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A point mutation in the 5′ splice site of the dystrophin gene first intron responsible for X-linked dilated cardiomyopathy

Jelena Milasin[1], Francesco Muntoni[2], Giovanni Maria Severini[1], Lucia Bartoloni[3], Matteo Vatta[1], Maja Krajinovic[1], Anna Mateddu[6], Corrado Angelini[3], Fulvio Camerini[4], Arturo Falaschi[1], Luisa Mestroni[1,4], Mauro Giacca[1,*] and the Heart Muscle Disease Study Group[+,4,5]

[1]International Centre for Genetic Engineering and Biotechnology, 34012 Trieste, Italy, [2]Department of Pediatrics and Neonatal Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 0NN, UK, [3]Department of Biology and Neurology, University of Padova, 35121 Padova, Italy, [4]Departments of Cardiology and [5]Morbid Anatomy, Ospedale Maggiore and University of Trieste, 34100 Trieste, Italy and [6]Department of Neuroscience, University of Cagliari, 09100 Cagliari, Italy

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X-linked dilated cardiomyopathy (XLDC) is a familial heart disease presenting in young males as a rapidly progressive congestive heart failure, without clinical signs of skeletal myopathy. This condition has recently been linked to the dystrophin gene in some families and deletions encompassing the genomic region coding for the first muscle exon have been detected. In order to identify the defect responsible for this disease at the molecular level and to understand the reasons for the selective heart involvement, a family with a severe form of XLDC was studied. In the affected members, no deletions of the dystrophin gene were observed. Analysis of the muscle promoter, first exon and intron regions revealed the presence of a single point mutation at the first exon-intron boundary, inactivating the universally conserved 5′ splice site consensus sequence of the first intron. This mutation introduced a new restriction site for *Mse*I, which cosegregates with the disease in the analyzed family. Expression of the major dystrophin mRNA isoforms (from the muscle-, brain- and Purkinje cell-promoters) was completely abolished in the myocardium, while the brain- and Purkinje cell- (but not the muscle-) isoforms were detectable in the skeletal muscle. Immunocytochemical studies with anti-dystrophin antibodies showed that the protein was reduced in quantity but normally distributed in the skeletal muscle, while it was undetectable in the cardiac muscle. These findings indicate that expression of the muscle dystrophin isoform is critical for myocardial function and suggest that selective heart involvement in dystrophin-linked dilated cardiomyopathy is related to the absence, in the heart, of a compensatory expression of dystrophin from alternative promoters.

INTRODUCTION

The dystrophin gene spans a ~3 megabase region of chromosome Xp21 and consists of at least 79 exons. The gene is characterized by a very complex developmental- and tissue-dependent regulation (1–4). It encodes for a high molecular weight cytoskeletal protein expressed primarily in skeletal and cardiac muscle and, to a lesser extent, in smooth muscle and brain tissue (5). Different isoforms are produced by alternative splicing events and by transcription initiating from at least seven distinct promoters. Three of these promoters, the brain-, muscle- and Purkinje cell-promoters, generate full length transcripts of 14 kb (reviewed in ref. 6).

Mutations of the dystrophin gene cause a severe degenerative disease, Duchenne muscular dystrophy (DMD) and its milder allelic variant, Becker muscular dystrophy (BMD). Deletions are the most common type of mutations responsible for both DMD and BMD (~65% of the cases). Almost invariably, the deletions found in DMD patients predict an out-of-frame and grossly altered product, while those found in BMD patients maintain the correct reading frame and lead to the production of a partially

*To whom correspondence should be addressed

[+]Heart Muscle Disease Study Group: Bruno Pinamonti, Gianfranco Sinagra, Andrea Di Lenarda, Furio Silvestri, Rossana Bussani and Milla Davanzo

functional protein, often also reduced in quantity (7). In addition to skeletal muscle involvement, a myocardial involvement is often present in both conditions.

