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Expression of the Murine Duchenne Muscular Dystrophy Gene in Muscle and Brain

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Complementary DNA clones were isolated that represent the 5′ terminal 2.5 kilobases of the murine Duchenne muscular dystrophy (Dmd) messenger RNA (mRNA). Mouse Dmd mRNA was detectable in skeletal and cardiac muscle and at a level approximately 90 percent lower in brain. Dmd mRNA is also present, but at much lower than normal levels, in both the muscle and brain of three different strains of dystrophic *mdx* mice. The identification of Dmd mRNA in brain raises the possibility of a relation between human Duchenne muscular dystrophy (DMD) gene expression and the mental retardation found in some DMD males. These results also provide evidence that the *mdx* mutations are allelic variants of mouse *Dmd* gene mutations.

DUCHENNE MUSCULAR DYSTROPHY (DMD) is a severe, X-linked recessive degenerative muscle disease frequently associated in humans with varying degrees of mental retardation (*1*). Genomic and complementary DNA (cDNA) clones for portions of the DMD gene have recently been isolated from humans and mice, which allows increasingly accurate prenatal diagnosis and carrier detection of DMD (*2–5*). A complete understanding of the function of the DMD gene product and the mechanism by which an abnormal gene leads to muscular dystrophy can be facilitated by the study of mice as a model system. The mouse *Dmd* gene is expressed in both skeletal and cardiac muscle at levels similar to those found in humans (*5*). In addition, the mouse *Dmd* gene has recently been mapped to a region of the X chromosome similar to that of the mouse muscular dystrophy mutation, *mdx* (*6, 7*). These mapping data together with pathological studies of three independently derived *mdx* mutants suggest that the *mdx* mutations could be within the mouse *Dmd* gene (*8*). The availability of mouse models for DMD would provide an important system for the study of DMD and for the development of experimental therapeutic procedures for muscular dystrophy.

We have isolated several cDNA clones for the mouse *Dmd* gene from an adult ICR mouse muscle cDNA library prepared in λgt11 (*9*). Together these clones span 2.5 kb at the extreme 5′ end of the murine Dmd messenger RNA (mRNA). Aside from two apparently polymorphic base substitutions, these skeletal muscle clones are identical to the cardiac muscle cDNAs previously reported (*5*), confirming that the skeletal and cardiac muscle transcripts are not differentially spliced over this portion of the *Dmd* gene.

We examined the expression of the *Dmd* gene using RNA prepared from several tissues of ICR mice, *mdx* mice, and two new *mdx* isolates (designated 467 and 551) (*8*). Dmd mRNA was detected by Northern analysis at low levels in ICR skeletal and cardiac muscle, and at very low levels in brain, but was not detected in liver, kidney, or spleen (Fig. 1A) (*10*). When skeletal muscle from the three *mdx* mouse strains was examined, Dmd mRNA of apparently normal size was detected in each (Fig. 1A). However, Dmd mRNA was not detected via Northern analysis in the brain of any of the *mdx* strains (Fig. 1A) (*10*). To control for the amount of RNA loaded in each lane and to examine the integrity of the RNA samples, we rehybridized the Northern blot shown in Fig. 1 with muscle-specific [γ-phosphorylase kinase (γ-Phk) (*11*)] and ubiquitously expressed [hypoxanthine phosphoribosyltransferase (HPRT) (*12*)] cDNA probes (Fig. 1, B and C). The results indicated that the mutant muscle RNAs were underloaded and slightly less intact than the control RNA, whereas the *mdx* brain sample appeared slightly overloaded but as intact as the control brain RNA. This experiment demonstrated that approximately normal-sized Dmd mRNA was detectable in brain samples of ICR mice and in the muscle samples of all three mutant *mdx* mice. However, we could not be sure whether the weaker hybridization observed for the Dmd cDNA with muscle and brain RNAs from *mdx* mice reflected a reduction of Dmd mRNA levels or was due to differences in the quality and quantity of the RNA samples.

