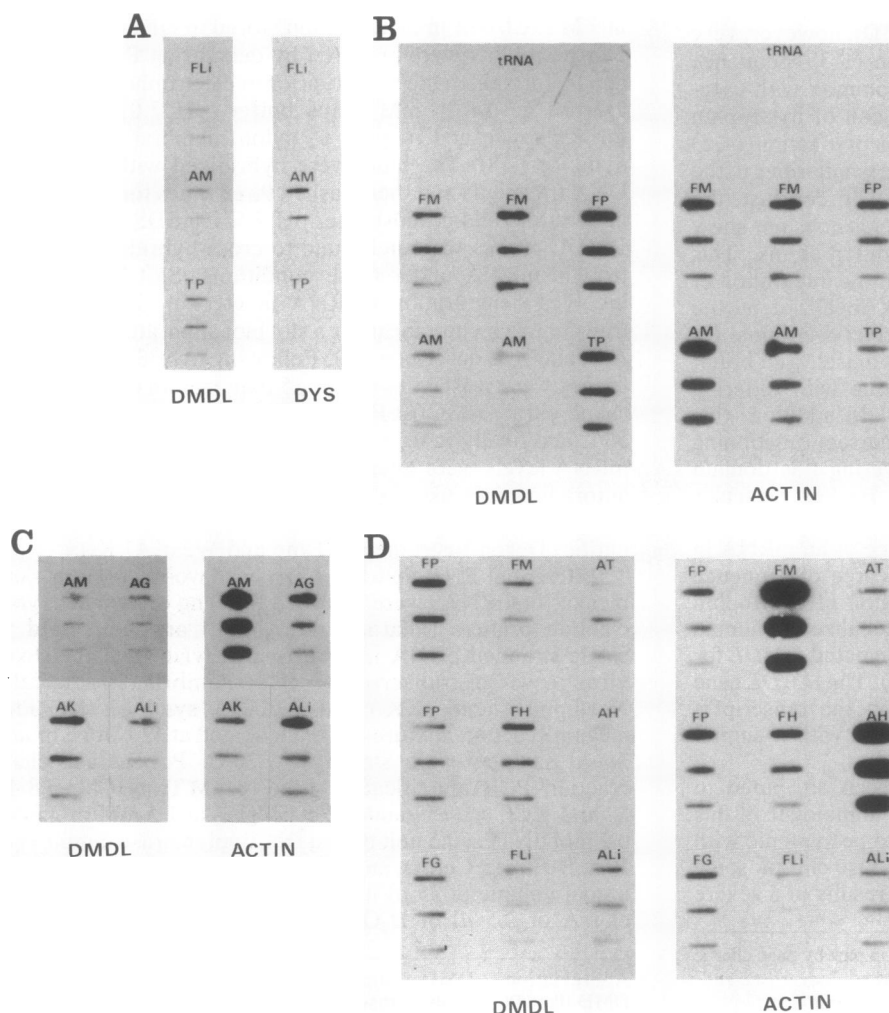


FIG. 2. Slot blot RNA analysis of DMDL transcript expression in human tissues. Five-microgram, 2-µg, and 1-µg samples of each RNA were applied to a slot blotting apparatus and hybridized with the 1.6-kb *EcoRI*-*HindIII* untranslated sequence of Bfm3 (DMDL, ref. 14), a 1.0-kb 3' end sequence of human skeletal muscle α-actin mRNA (pGF3, ref. 25), and the 5' coding sequence of dystrophin (DYS; Cf27, ref. 26). RNAs were isolated from the following human fetal (F) and adult (A) tissues: heart (H); intestine (I); liver (Li); kidney (K); muscle (M); placenta (P); and testis (T). RNA was also isolated from human term placenta (TP). The slot blots in A–D were hybridized twice, with the first probe removed (lefthand side of each panel) prior to the second hybridization (righthand side of each panel).

nonimmune serum (diluted 10^{-3}), followed by a biotin-avidin detection system for mouse immunoglobulins (Vectastain kit; Vector Laboratories).