Recently, it has been suggested that specific dystrophin defects could be responsible for a familial form of dilated cardiomyopathy, X-linked dilated cardiomyopathy (XLDC). This is a progressive heart disease, presenting generally with heart failure in young males in the absence of overt signs of skeletal myopathy. From the clinical standpoint, the disease in these patients does not differ from that found in other forms of familial dilated cardiomyopathy with different patterns of inheritance (8,9). In two kindred with XLDC, Towbin *et al.* (10) established linkage between the centromeric portion of the dystrophin gene and XLDC, but failed to find any specific defect in the gene. Evidence supporting the involvement of this gene was brought up by Muntoni *et al.* (11), who reported about a large deletion in the 5′ portion of the muscle isoform region, removing the muscle promoter and the first muscle exon. Similar findings were observed also by Yoshida *et al.* (12), who described a deletion in the first muscle exon and the 5′ portion of the first intron in two unrelated cases of XLDC.

In order to define the molecular basis of the disease, and to test the hypothesis of a 5′ end dystrophin gene defect in the pathogenesis of selective heart involvement, a clinical, immunohistochemical and molecular genetic study was carried out in a family with a severe form of XLDC.

RESULTS

Clinical and genetic studies

In 1987, a 24 year old male was admitted to the Cardiology Department in Trieste, Italy for a severe heart failure developed within the preceding 6 months. The diagnosis was idiopathic dilated cardiomyopathy. He was completely free of any clinical and laboratory sign of skeletal muscle disease, including increased serum creatine kinase (CK) level. Additionally, he had been a competitive basketball player for several years. He died 2 years later from post-surgical complications after heart transplantation. After several years, his brother was examined because of atypical chest pain and found to have a severely dilated and hypokinetic left ventricle. The levels of the MM isoform of creatine kinase (MM-CK) were moderately increased, without clinical evidence for muscle disease and with normal electromyography, despite some reported episodes of urine pigmentation after physical exercise and sporadic myalgias. These observations prompted us for a careful examination of the other family members. The pedigree of this family is reported in Figure 1A (the proband was individual II-1 and his brother II-2) and some of the relevant clinical and immunohistological findings of the affected and unaffected family members are reported in Table 1. The proband's mother (I-1) and the second brother (II-3) were found to be normal at physical examination, electrocardiography and echocardiography. Individual II-4 died at birth for unknown causes and individual I-2 died for a non-cardiac disease. The reported history of first- and second-degree relatives was completely negative.

The increase in serum CK in individual II-2 led us to examine the pattern of inheritance of the dystrophin gene in this family, by means of highly polymorphic microsatellite markers located within, or very close to the gene. This analysis demonstrated segregation of the same allele in the two affected brothers and the occurrence of a critical intragenic recombination in individual II-3, occurring in the interval between DYS MSB and DYS MSA (see Fig. 1B). Due to this recombination, the chromosomal region downstream of marker DYS MSA (telomeric to the muscle promoter) is shared by the unaffected (II-3) and the affected (II-1 and II-2) brothers, while the portion upstream of the marker DYS MSB (centromeric to the muscle promoter) is recombined in the healthy brother. Because of this finding, we focused our attention on the analysis of the 5′ end of the dystrophin gene, including the muscle promoter and first muscle exon.

