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Feature Article

A 5′ Dystrophin Duplication Mutation Causes Membrane Deficiency of α-Dystroglycan in a Family with X-linked Cardiomyopathy

Roger D. Bies[1], Masato Maeda[1], Steven L. Roberds[2], Emma Holder[1], Teresa Bohlmeyer[1], James B. Young[3] and Kevin P. Campbell[2]

[1]*Division of Cardiology, VA Medical Center and University of Colorado Health Sciences Center, Denver, CO 80262;* [2]*Howard Hughes Medical Institute and Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, IA 52242; and* [3]*Heart Failure and Transplant Division, Cleveland Clinic, Cleveland, OH 44195, USA*

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R. D. Bies, M. Maeda, S. L. Roberds, E. Holder, T. Bohlmeyer, J. B. Young and K. P. Campbell. A 5′ Dystrophin Duplication Mutation Causes Membrane Deficiency of α-Dystroglycan in a Family with X-linked Cardiomyopathy. *Journal of Molecular and Cellular Cardiology* (1997) **29**, 3175–3188. 5′-mutations in the dystrophin gene can result in cardiomyopathy without clinically-apparent skeletal myopathy. The effect of dystrophin mutations on the assembly and stability of the dystrophin associated protein (DAP) complex in human heart are not fully understood. The molecular defect in the dystrophin complex was explored in a family with an X-linked pedigree and severe dilated cardiomyopathy. Dystrophin gene analysis demonstrated a 5′ duplication involving exons 2–7, which encodes the N-terminal actin binding domain of dystrophin. Ribonuclease protection and PCR assays demonstrated a reduction in muscle promoter transcribed dystrophin mRNA in the heart compared to skeletal muscle. A deficiency of cardiac dystrophin protein was observed by Western blot and lack of membrane localization by immunocytochemistry. The cardiac expression of the dystrophin related protein utrophin was increased, and the 43 kDa (β-dystroglycan), 50 kDa (α-sarcoglycan) and 59 kDa (syntrophin) dystrophin associated proteins (DAPs) were co-isolated and present in nearly normal amounts in the membrane. However, cardiac dystrophin deficiency and increased utrophin expression were associated with loss of extracellular 156 kDa dystrophin associated glycoprotein (α-dystroglycan) binding to the cardiomyocyte membrane. α-Dystroglycan is responsible for linkage of the dystrophin complex to the extracellular matrix protein laminin. Therefore, 5′ dystrophin mutations can reduce cardiac dystrophin mRNA, protein expression, and dystrophin function in X-linked cardiomyopathy (XLCM). The presence of membrane-associated β-dystroglycan, α-sarcoglycan, syntrophin, and utrophin are insufficient to maintain cardiac function. This XLCM family has a 5′ dystrophin gene mutation resulting in cardiac dystrophin deficiency and a loss of α-dystroglycan membrane binding.

Key Words: Dystrophin; Utrophin; α-dystroglycan; Cardiomyopathy; Muscular dystrophy.

Introduction

Familial cardiomyopathy and idiopathic dilated cardiomyopathy (IDC) are conditions associated with a high mortality (Goldstein and Brown, 1988), and in some families appear to have a genetic etiology (Michels *et al.*, 1992). Duchenne and Becker muscular dystrophies (DMD/BMD) are examples where a mutation in the gene encoding the cytoskeletal protein dystrophin can cause muscular

Please address all corrrespondence to: Roger D. Bies, Cardiology Division, Department of Veterans Affairs Medical Center, 1055 Clermont Street, Denver, CO 80220, USA.

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dystrophy and severe heart failure (Perloff *et al.*, 1966; Palmucci *et al.*, 1992; Rees *et al.*, 1993). Patients with BMD and cardiomyopathy have been shown to have decreased localization of dystrophin protein at the cardiomyocyte membrane (Maeda *et al.*, 1995a). The degree of cardiac and skeletal involvement in BMD may differ, and severe cardiomyopathy may be present with either mild or clinically inapparent skeletal muscle disease. Patients in such families may be misdiagnosed with IDC. Families with heart failure and mutations in the dystrophin gene have been designated as BMD with dilated cardiomyopathy or X-linked cardiomyopathy (XLCM) (Muntoni *et al.*, 1993; Towbin *et al.*, 1993; Yoshida *et al.*, 1993; Franz *et al.*, 1995).

Several reports now suggest that most patients with severe heart failure and mild or inapparent skeletal myopathy have mutations in the 5′ region of the dystrophin gene. The proposed mechanisms which may explain the XLCM phenotype in these families include either mutations which cause diminished expression of cardiac dystrophin (Muntoni *et al.*, 1995, Milasin *et al.*, 1996), or cardiac sensitive changes in N-terminal amino acid sequence (Ortiz-Lopez *et al.*, 1997). What remains unclear, is whether some common molecular or biochemical feature could explain the similar phenotype in these different families. Are there other types of mutations which can result in XLCM? What role does the dystrophin related protein, utrophin, play in modulating disease phenotype? How is the stability and organization of the dystrophin associated protein complex affected in XLCM?

Dystrophin is found at the inner membrane surface of cardiac and skeletal muscle fibers and is bound to a dystrophin-associated protein (DAP) complex. This complex forms a structural network which stabilizes the muscle membrane and links intracellular actin microfilaments to the extracellular matrix protein laminin (Ervasti and Campbell, 1991). Loss of dystrophin can cause destabilization and loss of the DAP complex at the cell membrane (Ohlendieck *et al.*, 1991a, 1993). The importance of this complex has been demonstrated by the fact that mutations in either dystrophin or the dystrophin associated glycoproteins [50 kDa DAG (α-sarcoglycan), 43 kDa DAG (β-sarcoglycan), and 35 kDa DAG (γ-sarcoglycan)] can affect skeletal muscle causing muscular dystrophies (Hoffman *et al.*, 1987; Roberds *et al.*, 1994; Bonneman *et al.*, 1995; Lim *et al.*, 1995; Noguchi *et al.*, 1995). A review by Towbin (1995) suggested that a reduction in the abundance of cardiac 156 kDa α-dystroglycan may occur in some cases of XLCM. Thus, the organization of the DAP complex may also affect cardiac muscle and play an important role in XLCM. The autosomal homologue of dystrophin, dystrophin related protein (DRP) or utrophin, has also been shown to associate with the DAP complex in the membrane (Matsumura *et al.*, 1992). Data in small-caliber skeletal muscle in the *mdx* mouse has led to the hypothesis that utrophin may be substituted for dystrophin as an approach to therapy in DMD/BMD (Matsumura and Campbell, 1994). The organization of DAPs and utrophin may therefore influence the phenotypic presentation of dystrophin mutations in diseases like XLCM. In this report, we have analysed cardiac and skeletal muscle dystrophin, utrophin, and DAP expression and membrane affinity in a family with X-linked cardiomyopathy. Our results suggest that a mutation in the 5′ region of the dystrophin gene can cause muscle-promoter-derived mRNA and protein to be reduced in heart compared with skeletal muscle. This abnormality is associated with an increase in cardiac utrophin, and a loss of α-dystroglycan binding with the cardiomyocyte membrane.