RESULTS

Preliminary data on the expression of the *DMDL* gene have shown that the 3.5-kb cDNA fragment, designated Bfm3, identifies a 13-kb transcript in human skeletal muscle (14). The *DMDL* transcript migrates slightly ahead of dystrophin on Northern blots (Fig. 1) and shows ubiquitous tissue distribution (Fig. 2). The *DMDL* and dystrophin transcripts are expressed at relatively low levels in brain (results not shown) and, in contrast to dystrophin, skeletal muscle is not the major site of *DMDL* expression (Fig. 2A); dystrophin is expressed predominantly in muscle and neuronal cells (2, 27).

DMDL transcripts are detected at highest levels in placenta during gestation and at term (Fig. 2B). Furthermore, *DMDL* mRNA is found in a broad range of fetal and adult tissues, including liver, intestine (smooth muscle), skeletal muscle, testis, and kidney (fetal testis and kidney were not tested; Fig. 2). The *DMDL* transcripts are more abundant in certain fetal tissues such as placenta, skeletal muscle, intestine, and heart than in the corresponding mature tissue; in particular, expression is high in fetal heart but almost undetectable in adult heart. Among adult tissues, *DMDL* mRNA levels are highest in kidney, testis, and liver.

The pattern of hybridization shown by the actin probe was as expected (Fig. 2). This probe contains untranslated sequence that hybridizes specifically with human skeletal α -actins and coding sequences that are conserved among skeletal, cardiac, and smooth muscle actins and nonmuscle actins (25).

Differential splicing has been reported to occur in the carboxyl-terminal end of the dystrophin transcript in fetal tissue and it has been suggested that this yields protein isoforms that may interact differently with membrane proteins (9). We screened the corresponding region of the *DMDL* transcript for alternative splicing in muscle in order to determine if isoforms are also a feature. The PCR was used to amplify overlapping 5' and 3' segments of Bfm3, and the products were compared with those obtained from the corresponding region of the dystrophin transcripts (Fig. 3). The alternatively spliced products previously described for the 3' end of dystrophin in fetal muscle are clearly seen but only the single fragments predicted for each amplified segment of the *DMDL* transcript are found. Similar results were observed after amplification of brain RNA (results not shown). The PCR analysis also shows that differential splicing within the most 3' region of dystrophin is developmentally expressed. Two high molecular weight PCR products are seen after amplification of fetal muscle RNA, whereas only one of these products is amplified from adult muscle RNA within region D3' (Fig. 3). In the case of the 5' region of Bfm3 (B5'; Fig. 3), the low molecular weight fragment amplified from adult muscle RNA would appear to be an artefact because it did not hybridize with Bfm3. No evidence of alternative splicing was found when the entire coding region of Bfm3 was amplified from fetal muscle cDNA (results not shown).

The degree to which the 3'-end coding sequence of the *DMDL* gene is conserved among various vertebrate species was examined using a 1.9-kb *EcoRI-HindIII* fragment of Bfm3 that contains at least seven 3' exons (14). Fig. 4 shows that this region of the *DMDL* gene is very highly conserved since multiple homologous fragments are observed in all of the species tested, including the chicken, and that this homology extends to the nematode. In the case of Fig. 4 A and B, the hybridization conditions are such that dystrophin sequences are not detected in human DNA.

The mapping of the *DMDL* locus to the same region of mouse chromosome 10 as the *dy* mutation suggested the