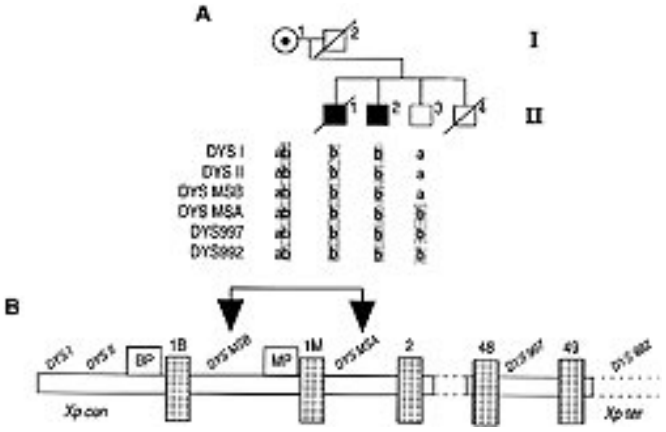

Figure 1. Pedigree of family XLDC1 and inheritance of the dystrophin gene. (**A**) Upper part: pedigree of family XLDC1. Individuals are indicated by generation and pedigree number. The affection status is indicated by solid symbols (affected), clear symbols (unaffected) and circle with a dot (carrier). Lower part: haplotypes of the family members, resulting from the analysis of six informative microsatellite markers located within or very close to the dystrophin gene. The gray boxed areas represent the haplotype cosegregating with the disease. (**B**) Schematic representation showing the genomic structure of the part of dystrophin gene that was analyzed for cosegregation. Exons are indicated by gray boxes (1B: first brain exon; 1M: first muscle exon) and promoters by clear boxes (BP: brain promoter; MP: muscle promoter). The recombination occurred in the healthy subject II-3 in the region included by the arrows, suggesting that the disease mutation could be located in the 5′ end of the gene, upstream of DYS MSA.

Mutation analysis

Sequential segments of the region including the muscle promoter, the first muscle exon and 250 bp of the first intron (13) were amplified by PCR and screened for single strand conformation polymorphism (SSCP). Individuals I-1, II-1, II-2 and II-3 were studied. The PCR products encompassing the second half of the first exon and the beginning of the first intron (nucleotides 215–324) had a different migration pattern. In individuals II-1 and II-2, two prominent bands were detectable, which were clearly different from those found in individual II-3. All four species were detected in the mother (I-1; Fig. 2A).

The nucleotide sequence of a 1387 bp region encompassing the muscle promoter, the first muscle exon and the first portion (250 bp) of intron 1 was determined. As predicted by SSCP analysis, the only mutation found was a G to T transversion at the 3′ first muscle exon-intron boundary in the genomic DNA from individual II-2. This mutation occurs at the first base of the GT dinucleotide consensus sequence of the 5′ splice site, which is absolutely conserved in all mammalian intron sequences (Fig. 2B). The unaffected brother II-3 showed a normal sequence.

Table 1. Clinical findings of affected and unaffected members of family XLDC 1

Subject	II-1	II-2	II-3	I-1
Status	Affected male	Affected male	Unaffected male	Carrier female
First symptom	Heart failure	Chest pain	–	–
Age at diagnosis (years)	24	32	35	67
Disease duration (months)	6	1	–	–
NYHA status	3	1	–	–
Serum CK (MM-CK)	Normal	242-488 (NV < 170 U/l)	–	–
ECG	Anterior-lateral 'necrosis'	Anterior-lateral 'necrosis'	Normal	Normal
Arrhythmia	Ventricular tachycardia	Ventricular tachycardia	–	–
LVEDD (cm)	7.8	6	4.9	4.6
FS (%)	9	15	37	30
EF (%)	12	34	–	72
Endomycardial biopsy	Hypertrophy, fibrosis	Hypertrophy, fibrosis, absence of dystrophin	–	–
Skeletal biopsy	–	Minimal histological changes, slight reduction of dystrophin	–	–
Follow-up	Heart transplantation	Stable	–	–

NYHA, New York Heart Association functional classification; CK, serum creatine kinase; MM, isoform; ECG, electrocardiography; NV, normal values; LVEDD, echocardiographic left ventricular end diastolic diameter; FS, fractional shortening; EF, ejection fraction.

The G to T mutation detected in individual II-2 introduces a new restriction site for *Mse*I. As expected, the restriction analysis of a PCR product encompassing the mutation site generates a single undigested band (of 110 bp) from the unaffected individual II-3 and two digested bands (of 60 and 50 bp respectively) from the two affected brothers II-1 and II-2, showing complete segregation of the mutation with the disease. Accordingly, three bands corresponding to the wild type and to the mutated alleles, were detected after restriction of the PCR product generated by the amplification of the genomic DNA from the mother (Fig. 2C).

Expression of dystrophin in heart and skeletal muscle

Analysis of dystrophin expression was performed in left ventricular endomyocardial and skeletal muscle biopsies obtained from individual II-2.

Histological examination of the skeletal muscle biopsy showed a mild variability in fiber size with the presence of mildly scattered atrophic fibers, rare splittings and no increase in interstitial connective tissue. The number of internal nuclei was slightly augmented. At immunocytochemistry with antibodies directed toward the N-terminus, mid-rod and C-terminus of dystrophin, all fibers appeared continuously labelled, but the intensity of fluorescent labelling was paler than in control muscle (Fig. 3A–C).