To explore the possibility of altered Dmd mRNA levels in the three strains of *mdx* mice, we elected to use the technique of ribonuclease A (RNase A) protection (*13*). This method is more sensitive than Northern analysis, is highly specific, and results in sharp bands due to protection from RNase A of a short, complementary RNA (cRNA) probe hybridized in solution to RNA. Figure 2A demonstrates that a cRNA probe transcribed from the 530-bp Hind III–Bgl II fragment at the 3′ end of the Dmd cDNA XD-1 (*9*) is protected from cleavage with a range of RNase A concentrations after hybridization with ICR skeletal muscle RNA (*14*). The probe is also protected by cardiac muscle RNA, to a lesser extent by brain RNA, but not by liver or transfer RNA (Fig. 2B) (*10*). Since an identical 530-bp fragment is protected from RNase A diges-

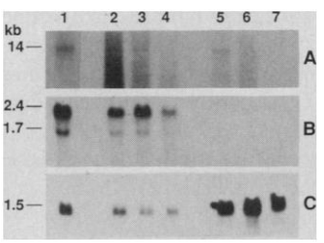

Fig. 1. Northern blot analysis of mouse RNAs. Samples (20 μg) of RNA from various tissues were isolated (*9*), separated by electrophoresis through 1% agarose/formaldehyde gels, transferred to GeneScreenPlus membranes (DuPont), and hybridized with cDNA probes essentially as described (*12, 20*). Autoradiograms from three successive hybridizations of the same blot with the indicated probes are shown. (**A**) Hybridization with Dmd cDNA XD-1 (*9*), (**B**) hybridization with mouse muscle γ-Phk cDNA Phk-2 (*11*), and (**C**) hybridization with mouse HPRT cDNA HPT5 (*12*). Lane 1, ICR skeletal muscle; lane 2, *mdx* skeletal muscle; lane 3, 467 skeletal muscle; lane 4, 551 skeletal muscle; lane 5, ICR brain; lane 6, *mdx* brain; and lane 7, ICR liver. Lane 1 in (A) and all lanes in (B) are from 24-hour exposures to film; the remaining lanes in (A) and those in (C) are from 72-hour exposures to film. Longer exposure of the blots in (B) reveals similar low levels of γ-Phk mRNA in ICR and *mdx* brain.

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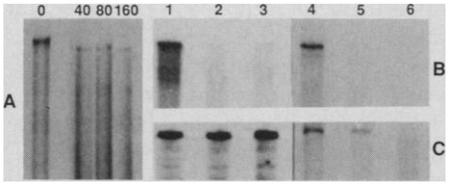

Fig. 2. RNase A protection assay of mouse RNAs. Total RNA [12.5 μg (A and C) or 25 μg (B)] was hybridized with cRNA probes labeled to a specific activity of 8.6×10^8 dpm per microgram, digested with RNase A, separated by electrophoresis through polyacrylamide gels, and exposed to x-ray film (*14*). (**A**) Shows the effects of various concentrations of RNase A (indicated at the top in micrograms per milliliter) on skeletal muscle RNA/Dmd cRNA hybrids. The samples in panels (B) and (C) were treated with RNase A (100 μg/ml). The indicated RNA samples (lane 1, ICR muscle; lane 2, *mdx* muscle; lane 3, 467 muscle; lane 4, ICR brain; lane 5, *mdx* brain; and lane 6, ICR liver) were hybridized with the Dmd cRNA (**B**) or the γ-Phk cRNA (**C**). Autoradiographic exposure times were as follows: (B) skeletal muscle lanes, 3 hours; brain and liver, 14 hours; (C) skeletal muscle lanes, 1.5 hours; and brain and liver, 6 hours.

tion by both muscle and brain RNAs, we conclude that this portion of Dmd mRNA is identical in both tissues (*15*).

Figure 2B also demonstrates the relative protection of the XD-1–derived cRNA probe after hybridization with ICR, *mdx*, and 467 mouse skeletal muscle RNA and with ICR and *mdx* brain RNAs. We determined the relative levels of γ-Phk mRNA in parallel using a cRNA probe transcribed from a 450-bp Bgl II–Bam HI fragment derived from the tenth exon of the mouse muscle γ-*Phk* gene (Fig. 2C) (*10*). To compensate for potential differences in the amount of RNA in each hybridization, the relative levels of Dmd mRNA between control and mutant mice were determined by densitometry, and this value was then normalized to the relative levels of the γ-Phk mRNA (*14*). As shown in Table 1, *mdx* mice contained approximately 16% of the normal levels of Dmd mRNA in skeletal muscle and 21% of the normal levels in brain, when compared with γ-Phk mRNA. The 467 mutant displayed a similar reduction of Dmd mRNA in muscle, as only 18% of the control levels were detected.

In a second experiment muscle RNA from the original *mdx* mutant contained 25% of the ICR Dmd mRNA levels when compared with the relative levels of γ-Phk mRNA (*10*). Because of the limited availability of the 467 and 551 mutants, we were not able to accurately quantitate the Dmd transcripts in the brains of these mutants nor those in the muscle of the 551 mice. However, in several different experiments we consistently detected similar levels of Dmd mRNA in all three of the *mdx* mutants (*10*), and we therefore conclude that the *mdx* mutants contain reduced levels of Dmd mRNA in both skeletal muscle and brain.