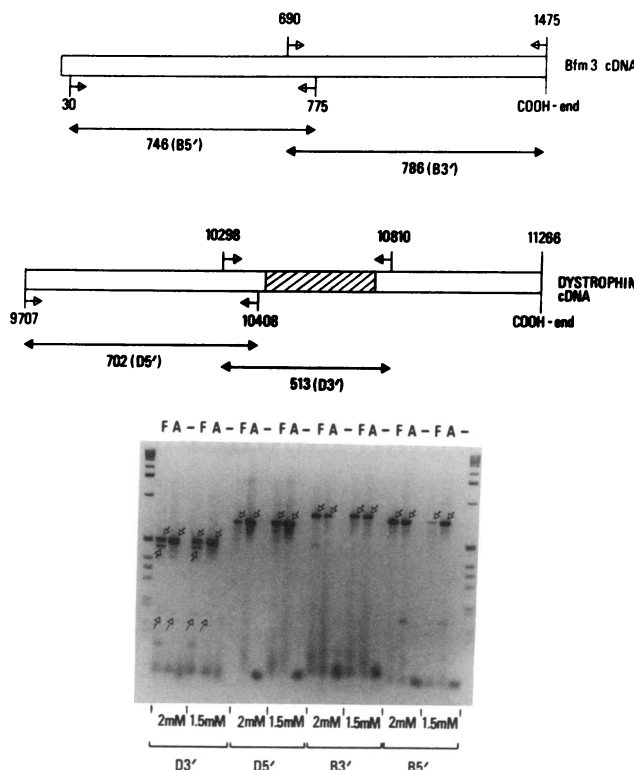


FIG. 3. PCR amplification of the 3' end of the *DMDL* and dystrophin transcripts. (Upper) Schematic representation of the 3' coding regions of the *DMDL* and dystrophin transcripts. The numbers denote nucleotide position from the 5' end of Bfm3 (14) and dystrophin (4), respectively. The open arrows indicate the direction in which oligonucleotide primers would be extended during PCR and the filled-in arrows denote the regions that would be amplified, with lengths of products indicated in base pairs. The hatched box denotes the region of the dystrophin transcript that exhibits differential splicing (9). (Lower) Photograph of the electrophoretic separation of PCR-amplified fragments from adult (A) and fetal (F) muscle RNA samples following the procedures outlined in the text. The size standard is the 1-kb ladder (BRL), and the negative control for amplification (denoted as -) consisted of samples with all ingredients and oligonucleotide primers but lacking exogenous cDNA. The arrows indicate those fragments that hybridized with the 1.9-kb *EcoRI-HindIII* fragment of Bfm3 (B5' and B3') and the 4.3-kb *EcoRI* insert of the 3' dystrophin cDNA, Cfl15 (ref. 24; D5' and D3'); the detection of D3' products also involved hybridization with an oligonucleotide that mapped within the D3' region (nucleotide positions 10,408-10,379). The concentration of $MgCl_2$ in each PCR is indicated under the photograph together with the regions of each transcript that were amplified.

Dmdl gene as a candidate for dystrophin muscularis. Prior to investigating this hypothesis, polyclonal antibodies to a fusion protein containing the carboxyl-terminal region of the human *DMDL* protein were used to examine the normal distribution of this protein in mouse tissue. The antibodies detect a protein in wild-type mouse muscle of 400,000 M_r (Fig. 5A), which is equivalent in size to dystrophin (Fig. 5B). A comparison of Fig. 5 A and B indicates that the 400,000 M_r protein is produced in *mdx* mouse muscle, which shows no detectable dystrophin. Therefore, a survey of tissue distribution of the 400,000 M_r protein was made in the *mdx* mouse in order to avoid problems of interpretation associated with cross-reactivity between *DMDL* antibodies and dystrophin.

The 400,000 M_r protein is ubiquitously expressed in the adult *mdx* mouse (Fig. 5C), a finding consistent with our slot blot assay of human *DMDL* mRNA. Comparisons of relative staining intensity indicate that expression is relatively high in lung and low in brain, with the remaining tissues (skeletal

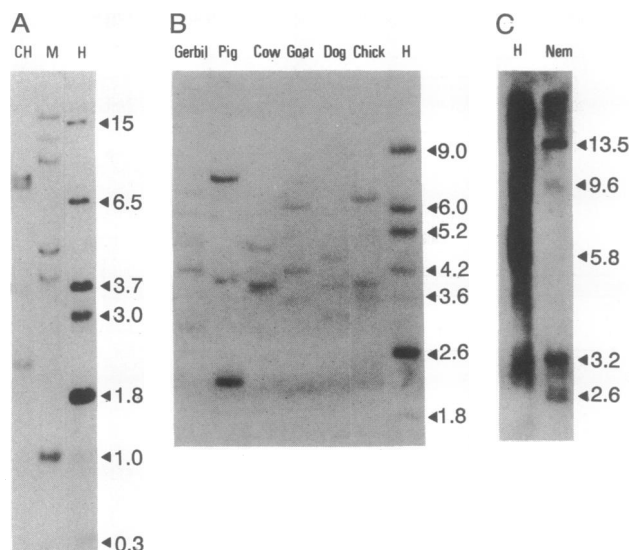


FIG. 4. Cross-species hybridization with human 3' DMDL cDNA (Bfm3). Five micrograms to 10 μ g of chromosomal DNAs from Chinese hamster (CH), mouse (M), human (H), nematode (Nem), and several other species was digested (*Eco*RI, A; *Hind*III, B; and *Hind*III, C), electrophoresed, and Southern blotted. The blots were hybridized with Bfm3 (A) and the 1.9-kb *Eco*RI-*Hind*III fragment of Bfm3 containing the 3' coding region of the DMDL transcript (B and C). The sizes of the hybridizing fragments in human and nematode DNAs are indicated in kb.