The endomyocardial biopsy showed a severe fibrosis and a marked variability of fiber size. Contrary to the pattern detected in the skeletal muscle, no immunoreactivity was found with antibodies directed toward the N-terminus and mid-rod region of dystrophin, while only a weak reactivity at the periphery of cardiomyocytes was observed using a C-terminal anti-dystrophin antibody (Fig. 3D and E).

The analysis of expression of the dystrophin mRNA was performed on total RNA isolated from skeletal and endomyocardial biopsies and reverse transcribed using random hexanucleotide primers. PCR amplification of this cDNA was performed using a muscle isoform-specific forward primer and a reverse primer located in exon 2 (Fig. 4A). No cDNA corresponding to the muscle isoform could be amplified either from the muscular or the cardiac tissues of the patient. Additionally, no PCR products of larger size (suggestive of the usage of alternative cryptic splice sites) were obtained, even after extended PCR cycling (not shown). When forward primers specific for the brain- and the Purkinje cell-dystrophin mRNA isoforms were used, clear amplification products were observed with the cDNA obtained from the muscle of the patient, but not from the heart (Fig. 4B). When primers specific for the Dp71 transcript were used (14), amplification was obtained for the cDNAs from the heart samples from both the control and the patient. The presence of the Dp71 mRNA (encoded by the 3′ region of the dystrophin gene (14,15), is consistent with the weak reactivity detected in the heart sample of the patients with antibodies directed exclusively against the C-terminal of the protein.

DISCUSSION

The present study reports the identification of a splice-site point mutation at the 5′ end of the dystrophin gene, responsible for dilated cardiomyopathy in a family. Our findings, in agreement with other studies (10–12), indicate a specific involvement of the 5′ end of the dystrophin gene in determining isolated heart 'dystrophy'. It should be further stressed that the phenotype examination of the two affected family members did not reveal signs of skeletal muscle disease and, unlike all previously reported cases (10–12), the proband (II-1) had constantly normal creatine kinase values. On the other hand, clinical investigations were indicative of a severe myocardial dilatation and dysfunction.