Materials and Methods

Case descriptions

The family's pedigree is shown in Figure 1. The propositus (W.W.) was a 20-year-old male stock clerk suffering from dyspnea, fatigue and weight loss. Physical examination showed bibasilar rales

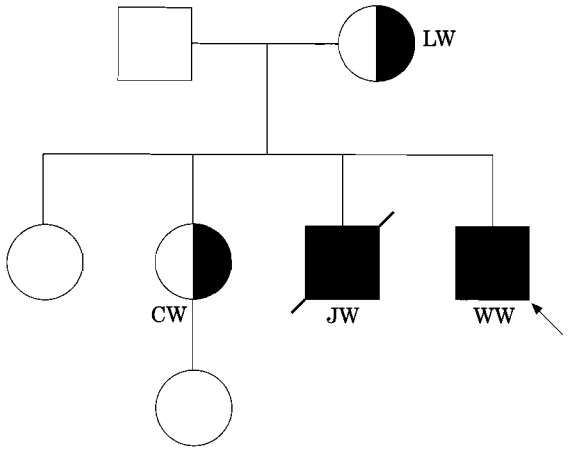

Figure 1 Pedigree of a family with X-linked (Becker) dilated cardiomyopathy. Darkened squares indicate affected males and half darkened circles indicate carrier females. The open circles and square are unaffected females and male respectively. Clinical data from subjects L.W., C.W., J.W. and W.W. are described in Materials and Methods. Arrow indicates proband who underwent heart transplantation. Diagonal slash indicates death from heart failure.

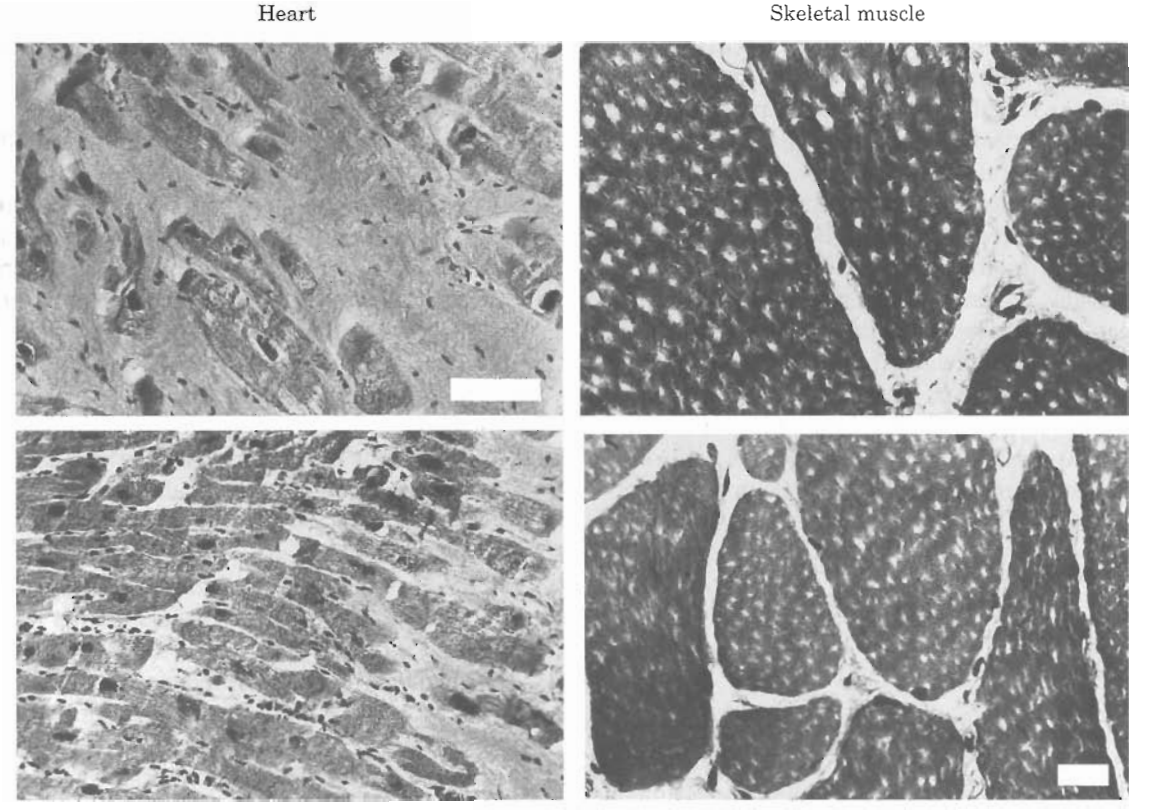

Figure 2 Hemotoxylin and Eosin stain of tissue sections of XLCM skeletal muscle and heart. Biceps (skeletal muscle) biopsy was remarkable for fiber size variation, scattered internal nuclei, and an increase in the interstitial connective tissue. Endomyocardial (heart) biopsy showed focal replacement by fibrous tissue and variation in myofiber size, some exhibiting hypertrophy, enlarged irregularly shaped nuclei, and others atrophic. Bar = 30 μm.

and an S3 gallop, and chest x-ray film indicated cardiomegaly and pulmonary congestion. Serum CPK (MM) was elevated at 2533 IU/ml, but there was no significant skeletal muscle weakness. Skeletal muscle biopsy sections showed fiber size variation and a slight increase in the interstitial connective tissue, with rare internal nuclei (Fig. 2). Echocardiogram showed left ventricular dilatation with an ejection fraction of less than 20%. Endomyocardial biopsy showed focal fibrous tissue replacement of myocytes and variation in myofiber size, with hypertrophic and atrophic myocytes intermixed. Some cells showed enlarged irregularly shaped nuclei and perinuclear vacuolization, and others were atrophic (Fig. 2). This pattern is not disimilar to that observed in idiopathic dilated cardiomyopathy. The subject subsequently developed end-stage heart failure requiring cardiac transplatation, and has since regained normal functional activity. The 17-year-old younger brother (J.W.) was evaluated when W.W. presented with heart failure. Initially, he complained only of a dry cough and mild dyspnea on exertion. Echocardiogram showed a dilated cardiomyopathy with a left ventricular ejection fraction of 20–25%. Serum CPK (MM) was 5100 IU/ml, and he had no significant skeletal muscle weakness. Within 1 year of medical treatment, he also developed intractable heart failure and died while awaiting a donor heart. The 42-year-old mother (L.W.) of W.W. and J.W. was a genetic carrier and had no muscle weakness or cardiac symptoms with the exception of rare palpitations. Echocardiogram revealed a mildly dilated left ventricle and a moderately depressed ejection fraction of 35%. The 25-year-old female sister (C.W.) was a genetic carrier who had no muscle weakness or cardiac symptoms except for occasional episodes of palpitations, with no arrhythmia documented by Holter monitor. Echocardiogram showed normal chamber size and valves, and a left ventricular ejection fraction of 65%.