As this manuscript was being prepared, Hoffman *et al.* (*16*) reported that the Dmd gene product, dystrophin, was detectable via immunoblot analysis with specific antiserum in control mouse muscle but not in muscle from either the original or the 467 *mdx* mutants. Our observations of reduced Dmd mRNA levels in three strains of *mdx* mice considered with the observation of Hoffman *et al.* (*16*) that dystrophin is undetectable in two of the *mdx* mutants strongly argues that the *mdx* mice contain mutant *Dmd* genes. This conclusion is also compatible with previous reports that the *mdx* and *Dmd* genes map to a similar region of the mouse X chromosome (*6, 7*).

A mutation that leads to reduced levels of normal-sized mRNA but no detectable protein would likely arise from a point mutation or small deletion resulting in premature chain termination and ribosomal release. Such is the case with several reported β-thalassemia mutations (*17*). In this regard we have demonstrated that no gross abnormalities of the *Dmd* gene (such as deletions or rearrangements) are detectable via Southern analysis with the 5′ terminal 2.5 kb of the mouse Dmd cDNA in any of the strains of *mdx* mice (*6, 10*). Each of the *mdx* mutants displays a very mild dystrophy (*8*), which might suggest that they are a more appropriate model for Becker, rather than Duchenne, muscular dystrophy. However, the absence of dystrophin in *mdx* mice suggests that the *mdx* mutations are more similar to Duchenne mutations (*16*). Similar mutations in homologous genes of different species do not always produce similar phenotypes, as is the case with the HPRT-deficient Lesch-Nyhan mouse (*18*) and the ornithine transcarbamylase–deficient *spf* mouse (*19*). Nonetheless, the availability of mouse models containing mutant *Dmd* genes should facilitate studies of the etiology and potential for therapy of DMD.

The observation of *Dmd* gene expression in mouse brain raises the possibility that the human DMD gene is directly involved in DMD-associated mental retardation. Although the involvement of DMD gene expression in mental retardation has been questioned by earlier reports that Dmd mRNA is not detectable in brain (*3, 5*), our results with more sensitive assays demonstrate unequivocal low-level expression in mouse brain. Further experiments will be required to determine whether mental retardation results directly from altered DMD gene expression in the central nervous system or is a secondary consequence from alterations in other tissue types.

Table 1. Relative levels of Dmd and γ-Phk mRNAs. Optical densities were obtained from densitometric analysis of the data shown in Fig. 2, B and C. %ICR indicates the density of a given band relative to the appropriate muscle (M) or brain (B) control (ICR) band and is given in parentheses in the top of the table.

Probe	Source of RNA				
	ICR-M	*mdx*-M	467-M	ICR-B	*mdx*-B
	Integrated optical densities				
Dmd	152 (100)	25.9 (17.0)	31.7 (20.8)	103 (100)	9.2 (8.9)
Phk	252 (100)	261 (103)	300 (119)	93.3 (100)	40.4 (43.3)
	Normalized data (%)				
%ICR (Dmd/Phk)*		15.9	17.6		20.6

*Relative level of mutant versus ICR Dmd mRNA normalized to the relative level of γ-Phk mRNA in the same sample.

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9. RNAs for all experiments were isolated from fresh mouse tissues by means of the guanidine isothiocyanate/CsCl procedure [J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. E. Rutter, *Biochemistry* **18**, 5294 (1979)]. A cDNA library was prepared in λgt11 essentially as described [U. Gubler and B. J. Hoffman, *Gene* **25**, 263 (1983); R. A. Young and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1194 (1983)]. XD-1 was identified by low-stringency screening of the library (20% formamide, 0.75*M* NaCl, 75 m*M* sodium citrate, 42°C) with a 31-base synthetic oligonucleotide corresponding to a portion of a mouse *Dmd* exon (*3*). The oligomer had the sequence 5′-CAGGGCCTGAGCTGATCTG-CTGGCATCTTGC-3′ and was synthesized on an Applied Biosystems model 380B DNA synthesizer as described by the manufacturer. Purified oligomers were labeled to a specific activity of 1×10^9 dpm/μg with T4 polynucleotide kinase (New England Biolabs). The original isolate, XD-1, extended 897 bp in the 5′ direction from the first Eco RI site at the 5′ end of the murine Dmd cDNA as reported by Hoffman *et al.* (*16*). Several additional clones were isolated from the library by means of XD-1 as a probe, and sequence analysis revealed the 5′-most