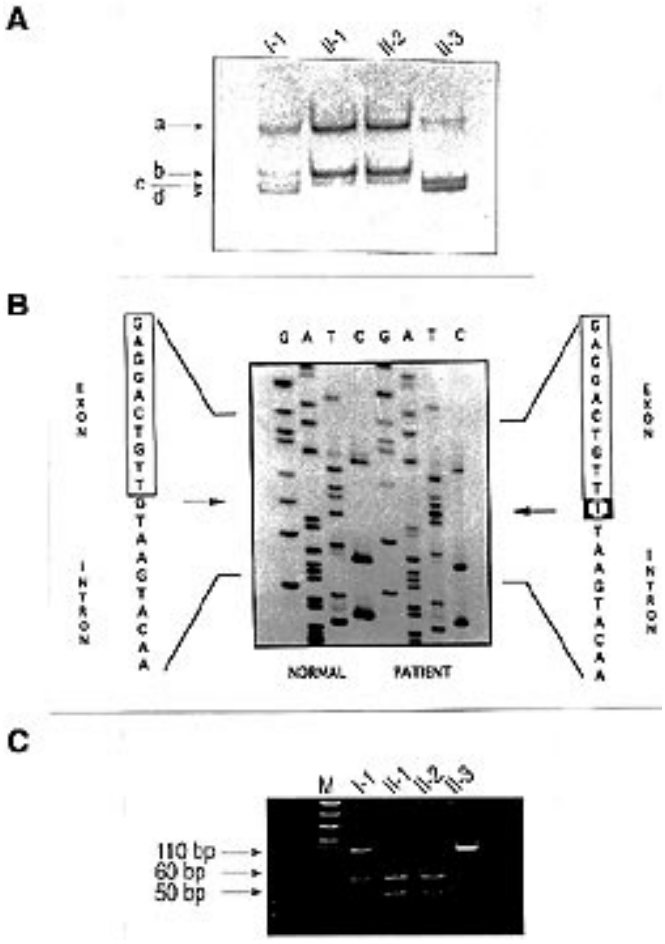

Figure 2. Mutation analysis. (**A**) SSCP analysis of the PCR product encompassing the first muscle exon-intron junction. Individual ssDNA strands are indicated by arrows (a–d). Individuals II-1 and II-2 (affected) carry the same dystrophin allele, that is different from the one of individual II-3 (normal). The mother (I-1) is heterozygous, as expected. (**B**) Sequence analysis of the first muscle exon-intron junction of dystrophin gene. In the patient II-2, a G→T point mutation eliminates the 5′ splice site consensus sequence. The normal control is represented by the healthy brother (II-3). (**C**) Restriction analysis of PCR products from individuals of family XLDC1. The G→T point mutation introduces a new restriction site for *Mse*I. The *Mse*I digestion analysis showed an undigested fragment of 110 bp corresponding to the normal allele (healthy individual II-3 and carrier I-1) and two digested fragments of 60 and 50 bp, corresponding to the mutant allele (affected individuals II-1, II-2 and carrier I-1).

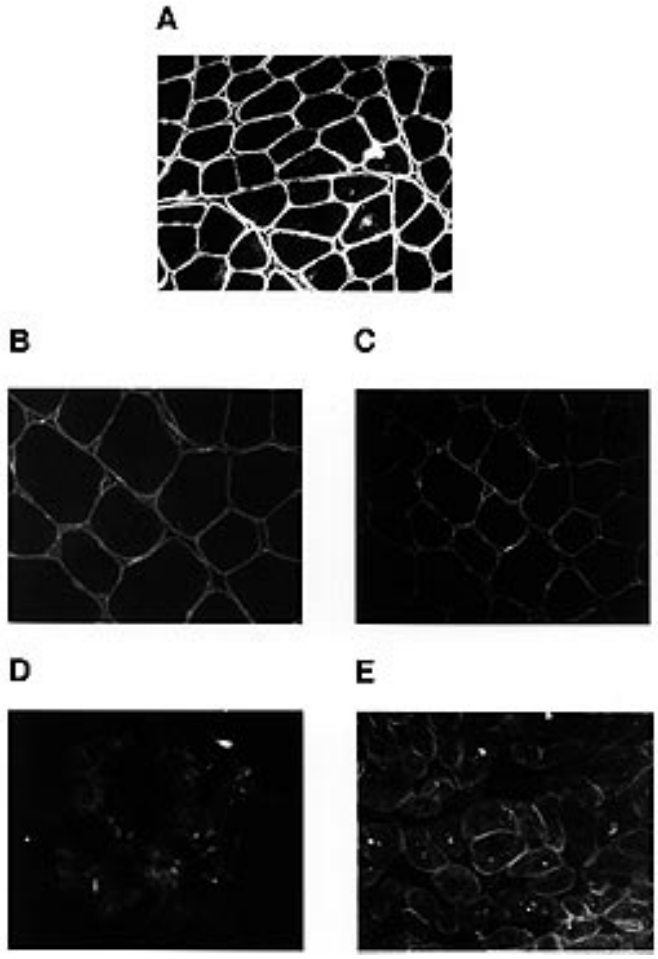

Figure 3. Immunocytochemistry of skeletal and heart muscle. Cryostat sections of skeletal muscle from a normal individual (**A**), were immunolabelled with antibodies to C terminal region of dystrophin. The cryostat sections of the patient's skeletal muscle were labeled with antibodies against both N- (**B**) and C- terminus (**C**). These antibodies show reduced but structurally preserved staining of the muscle of the patient as compared to that of the normal control (×180). The cryostat section of the cardiac muscle from the patient immunostained with antibodies to the N-terminal (**D**) shows complete absence of dystrophin (×250). Only a weak reactivity could be detected with antibodies against the C-terminus of the protein (**E**) (×250).