DNA analysis

Genomic DNA from blood lymphocytes of family members was used for Southern blot analysis using cDNA probes spanning the entire dystrophin gene

as previously described (Bies *et al.*, 1992a). Dystrophin cDNA probe 9.7 (ATCC, Rockville, MD, USA) which spans exons 1–9 (cDNA bp 1–1538), detected the mutation, and the other probes showed no abnormality. Purified DNA was digested with Hind III, separated by 1% agarose gel electrophoresis, and transferred to nitrocellulose membrane (Genescreen, Dupont, Boston, MA, USA). Probes used in this study were labeled with ^{32}P-dCTP (New England Nuclear, Boston, MA, USA) using a random hexamer priming kit (Pharmacia Biotech Inc., Piscataway, NJ, USA). Exon numbering of Hind III fragments detected on Southern blot follows the convention established by Koenig *et al.*, (1989). Analysis of band intensity was performed using a scanning densitometer (Helena Labs, Beumont, TX, USA) on the autoradiograms.

Tissue sources

Fresh heart muscle was obtained at the time of cardiac transplantation and immediately frozen in liquid nitrogen-colled isopentane. Biopsy specimens from left biceps muscle were prepared in a similar fashion. Control skeletal muscle samples were obtained from gastrocnemius muscle from two diabetic subjects post-leg-amputation. Control cardiac tissues were obtained from three explanted hearts at the time of transplantation from non-dystrophic (ischemic) cardiomyopathy. All tissues were pathological specimens obtained at the time of surgery with informed consent per institutional guidelines.

PCR analysis of dystrophin cDNA

Evaluation of dystrophin mRNA transcribed from the genomic mutation (exon 2–7 duplication) was performed by reverse transcription of heart and skeletal muscle RNA into cDNA with a dystrophin-specific primer, followed by nested PCR amplification. Primers flanking the duplication (in exons 1 and 10) were selected for an initial round of reverse transcription PCR. Total RNA was prepared as described by Chirgwin (1979). Five μg of RNA was incubated with 50 ng of reverse primer (bases 1229–1253) in exon 10 for reverse transcription into cDNA (Bies *et al.*, 1992). The cDNA product was diluted to 5 μl and PCR performed using 500 ng exon 1 forward primer (cDNA sequence 36–61) and 450 ng exon 10 reverse primer (cDNA sequence 1229–1253) for 30 cycles. This reaction was diluted 1 to 50 and a 1 μl aliquot was used as a template for subsequent PCR reactions.

Figure 4 shows the location of primers used in PCR reactions 1–4. Reaction 1 utilized primers in exon 1 (cDNA sequence 218–242) and exon 8 (cDNA sequence 967–986), spanning the duplication, to synthesize a large 1387 base pair fragment. Reaction 2 utilized a forward primer in exon 1 (cDNA sequence 218–242) and a reverse primer in duplicated exon 4 (cDNA sequence 428-452) to synthesize both 852 bp (2a) and 235 bp (2b) fragments from each of the exon 4 annealing sites. Reaction 3 utilized a reverse primer in exon 8 (cDNA sequence 967–986) and a forward primer in duplicated exon 7 (cDNA sequence 784–808) to synthesize both 821 bp (3a) and 203 bp (3b) fragments from each of the exon 7 annealing sites. Reaction 4 utilized a forward exon 7 primer (cDNA sequence 784–808) and a reverse exon 4 primer (cDNA sequence 428–452) predicted to synthesize a 286 bp fragment from the center of the duplication.

Dystrophin gene transcription is regulated in a tissue and developmentally specific manner by multiple promoter/enhancer elements in the 5′ region. The majority of cardiac and skeletal muscle dystrophin expression is driven by the "muscle" promoter, though expression from "brain" promoters has been described (Feener *et al.*, 1989; Nudel *et al.*, 1989; Bies *et al.*, 1992b). To test for transcripts derived from muscle and non-muscle dystrophin promoters, PCR amplification was performed with promoter-specific exon 1 forward primers for the muscle, cortical brain, and Purkinje transcripts according to previous reports (Bies *et al.*, 1992b; Holder *et al.*, 1996). Ten μl of all PCR reactions were analysed on 3% NuSieve agarose gels (FMC Bioproducts, Rockland, ME, USA) containing 0.2 mg/ml of ethidium bromide prior to photography.

Subcloning and sequencing

The PCR products were subcloned directly into the TA cloning vector kit (Invitrogen Cor., San Diego, CA, USA). Plasmid DNA containing dystrophin cDNA inserts were purified (Maxiprep, Promega Cor., Madison, WI, USA) and sequenced using T7 and SP6 primers with a Sequenase version 2.0 sequencing kit (United States Biochemical Cor., Cleveland, OH, USA).

Ribonuclease protection assay

A 235 nt riboprobe spanning dystrophin exons 1–4 (cDNA bases 218–452) was selected to determine the abundance of dystrophin mRNA in affected heart and skeletal muscle (Holder *et al.*, 1996). The probe starts with the last 22 bp from the muscle promoter exon 1, and ends with the first 58 bp from exon 4. Hybridization to the duplicated dystrophin transcript should protect two equal fragments; one normal sized (235 nt) fragment corresponding to hybridization to the 5′-end of the mutant transcript containing exon 1–4 sequence, and one smaller 213 nt fragment of approximately equal intensity hybridizing to the downstream duplication sequence junction containing exons 2, 3 and 4. The 22 nt of sequence in the riboprobe from exon 1 should not hybridize to the exon 7 sequence which preceeds exon 2 at the central portion of the duplicated transcript.

Hybridization to dystrophin transcript in control tissues should detect a 235 nt fragment. The low level transcription from the brain (Bies *et al.*, 1992b) and Purkinje promoter (Holder *et al.*, 1996) in control muscle tissues should also detect a small amount of the 213 nt (exon 2–4) fragment due to mismatch from brain exon 1 and Purkinje exon 1 (Holder *et al.*, 1996). Ribonuclease protection assay (RPA II kit, Promega Corp.) of dystrophin transcripts was performed using 5 μg of total RNA as previously described (Holder *et al.*, 1996). To assess equal RNA loading, 5 μg of total RNA from affected and control heart and skeletal muscle was examined by Northern blots using a GAPDH ^{32}P-labeled cDNA probe (ATCC, Rockville, MD, USA). RPA samples were analysed by electrophoresis in an 8% polyacrylamide gel and exposed to x-ray film for 4 days.

Western blot analysis

Whole tissue heart and skeletal muscle tissue homogenates (Bies *et al.*, 1992c), and microsomal membranes from human cardiac tissue were prepared (Ohlendieck *et al.*, 1991b). Proteins were resolved electrophorectically in 3–12% polyacrylamide gradient gels which were then electrophoretically transferred to nitrocellulose membrane (0.2 μm, Bio-Rad Laboratories Inc., Hercules, CA, USA) (Bies *et al.*, 1992c). The antibodies used in this study are as follows: rabbit polyclonal anti-dystrophin antibody (Bies *et al.*, 1992c) at 1:500 dilution in Tris (10 mM; ph 8.0) buffered saline, 0.05% Tween 20 (TBST), anti-utrophin (Ohlendieck *et al.*, 1991a) rabbit polyclonal antibody at 1:100, anti-156 DAG (Ibraghimov-Beskrovnaya *et al.*, 1992) sheep polyclonal antibody at 1:50, anti-59 DAP (Ohlendieck *et al.*, 1991b) sheep polyclonal antibody at 1:50, anti-50 DAG (Roberds *et al.*, 1993) sheep polyclonal antibody at 1:500, and anti-43 DAG (Ohlendieck *et al.*, 1991b) sheep polyclonal antibody at 1:50 dilution. Immunoreactive bands were detected with secondary antibodies coupled to alkaline phosphatase (Sigma Chemical Co.) (Bies *et al.*, 1992c) or to horseradish peroxodase using the enhanced chemiluminescent method (Amersham Corp.). Protein concentrations of tissue homogenates were determined by BCA protein assay (Pierce, Rockford, IL, USA) and 24 μg was loaded in each lane. Equal sample loading was assessed by staining the gel with Coomassie blue and insuring a consistent density of the myosin band in each lane as previously described (Maeda *et al.*, 1996). A scanning densitometer (Bio-Rad Laboratories Inc.) was used to analyse dystrophin, utrophin, and DAP band intensity relative to controls.