muscle, heart, spleen, liver, and kidney) showing approximately equal amounts of the 400,000 M_r protein.

The DMDL antiserum was used to compare the levels of expression of the 400,000 M_r protein in *mdx* and *dy*^{2J} mouse

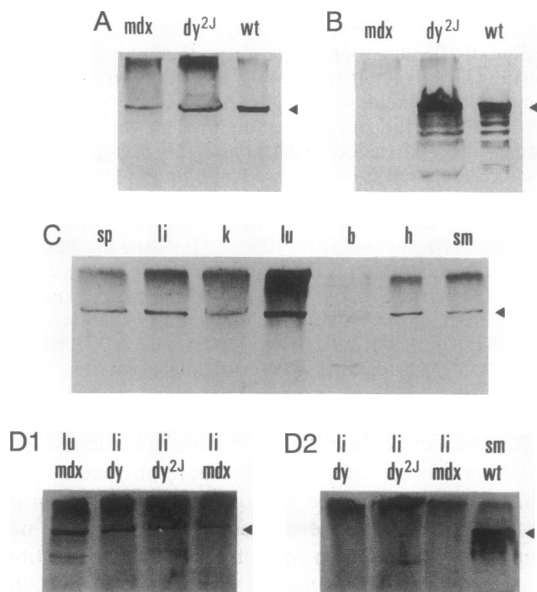


FIG. 5. Western blot analysis of mouse tissues with polyclonal antibodies to the DMDL protein. (A) Muscle tissues from *mdx*, *dy*^{2J}, and wild-type (wt) mice were screened with polyclonal antibodies against the DMDL protein. (B) Same as A, except that a monoclonal antibody against dystrophin (MANDYS 1; ref. 23) was used. (C) *mdx* adult mouse tissues (spleen, sp; liver, li; kidney, k; lung, lu; brain, b; heart, h; skeletal muscle, sm) were screened with polyclonal antibodies against the DMDL protein. (D1 and D2) Tissues were screened with polyclonal antibodies against the DMDL protein (D1) and MANDYS 1 (D2). The arrowhead indicates the mobility of a 400,000 M_r protein. Apart from dystrophin degradation products (B), the lower molecular weight proteins detected in this analysis are nonspecific, as they are also stained when control nonimmune serum was used.

muscle and liver; the latter tissue was tested because dystrophin is not expressed in liver (Fig. 5D2). The *dy*^{2J} mouse produces approximately the same amounts of the 400,000 M_r protein in both tissues as the *mdx* mouse (Fig. 5A and D1); the staining in *dy*^{2J} muscle, however, may be complicated by cross-reactivity between the antibodies and dystrophin. The *dy*^{2J} mutation is one of at least five mutant alleles at the *dy* locus and gives rise to the less severe phenotype compared with *dy* mice (28). The analysis of the *dy* mouse shows no significant difference between the levels of expression of the 400,000 M_r protein in liver (Fig. 5D1) and muscle (results not shown) compared with the same tissues in *dy*^{2J} and *mdx* mice.

DISCUSSION

The data presented here show that the *DMDL* gene is ubiquitously expressed in humans and mouse, although the level of expression is tissue dependent; placenta and lung express the *DMDL* gene at relatively high levels, whereas the brain expresses this gene at relatively low levels. In view of the homology between dystrophin and the *DMDL* gene, RNA and Western analyses were used to examine tissue distribution in order to obtain consistent data. The polyclonal antisera to the DMDL protein appear to be specific for the product of the mouse chromosome 10 (human chromosome 6) *Dmdl* gene for two reasons. (i) The 400,000 M_r protein detected in *mdx* mice is equivalent in size to that predicted from the length of the transcript. (ii) The tissue distribution determined by Western analysis of *mdx* mouse tissue parallels that observed in the human mRNA analysis, although direct comparisons of the steady-state levels of mRNA and protein are not straightforward. The hybridization and washing conditions for the mRNA study were such that dystrophin transcripts were not detected. This analysis also showed the temporal expression of the DMDL transcript, especially in human heart, suggesting that this protein may play a role in mammalian development.