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15. A comparison of several mouse genomic clones with the XD-1 sequence demonstrates that the Bgl II–Hind III cRNA probe overlaps four exon/intron boundaries, and thus spans portions of five exons (J. S. Chamberlain, unpublished observations). If there were any differences in the exon composition of this portion of the brain and muscle Dmd transcripts, a dramatically altered RNase A protection pattern would have been obtained with brain RNA.
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Duchenne Muscular Dystrophy Gene Expression in Normal and Diseased Human Muscle

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A probe for the 5′ end of the Duchenne muscular dystrophy (DMD) gene was used to study expression of the gene in normal human muscle, myogenic cell cultures, and muscle from patients with DMD. Expression was found in RNA from normal fetal muscle, adult cardiac and skeletal muscle, and cultured muscle after myoblast fusion. In DMD muscle, expression of this portion of the gene was also revealed by in situ RNA hybridization, particularly in regenerating muscle fibers.

Duchenne muscular dystrophy (DMD) is a severe hereditary degenerative disease of muscle with an incidence of about 1 in 3500 live male births. Cytogenetic and linkage studies have localized the genetic defect to the middle of the short arm of the X chromosome (*1*), and two approaches have led to the identification of a segment of DNA deleted in many patients with the disease (*2*). Portions of this DNA segment have been used to isolate complementary DNA (cDNA) from a 14-kb transcript specifically expressed in fetal and adult skeletal muscle (*3–5*). The protein encoded by this transcript has recently been identified and given the name dystrophin (*6*). We sought to determine whether expression of the dystrophin gene is detectable in muscle from DMD patients. We found that at least a part of the gene was expressed in every patient studied.

The probe used in this study was a 1.4-kb subclone of a 2.0-kb cDNA isolated with genomic DNA from the region of an X;21 translocation that produced symptoms of DMD in a female patient (*4*). The portion of the transcript identified by this probe is at the 5′ end of the gene (*4, 5*). The cDNA was subcloned into a Bluescript vector (Stratagene), which allowed production of labeled strand-specific RNA probes. Strand polarity was identified by sequence analysis; experiments done with antisense probes included controls done with probes of the opposite polarity. Sense and antisense probes had specific activities that differed by less than 20%, and equal counts of each probe were used.

The probe identified a messenger RNA (mRNA) transcript of about 14 kb in Northern blots of total RNA from human fetal muscle and adult cardiac and skeletal muscle (Fig. 1A). Detection of this signal depended on isolation of high-quality (nondegraded) RNA. RNA isolated from human muscle cells grown in monolayer culture also showed a 14-kb transcript after 23 days of growth from primary explants. The signal was noted only after the appearance of myotubes in the culture plates.

Muscle specimens were taken from patients with typical clinical manifestations of DMD. Each patient had the usual hereditary pattern, including distribution of weakness, elevation of serum creatine kinase, and muscle biopsy findings that are characteristic of this disease. None of the patients studied had deletions of genomic DNA on Southern blot with the cDNA probe we were using. Total cellular RNA isolated from the muscle

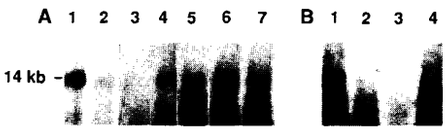

Fig. 1. Northern blot analysis of human muscle RNA with a probe for the DMD gene. (**A**) Samples from normal muscle; lane 1, quadriceps muscle from a 29-year-old male, lane 2, skeletal muscle from an 18-week fetus; lane 3, tissue culture 17 days; lane 4, tissue culture 23 days, lane 5, tissue culture 43 days; lane 6, cardiac muscle (atrium); lane 7, cardiac muscle (ventricle). (**B**) Quadriceps muscle samples from patients with DMD, aged 4 to 10 years: a transcript is seen in lane 1 but not in lanes 2 to 4. Total RNA was isolated from human muscle by means of a guanidine isothiocyanate/cesium chloride gradient technique (*9*). RNA was isolated from tissue culture preparations with a guanidine hydrochloride precipitation procedure (*10*). The RNA was separated in a 0.8% agarose-formaldehyde gel and transferred onto Biotrans nylon membranes (ICN) after a water rinse to remove excess formaldehyde (*9*). The RNA was not alkali-treated before transfer. Antisense probes were labeled with [^{32}P]UTP by means of T3 polymerase (Stratagene). The blots were hybridized in 5× standard saline citrate (SSC) (1× SSC is 0.15*M* NaCl, 0.015*M* sodium citrate), 50% formamide, and 10% dextran sulfate at 52°C and washed in 0.1× SSC, 0.1% SDS at 65°C (*11*). The mRNA size is based on comparison with a standard RNA ladder (BRL).

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