Immunocytochemistry and histopathology

Unfixed frozen samples of heart and skeletal muscle were cut into 6 μm cryosections and placed on Superfrost microscope slides (Fischer Scientific, Pittsburgh, PA, USA). The slides were blocked with 3% BSA in phosphate buffered saline (PBS, pH 7.4) for 20 min at room temperature and then incubated with anti-dystrophin antibody (Bies *et al.*, 1992c) or anti-vinculin antibody (Maeda *et al.*, 1997) for 2 h. Slides were incubated with secondary antibody for 1 h; tetramethylrhodamine isothiocyanate (TRITC) conjugated anti-rabbit IgG antibody (1:80, Sigma Chemical Co.) The slides were washed with PBS and mounted with 90% glycerol in PBS. Staining with secondary antibodies alone was used as a control for non-specific fluorescence. Stained sections were photographed under uv light with a Nikon microscope. Light microscopy of hemotoxylin and eosin stained sections were performed on 6-μm sections of 10% buffered neutral formalin fixed paraffin blocks of affected tissue.

Results

DNA analysis

Southern blot analysis using dystrophin cDNA probes showed a 5′ gene duplication. Probe 9.7 demonstrated the presence of nine Hind III

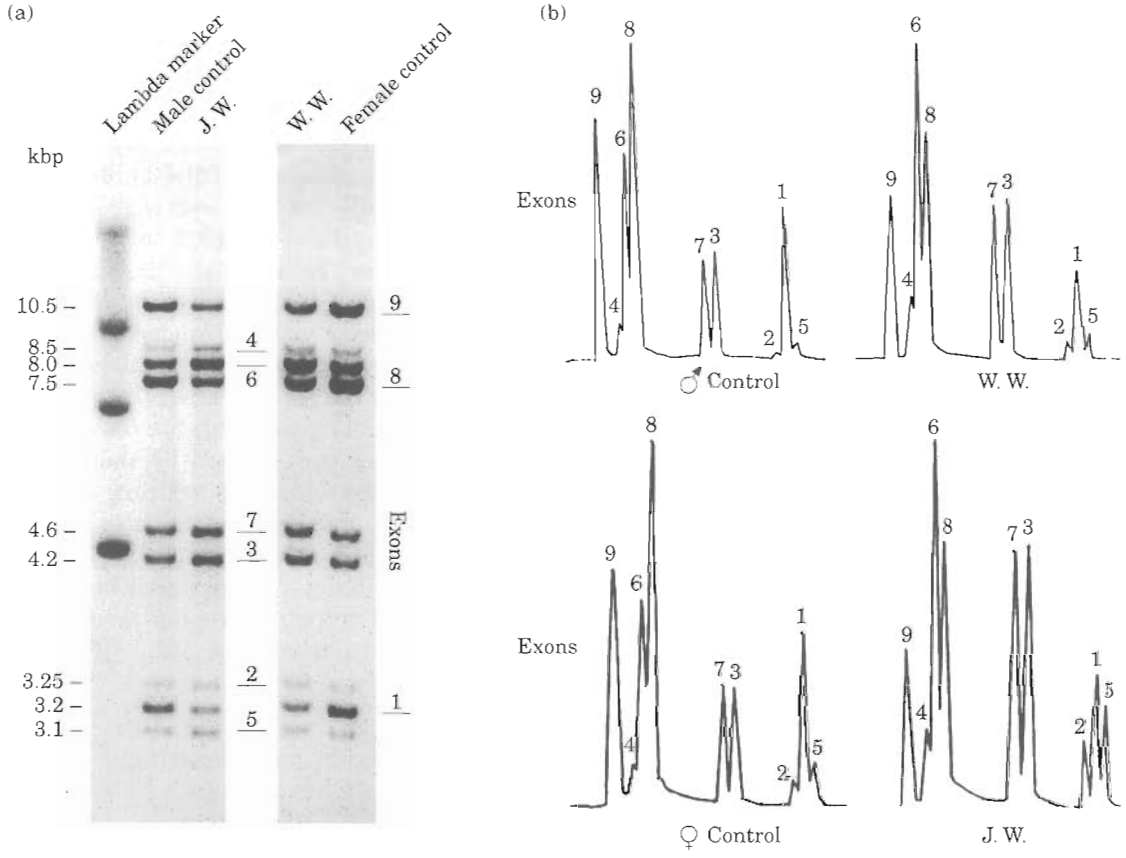

Figure 3 Southern blot analysis with dystrophin cDNA probe 9.7. (a) Duplication of Hind III fragments (3.25, 4.2, 8.5, 3.1, 8.0, 4.6 kbp) containing exons 2–7 in this family is shown by a relative increase in hybridization signals, compared to fragments containing exons 1,8, and 9 in brothers W.W. and J.W. The fragment size are labeled on the left, and the corresponding exons are also labeled. The exons involved in the duplication are labeled in the center of the figure, and non-duplicated exons are labeled on the right. Male and female controls are shown. (b) The original densitometry scanning of the autoradiogram is shown, suggesting a duplication (double density) in exons 2, 3, 4, 5, 6 and 7 signals. There is a relative underloading of W.W. and J.W. DNA, seen by comparing signals from bands corresponding to the fragments containing exons 1, 8, and 9 with controls.

fragments (3.2, 3.25, 4.2, 8.5, 3.1, 8.0, 4.6 and 10.5 kb), representing exons 1–9, respectively (Fig. 3). W.W. and his brother J.W. showed an increased hybridization intensity for fragments containing exons 2–7, compared to controls (normalized to unaffected exon fragments 1, 8, and 9). Comparative analysis of densitometric peaks from the autoradiograms shows a double increased density for fragments 2–7, suggesting a duplication in this portion of the dystrophin gene (Fig. 3).