The *DMDL* gene is very highly conserved in evolution and it is multiexonic in species as diverse as gerbil and nematode. Sequence comparisons of the *DMDL* gene among species, therefore, may indicate conserved regions that are functionally important domains of the DMDL protein. Mutational analysis of the nematode might also aid in identifying the biological consequences of alterations in the *DMDL* gene, although the evolutionary distance between human and nematode may mitigate against predicting pathophysiological effects in humans.

The lack of detectable isoforms inferred from the PCR analysis of the 3' end of the DMDL transcript contrasts with that of dystrophin. The functional significance of the proposed dystrophin isoforms has not been determined and the consequence of developmental regulation of alternative splicing within the 3' end of dystrophin remains unclear. The difference in detectable splicing at the 3' end of dystrophin and the DMDL transcripts may indicate varying protein interactions. It remains to be determined if the DMDL protein is associated with membranes in the same way as dystrophin (7, 8); however, studies with monoclonal antibodies that react specifically with the DMDL protein should enable its localization to be determined and hence provide clues as to its function.

Although our immunological studies do not provide evidence that mutations within the *dy* locus map to the *DMDL* gene, we cannot unequivocally exclude the possibility that *dy* and *dy*^{2J} mice are synthesizing an abnormal DMDL protein. Both mice carry allelic mutations that result in different pathological symptoms (28). These symptoms, therefore, do not correlate with large intragenic deletion events within the *DMDL* gene such that, as is found for dystrophin in Becker muscular dystrophy and DMD patients, the severity of the

phenotype can be predicted according to the size and abundance of the protein produced from the mutated locus (3). Mutations within the *dy* locus may be microdeletions or point mutations that do not disrupt the translational reading frame. It is relevant that a single base change in the coding region of the cardiac β -myosin heavy chain results in familial hypertrophic cardiomyopathy in humans (29, 30). In this disorder, initial analysis of the expression of cardiac β -myosin using immunohistochemical techniques failed to reveal changes in patients (31, 32). A detailed comparison of the sequence of the wild-type mouse *DMDL* gene with that of the *dy* mouse will be needed to determine the involvement, if any, of the *DMDL* protein in the *dy* phenotype.

Recently, dystrophin-related proteins of 400,000 M_r have been detected using polyclonal antibodies against dystrophin (33, 34) and the *DMDL* gene product (35). In the case of dystrophin antisera, immunolocalization studies show staining in the pia mater of *mdx* mouse brain (33) and the neuromuscular junctions of DMD patients and *mdx* mice (34). These studies compare with the sarcolemma localization determined for dystrophin in human and mouse muscle (36, 37) and indicate cross-reactive protein(s) with different subcellular interactions from dystrophin. It is unclear if these proteins are products of the *DMDL* gene because these studies have used polyclonal antibodies to dystrophin that may show cross-reactivity to a number of polypeptides. The preliminary work reported for the chromosome 6-encoded *DMDL* protein (35) indicates ubiquitous expression in all tissues tested in DMD patients. Although this study is confirmed by our results, RNA quantitation was not performed to support the view that no cross-reactive proteins were detected. A more rigorous analysis would require a null mutation within the *DMDL* gene and monoclonal antibodies specific to the *DMDL* protein. The data reported by us and others do not rigorously exclude the possibility that further dystrophin homologues exist that are themselves members of a family of high molecular weight proteins that share epitopes with the carboxyl-terminal region of dystrophin. Monoclonal antibodies will be needed to determine the localization of the protein product of the *DMDL* locus, and mutation studies of the *DMDL* gene should aid in assigning a function to this protein.

We are grateful to Barbara Byth and Derek Blake for helpful discussions and we thank Helen Blaber for help with this manuscript. This work was funded by the Medical Research Council, The Muscular Dystrophy Group of Great Britain and Northern Ireland, and The Muscular Dystrophy Association, U.S.A.

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