Standard multiplex PCR method (16,17) failed to detect any typical deletion of the dystrophin gene in these patients. However, the selective involvement of the dystrophin gene 5′ end was strongly suggested by the finding of a critical recombination in the unaffected subject II-3, detected by haplotype analysis. Sequence analysis of the DNA fragment corresponding to the first muscle exon-intron junction demonstrated a single base substitution. This mutation replaces the invariant G at the position +1 of the splice donor sequence G_{100} T_{100} A/G_{95} A_{70} G_{80} T_{45} [where the numbers indicate the percentage of nucleotide conservation (18)], with a T. Since the G at position +1 of the 5′ splice site consensus sequence is completely conserved in eukaryotes, this mutation is undoubtedly affecting mRNA maturation. The abolition of the 5′ splice site of the very large first intron [>200 kb (21)] predicts the production of an unstable mRNA, with consequent absence of the muscle isoform expression. The utilization of an alternative cryptic splice site located at a downstream position of the first intron could not be detected by RT-PCR amplification, neither in heart nor in muscle samples, using a pair of primers located in the first and second exons. Although this does not exclude that an aberrant mRNA is produced longer than the maximum PCR amplification limit, this would be expected to lead to the production of a grossly altered protein.

The discrepant clinical phenotype of the patients (apparent normal skeletal muscle and selective heart involvement) is consistent with the normal distribution of dystrophin in skeletal muscle biopsy, where only its quantitative reduction could be suggestive of a dystrophinopathy, in contrast to the absolute absence of the protein in the heart. Production of the protein is

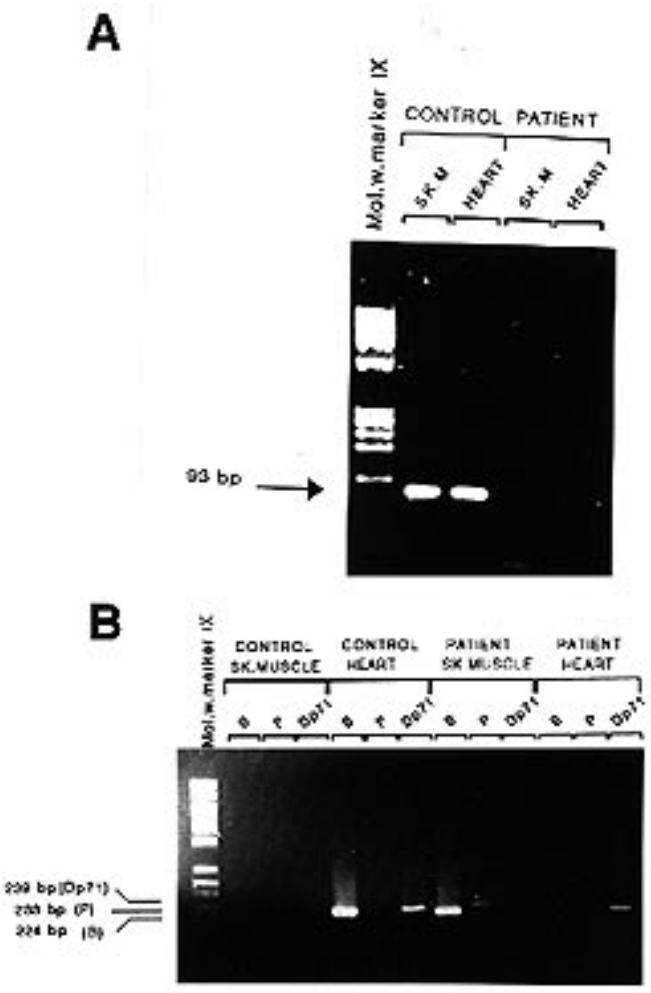

Figure 4. RT-PCR of dystrophin mRNAs. (**A**) Amplification of the muscle isoform of dystrophin. The 93 bp band corresponding to the amplification of the muscle isoform was obtained in normal skeletal and cardiac muscles, but not in the corresponding tissues from the II-2 patient. (**B**) Amplification of the brain (B), Purkinje-cells (P) and Dp71 dystrophin isoforms from skeletal and heart muscle of a normal individual and of patient II-2. In the control individual, transcription of the brain isoform could be detected in the heart, while it was completely absent in the heart of the patient. On the contrary, both the brain and Purkinje-cells transcripts could be obtained from the skeletal muscle of the patient. In both the patient's and the control's hearts, amplification from the Dp71 cDNA could be obtained.