PCR analysis of mutant dystrophin transcripts

Transcription of the duplicated segment was confirmed by reverse transcription PCR and sequencing. A diagram of the PCR primers is displayed in Figure 4(a). The different dystrophin mRNAs detected by PCR in cardiac and skeletal muscle were similar [Fig. 4(b)]. The results demonstrated tandem in-frame transcription of the duplicated exon 2–7 segment, as well as the synthesis of three smaller products in both tissues. The PCR products were all cloned and sequenced to confirm identity. Transcription of the 5′ tandemly duplicated segment was confirmed by three separate PCR reactions amplified across the duplication [Fig. 4(a), primer sets 2, 3, and 4]. These reactions detected the central mutation junction (Reaction 4), as well as the 5′ (Reaction 2) and 3′ (Reaction 3) ends of the duplicated transcript [Figs 4(b), 4(c)]. The latter two reactions produced two fragments corresponding to the two primer binding sites for exons 4 and 7, respectively, contained in the duplication [Figs 4(b)(lanes 2 and 3), 4(c)(primer set 4)]. Reaction

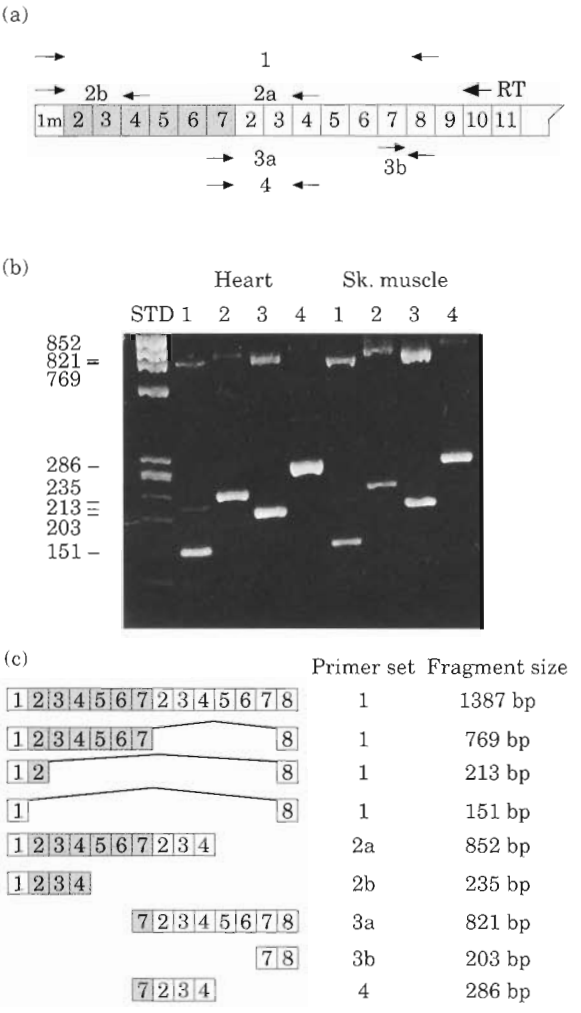

Figure 4 Schematic representation of the duplicated mRNA transcript and location of PCR primers. The exons are designated by a number in each box. Reverse transcription of total RNA was performed with a reverse primer located downstream in exon 10 (large arrowhead). The PCR primers for each of four numbered reactions are denoted by arrows over the appropriate exons. Reaction 1 spans the duplicated segment. Reactions 2 and 3 are predicted to have two products (2a and 2b, 3a and 3b) because of duplicated annealing sites for exon 4 and exon 7 in each respective primer set. The orientation of primers in reaction 4 should only synthesize a product if the duplicated segment is present. (b) XLCM cardiac and skeletal muscle dystrophin cDNA were amplified by PCR. Reaction 2 synthesized 852 bp (2a) and 235 bp (2b) products, reaction 3 synthesized 821 bp (3a) and 203 bp (3b) products, and reaction 4 synthesized a 286 bp product, all confirming the presence of the duplication transcript. Reaction 1 demonstrates synthesis of three products at 769 bp, 213 bp, and 151 bp corresponding to unusual dystrophin splice forms within the duplication. The 769 bp band corresponds to normal dystrophin transcript, and the 213 and 151 bp bands are transcripts deleted for the duplicated exons, with or without exon 2 [see Fig. 4(c) for explanation]. The reaction products from cardiac and skeletal dystrophin cDNAs were similar. (c) Sequence analysis from mutant cDNAs detected by PCR. The top four bar diagrams represent the four distinct dystrophin transcripts derived from the mutant gene. The bottom five bar diagrams indicate various PCR products sequenced from the duplicated transcript. The PCR fragment size and the reaction number [see Fig. 4(a)] is designated on the right.

4 synthesized the (exon 7 followed by exon 2) junction fragment in the middle of the duplication in both heart and skeletal muscle [Figs 4(b) (lane 4), 4(c) (primer set 4)]. PCR amplification across the entire duplication (reaction 1) detected the duplicated transcript and three smaller transcripts with a similar distribution in heart and skeletal muscle [Fig. 4(b), lane 1], presumably occurring via alternative mRNA splicing. Figure 4(c) schematically illustrates these alternatively spliced mRNAs which include: (1) the duplicated transcript; (2) a normal (769 bp) transcript; (3) a deletion (151 bp) transcript excising both duplicated segments (i.e. exon 1-8-9...); and (4) a deletion

(213 bp) transcript which excised the duplication but retained a single exon 2 (i.e. exon 1-2-8-9...). The ~1.4 kilobase reaction 1 PCR product spanning the duplication transcript, was not efficiently amplified by PCR, because of its large size and the presence of shorter products in the reaction. It was, however, seen by both overloading the gel and Southern blot of the reaction (data not shown). Sequence analysis of the duplication transcript predicts a duplicated actin binding domain, and a single missense mutation at the middle of the duplication exon 7/exon 2 junction, from Tyr to Asp.

PCR analysis of dystrophin promoter utilization

Dystrophin is normally transcribed from the muscle promoter, and at moderate to low levels in muscle tissues from two other promoters active in brain (Feener *et al.*, 1989; Bies *et al.*, 1992b; Gorecki *et al.*, 1992; Holder *et al.*, 1996). The main finding was that PCR of dystrophin muscle promoter transcripts showed a weak amplification product in XLCM heart, and synthesis of a strong band in XLCM skeletal muscle (Fig. 5). We also tested the possibility that differential activation of transcription from the dystrophin brain or Purkinje promoter in affected heart of skeletal muscle accounted for some of the tissue differences in dystrophin expression. Transcripts from the cortical brain promoter in affected heart and skeletal muscle were not detected by PCR and Purkinje promoter transcripts (compared to control reactions) did not appear to account for differences in cardiac *v* skeletal muscle transcription (Fig. 5). The two bands in the Purkinje promoter reaction are alternatively spliced mRNA isoforms, and only the lower ban encodes for the full length dystrophin protein (Holder *et al.*, 1996).

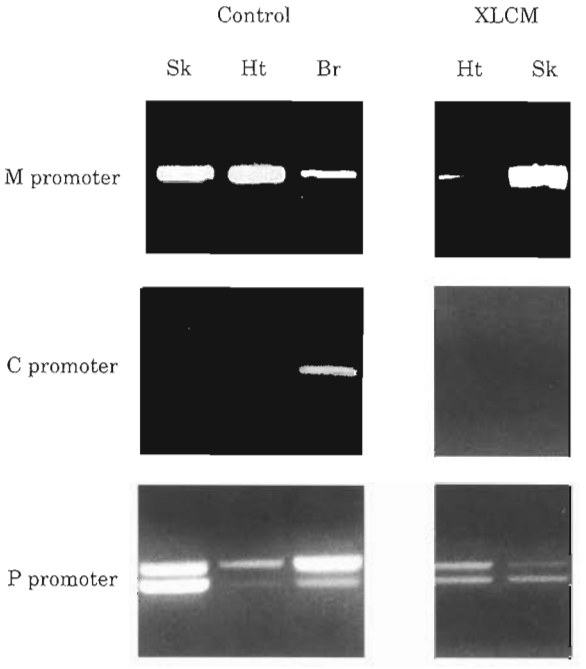

Figure 5 PCR of dystrophin muscle, brain, and Purkinje promoter transcripts. XLCM heart (Ht) shows a reduction in muscle promoter (M promoter) transcript compared to XLCM skeletal muscle (Sk) and controls. Reduced Purkinje promoter transcripts (P promoter) were detected in XLCM tissues, and the cortical brain promoter (C promoter) transcript was absent in XLCM. Differential activity of non-muscle promoter transcripts did not appear to significantly contribute to a difference in dystrophin mRNA in XLCM heart and skeletal muscle. Human brain cDNA (Br) was amplified as a control for both brain and muscle promoter activity.

Ribonuclease protection of cardiac and skeletal muscle dystrophin mRNAs

Ribonuclease protection assay (RPA) was performed to further analyse differences in dystrophin mRNA abundance in XLCM heart and skeletal muscle. Figure 6 shows a marked reduction of muscle promoter transcribed dystrophin mRNA abundance (235 nt fragment) in XLCM cardiac tissue compared to XLCM skeletal muscle. The lower (213 nt) fragment detected in XLCM skeletal muscle (and heart) probably represents hybridization to the middle of the duplication transcript where exon 2 is preceded by exon 7 rather than exon 1, causing the probe to match a segment 22 ng shorter. The small amount of the 213 nt fragment detected in normal tissues most likely represents the low level of transcripts from the cortical brain or Purkinje promoter which contain different exon 1 sequences (Bies *et al.*, 1992b; Holder *et al.*, 1996). The brain transcript does not appear to significantly contribute to dystrophin mRNA in XLCM tissue, since it was not well amplified in our PCR reactions. However, some of the 235 nt fragment in XLCM tissue may be from normal transcript detected by PCR, and some of the 213 nt fragment may be from Purkinje promoter transcript. The smaller deletion transcripts detected by PCR in reaction 1 of Figure 4 (transcripts which delete the duplicated segment) were not detected by RPA.

Western blot analysis of dystrophin, utrophin, and DAPs in XLCM

Dystrophin protein was analysed in XLCM and control heart and skeletal muscle (Fig. 7). Western

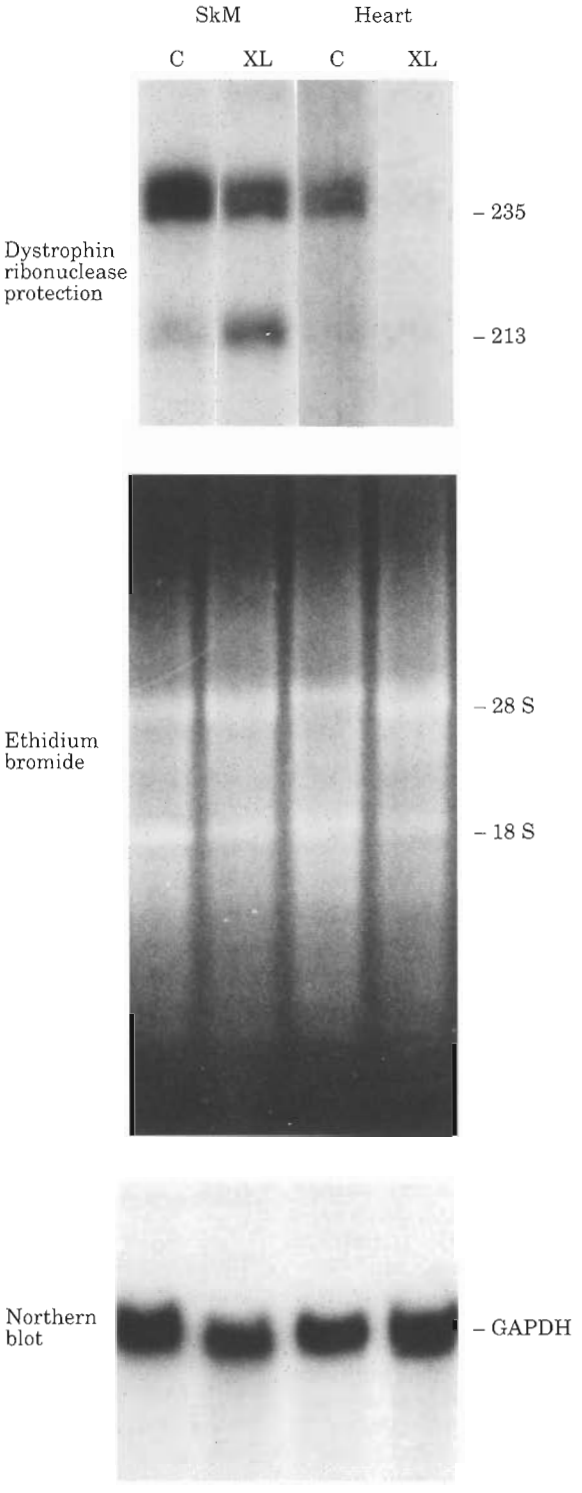

Figure 6 Ribonuclease protection assay of dystrophin mRNA abundance in XLCM heart and skeletal muscle. The riboprobe detects muscle promoter dystrophin transcript (235 nt fragment) in all four samples. There is a noticeable reduction of dystrophin mRNA in heart tissue from the subject with XLCM (XL). there is an approximately equal amount of a smaller 213 nt protected fragment in XLCM skeletal muscle (Sk M) and heart. The 213 nt fragment probably represents either hybridization to downstream duplicated exons 2, 3, and 4 (preceded by exon 7) and/or small amount of Purkinje promoter transcript. The 22 nt muscle exon 1 portion of the probe would not be protected in either of these hybridization reactions, and would give exactly a 213 nt fragment. The small amount of 213 nt fragment in control (C) heart and skeletal muscle represents hybridization to the small amount of cortical brain and Purkinje promoter transcripts in normal muscle (33). Ethidium bromide staining of RNA electrophoresis and Northern blot analysis using a P^{32}-GAPDH probe are shown as loading controls. 5 μg of RNA was loaded in each lane.