likely to be sustained in the muscle by translation of the brain- and the Purkinje cell-isoform mRNAs. Thus, it is conceivable that, in the skeletal muscle, the absence of the muscle dystrophin isoform can drive a compensatory overexpression of the other full-length isoforms, able to prevent the myopathy in the affected males. Surprisingly, transcription of these isoforms is absent in the heart, where even the normal expression of the brain mRNA isoform is suppressed. This finding is consistent with the selective cardiac disease in these patients. These observations point to the existence of selective mechanisms of regulation of transcription initiation at the different dystrophin promoters in these two tissues. It is still unclear by which mechanisms the detected single point mutation, that is expected to act primarily at the post-transcriptional level, is able to exert such effects on the dystrophin promoters. Similar findings have been recently reported for another family with XLDC, characterized by a large deletion in the 5′ end of the gene (19). In this case, it was hypothesized that deletions involving the muscle promoter, the first muscle exon and part of the intron 1, could remove a distinct cardiac enhancer responsible for transcription also from the brain and Purkinje cell promoter (12,19). However, the present report does not support this idea. In fact, it would be extremely unlikely that a putative cardiac specific enhancer is situated at the intron-exon splice site and that a point mutation at this level could completely knock out its function. Thus, the molecular reasons why the compensatory transcription mechanism from the brain and Purkinje cell promoters is occurring in the skeletal muscle but not in the myocardium still remain to be elucidated. Additionally, it is also intriguing to observe that, in other families, the deletion of the first muscle exon of dystrophin is associated with a clear muscular dystrophy phenotype of different severity (20–23). A better understanding of the genetic differences between these cases will await the definition of the exact boundaries of these deletions, of the transcriptional patterns of dystrophin in these patients and the identification of the *cis*-elements involved in transcriptional control of the different gene isoforms.

In the heart of the patient analyzed in this study, as well as in patients of another family with XLDC (24), the only detectable dystrophin mRNA was the one coding for Dp71, indicating that there is no transcriptional interference between the 5′ promoters and the Dp71 promoter. Recent published work on transgenic animals indicates that the expression of Dp71 fails to compensate for the absence of the dystrophin in the skeletal muscle (36,37). These data are in accordance with the presence of a severe cardiac impairment in our XLDC patients despite Dp71 expression in the heart. However, since the *mdx* mouse does not necessarily represent the best model to study the involvement of dystrophin in heart muscle disease, it is still possible that the presence of Dp71 could play a role in XLDC, partially compensating for the absence of the major isoforms, thus delaying the onset and mitigating the progression of the disease.

In conclusion, the data presented in this work define for the first time a precise point mutation at the 5′ end of the dystrophin gene as responsible for a selective dilated cardiomyopathy. Since the most apparent phenotype for this mutation is the absence of the muscle dystrophin isoform mRNA, it appears that expression of this isoform is essential for the heart function. Therefore, it is conceivable that, in addition to splice junction defects, also different types of mutations resulting in altered levels of this mRNA isoform could lead to selective heart myopathy, since its absence can not be compensated in the heart by transcriptional initiation at alternative promoters.

MATERIALS AND METHODS

Family studies

A detailed history of the family was taken and all the family members included in the study were evaluated with physical examination, resting electrocardiogram, M-mode and cross sectional echocardiography, as previously described (8). Normal values for echocardiographic measurements were determined according to standard protocols (25). Relatives showing signs of cardiomyopathy (II-1 and II-2, Fig. 1) underwent a comprehensive clinical evaluation including ventriculography, coronary

angiography and endomyocardial biopsy. Patient II-1 underwent also neurological evaluations, including electromyography and skeletal muscle biopsy.

Histology and immunochemistry

A needle biopsy of skeletal muscle and a left ventricular endomyocardial biopsy were obtained from individual II-2, after informed consent. The samples were studied according to standard techniques (26), including immunocytochemistry using a biotin-streptavidin Texas-red method. Six micron unfixed cryostat sections were immunostained using a panel of antibodies to dystrophin (Novocastra Laboratories, Newcastle-upon Tyne, UK), as described (11).