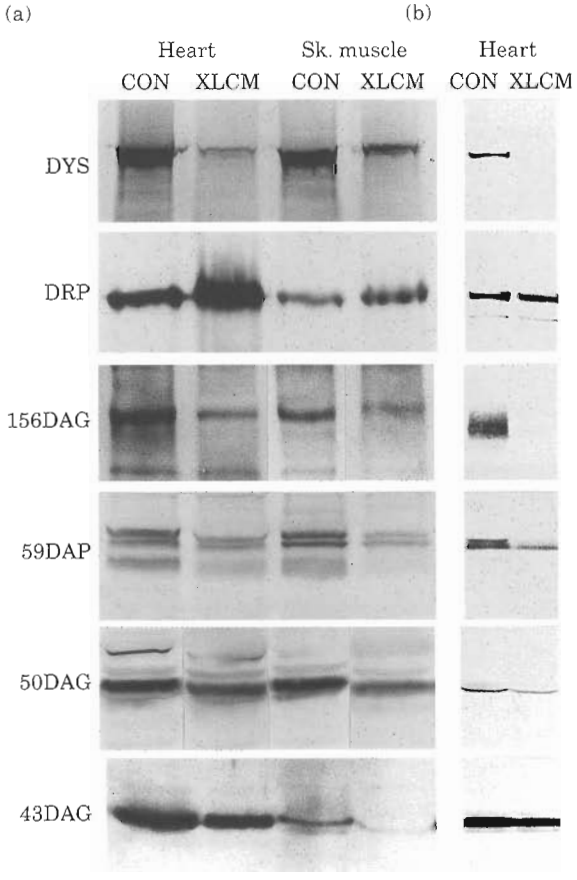

Figure 7 Western blot analysis for dystrophin, utrophin (DRP) and DAPs in whole tissue homogenates (a) and membrane preparation (b) of heart and skeletal muscle from control and XLCM subjects. (a) Anti-dystrophin antibody demonstrated dystrophin (DYS) immunoreactivity in control (CON) heart and skeletal muscle. The XLCM subject showed a very low amount of dystrophin protein in heart, and moderately reduced amount of dystrophin in skeletal muscle. (Sk. muscle). An increased intensity of utrophin (DRP) staining was observed in XLCM heart and skeletal muscle compared with control. Antibodies against α-dystroglycan (156 DAG), β-dystroglycan (43 DAG), α-sarcoglycan (50 DAG), and syntrophin (59 DAP) proteins demonstrated mild to moderately reduced abundance in both heart and skeletal muscle homogenates from an XLCM subject compared to control tissues. 24 μg of protein was loaded in each lane. (b) Membrane preparations from normal human heart showed an intact complex with co-isolation of DYS, utrophin (DRP), and α-dystroglycan (156 DAG), β-dystroglycan (43 DAG), α-sarcoglycan (50 DAG), and syntrophin (59 DAP). In contrast, XLCM heart membrane showed little or no immunoreactivity for either dystrophin or 156 DAG proteins. Utrophin (DRP), and the content of 59 DAP, 50 DAG, and 43 DAG proteins were co-isolated in the membrane preparation, despite dystrophin loss. The loss of 156 DAG (α-dystroglycan) suggests an uncoupling of the DAP membrane complex.

blots of whole tissue homogenates [Fig. 7(a), top panel] showed a reduced abundance of dystrophin in affected heart (~15% of control) compared to affected skeletal muscle (~40% of control). Equal loading was confirmed as described in methods. The mutant dystrophin is predicted to be ~20 kDa larger than normal dystrophin. However the difference can not be well resolved in polyacrylamide gels. Figure 7(a) also shows an increase in utrophin (DRP) in both heart (~230% of control) and skeletal muscle (~165% of control) in XLCM. Analysis of tissue homogenates for 156 kDa (α-dystroglycan), 59 kDa (syntrophin), 50 kDa (α-sarcoglycan), and 43 kDa DAP (β-dystroglycan) content showed mild to moderately reduced immunoreactivity in both XLCM heart and skeletal muscle compared to control [Fig. 7(a)]. Because protein content may not represent functional organization, we examined the effect of dystrophin deficiency and utrophin excess upon membrane stability of the DAP complex by analysing cardiac microsomal membranes [Fig. 7(b)]. Dystrophin and α-dystroglycan were absent from the cardiac membrane, while utrophin and the other dystrophin associated proteins (59, 50, and 43 kDa) were co-isolated in the membrane preparation [Fig. 7(b)]. Comparison between the whole tissue homogenates and the membrane preparation suggests that although α-dystroglycan is present in the cell, cardiac dystrophin deficiency results in a poor association of α-dystroglycan with the other proteins in the membrane complex. Sufficient biopsy material was not available for analysis of microsomal membranes from XLCM skeletal muscle.

Dystrophin immunocytochemistry in XLCM cardiac and skeletal muscle

Tissue sections were analysed to compare the membrane localization of dystrophin in control and XLCM tissues (Fig. 8). In XLCM heart, dystrophin was absent from the membrane of most cardiac cells. In a few sections rare cardiomyocytes were found with membrane staining of dystrophin. This pattern of "revertant fibers" with discontinuous membrane staining suggests expression of some dystrophin via either an alternative splicing mechanism (Winnard *et al.*, 1995), or somatic cell mosaicism. In XLCM skeletal muscle, all myofibers showed a uniform staining of dystrophin at the membrane surface (Fig. 7). Control heart dystrophin was localized at the membrane and T tubules. In control skeletal muscle dystrophin localization was at the sarcolemma.

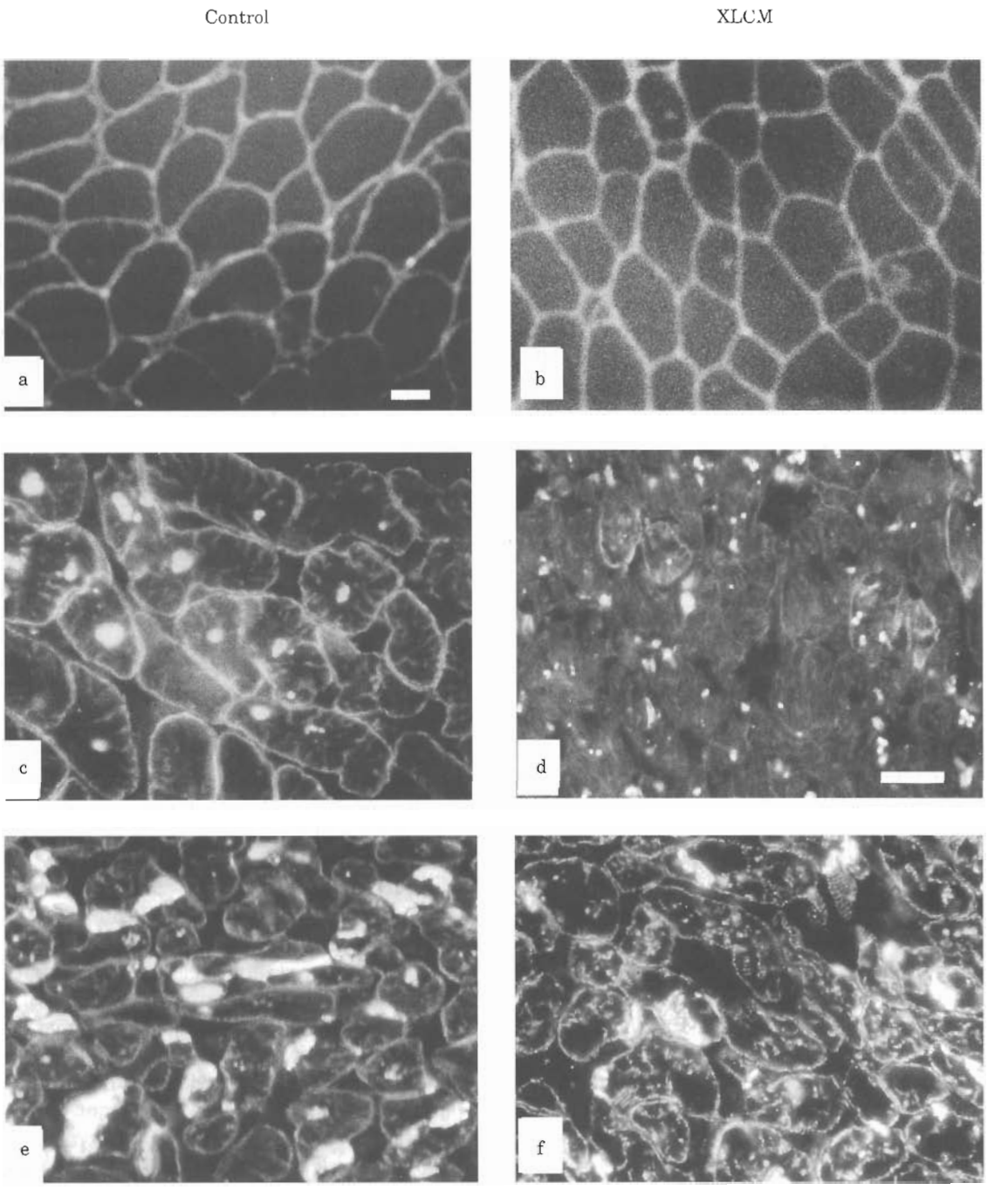

Figure 8 Immunofluorescent staining of XLCM tissue. Displayed are cross sections of heart tissue and skeletal muscle. Normal control subjects showed dystrophin membrane staining in skeletal muscle (a) and membrane and T-tubule staining in heart (c). In XLCM, normal and uniform dystrophin membrane staining was observed in all skeletal muscle cells (b). Dystrophin membrane staining in XLCM heart was markedly diminished or absent (d). In rare sections such as the one shown, a few cardiocytes showed membrane localized dystrophin in a discontinuous staining pattern. The white granules represent background staining artifact. Control staining of vinculin showed a normal distribution along membrane costomeres and T-tubules in both control (e) and XLCM (f) heart. The intense areas of staining represent cross sections of intercalated discs. Bar = 30 μm.

Discussion

This report describes an alteration of the dystrophin protein complex in a family with a 5′ dystrophin mutation presenting as a dilated cardiomyopathy. The severe cardiac failure is characterized by a marked decrease of dystrophin mRNA and protein and loss of dystrophin membrane localization in

the heart. This abnormality was associated with a loss of α-dystroglycan association with the cardiomyocyte membrane. Skeletal muscle dystrophin mRNA, protein abundance, and localization to the sarcolemma were not as severely afffected. Sufficient dystrophin expression in skeletal muscle accompanied by continuous immunolocalization at the sarcolemma in all myocytes was probably capable of maintaining partial integrity of the skeletal muscle DAP complex. The concept that some threshold amount of dystrophin can attenuate phenotype in *mdx* mice (Wells *et al.*, 1995). Therefore, differences in dystrophin abundance and membrane association probably account for most of the differences in disease severity in heart *v* skeletal muscle.

Utrophin can also bind and maintain membrane association of the DAP complex in skeletal muscle (Tinsley *et al.*, 1996). Skeletal muscle utrophin was mildly increased in the family studied, and therefore could potentially stabilize the skeletal muscle DAP membrane complex and attenuate the development of skeletal myopathy. However, we found cardiac utrophin expression was significantly increased and co-isolated in cardiac microsomal membranes with some components of the DAP complex. The cardiac utrophin/DAP complex appeared unable to maintain the membrane binding affinity of α-dystroglycan, or prevent the development of cardiomyopathy. This observation could be due to possible differences in utrophin expression in cardiac and skeletal myocytes. α-Dystroglycan is localized to the cardiac membrane, and is believed to link the dystrophin cytoskeletal complex to the extracellular matrix protein laminin (Ervasti and Campbell, 1991). Utrophin has been localized to the intercalated disc and T-tubules of bovine and mouse heart (Klietsch *et al.*, 1993; Pons *et al.*, 1994), and laminin is absent from the intercalated disc (Klietsch *et al.*, 1993). Therefore, the interaction of DAPs with other cytoskeletal elements like utrophin at the intercalated disc may lack the appropriate structural organization or subcellular localization to maintain DAP (α-dystroglycan) binding in the heart. The recent observation that a truncated utrophin transgene is capable of maintaining skeletal muscle membrane DAPs, and reduce the dystrophic pheneotype in *mdx* mouse, has not been proven to be effective in cardiac tissues (Tinsley *et al.*, 1996).

There is growing evidence that XLCM is commonly associated with 5′ dystrophin gene mutations. The 5′ mutation in this study is located in the same general region of the dystrophin gene described in other XLCM reports (Muntoni *et al.*, 1993; Milasin *et al.*, 1995; Ortiz-Lopez *et al.*, 1997), and had likely occurred via a cross-over event with breakpoints in intron 1 and intron 7. As in our report, the other families studied had some elevation of serum CK and subclinical muscle degeneration/regeneration was probably present. However, the lack of severe skeletal muscle symptoms suggests that the dystrophin complex in skeletal muscle function in the heart has been reported as one possible mechanism (Ortiz-Lopez *et al.*, 1997). In two other families, complete loss of muscle promoter activity may explain the phenotype. In these cases, selective activation of the brain or Purkinje promoters appeared to provide adequate dystrophin transcription in skeletal muscle, but not in the heart (Milasin *et al.*, 1995; Mutoni *et al.*, 1995b).

The current study expands the possible mechanisms involved in phenotypic heart and skeletal muscle disease expression. The same mutant dystrophin transcript was present in both tissues, yet dystrophin immunolocalized at the membrane in skeletal muscle, but was almost absent in heart. The mutation may have caused a cardiac-sensitive change in dystyropin function. However, part of the difference was also likely due to an alteration of dystrophin abundance in the heart. In other XLCM reports, cardiac dystrophin expression was selectively reduced (and skeletal muscle expression maintained) due to differential activity of non-muscle promoters in the absence of normal muscle promoter activity. This mechanism was probably not the cause of low cardiac dystrophin mRNA in our family. In this study, the amount of muscle-promoter-derived dystrophin mRNA in heart and skeletal muscle was different. Cardiac dystrophin mRNA was almost undetectable, and skeletal muscle dystrophin mRNA was present in near normal amounts. Differential activity of non-muscle promoters did not appear to play a major role in dystrophin expression. This observation suggests that the mutation breakpoints could have involved unidentified regulatory elements for dystrophin muscle promoter activity or dystrophin mRNA stability in the heart.

Therefore, several potential mechanisms have been identified which could contribute to the unusual XLCM phenotype. These include a reduction in cardiac dystrophin mRNA expression or stability,

abnormal dystrophin protein stability/function, and the potential salutary effect of increased skeletal muscle utrophin. The current literature for XLCM does not allow us to conclude that any one of these mechanisms predominate. However, when dystrophin is abnormally expressed in the heart, the final common pathway may be a loss of membrane bound α-dystroglycan and development of cardiomyopathy. Studies on other families will be required to determine whether a defect in cardiac membrane α-dystroglycan binding is a unique feature of XLCM.

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