Reverse transcription and PCR

Total RNA was isolated from frozen samples according to a published procedure (27) and cDNA synthesis was performed using random hexanucleotide primers. PCR was carried out in a reaction volume of 25 µl containing the cDNA template (2 µg) and oligonucleotide primers designed to amplify the muscle isoform of dystrophin (forward oligonucleotide in the first muscle exon and reverse oligonucleotide located in exons 2 or 4) and the Dp71 transcript (19,24). PCR reactions (25 µl) utilized 0.5 U *Taq* DNA polymerase, 0.25 µM each primer, 200 µM each dNTP, in 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$. After 10 min denaturation at 94°C, PCR amplification was carried out with the following cycle profile: denaturation at 94°C for 30 s; annealing at 58°C for 60 s; extension at 72°C for 120 s; 26 cycles. Nine microlitres of the reaction were resolved on 2.5% agarose gels containing 0.2 g/ml of ethidium bromide.

Genetic studies

DNA samples from individuals I-1, II-2 and II-3 (Fig. 1) were extracted according to the salting out procedure (28), either from fresh blood samples or from continuous B-lymphocyte cell lines immortalized with the Epstein–Barr virus (29). In the case of the deceased patient II-1, DNA was extracted from a paraffin-embedded myocardial tissue sample (30).

The screening for deletions of the dystrophin gene was performed using multiplex PCR, according to methods described elsewhere (16,17). Amplifications of the first muscle exon, muscle promoter, brain promoter and second exon were carried out as reported (2,13,21,31).

For the haplotype analysis, the polymorphic microsatellite sequences (CA repeats) DYS-I, DYS-II, DYS-III, DYS MSB, DYS MSA, DXS 997, DXS 992 were PCR amplified using described conditions (32–35). PCR products were resolved by a long run on a 10–12% polyacrylamide native gel and visualized by ethidium bromide staining.

PCR-SSCP analysis

For point mutations screening, the region including the muscle promoter, the first muscle exon and 250 bp of the first intron (13) were amplified by PCR using serial pairs of primers to obtain products of ~100–300 bp. PCR amplifications were carried out in 50 µl of a solution containing Tris-HCl 10 mM (pH 8.0), KCl 50 mM, $MgCl_2$ 1.5 mM, gelatin 0.01%, each dNTP 200 mM, each primers 0.1 µM, 200 ng of template DNA and 2.5 U of *Taq* DNA polymerase (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ), with 40 repetitions of the following cycle: 30 s at 94°C, 30 s at the annealing temperature and 30 s at 72°C. Annealing temperatures varied from 56 to 62°C, according to the different primer pairs. The PCR product encompassing the 3′ end of the muscle first exon and the beginning of intron 1 was obtained using the following primers: forward primer, 5′-TATCGCTGCCTTGATATACA-3′; reverse primer, 5′-ACTAAACGTTATGCCACAGT-3′, with an annealing temperature of 60°C. All the oligonucleotides were synthesized by the ICGEB Oligonucleotide Synthesis Service on a Applied Biosystem 380B synthesizer.

PCR-SSCP samples were prepared by denaturing 5 µl of PCR product with 7 µl of formamide dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) at 94°C for 8 min. After cooling in ice, the samples were quickly loaded on 10–20% non-denaturing polyacryamide gels with or without glycerol and run for 12–16 h at room temperature or at 4°C. DNA fragments on SSCP gels were visualized by silver staining using a commercial procedure (BioRad, Richmond, VI).

Cloning and sequencing of PCR products

The PCR amplified fragments were eluted from polyacrylamide gels and directly cloned in a commercial vector (TA Cloning Kit, Invitrogen Corporation, San Diego, CA). Sequence analysis was performed on plasmid DNA extracted from at least three individual bacterial clones by the dideoxinucleotide chain termination method using a T7-based DNA sequencing kit (Pharmacia, Uppsala, Sweden). Sequence data were obtained for both strands of the insert by extension of the universal and reverse primers of the vector, as well as by using internal primers.

Since the mutation found at the 5′ splice site of the first dystrophin intron predicts the generation of a new restriction site for *Mse*I, the 110 bp fragment encompassing the mutation was digested with this enzyme to produce two fragments of 60 and 50 bp, that were visualized following electrophoresis on an ethidium bromide-stained 8% polyacrylamide gel.

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ABBREVIATIONS

XLDC, X-linked dilated cardiomyopathy; DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy; CK, creatine kinase; SSCP, single strand conformation polymorphism; PCR, polymerase chain reaction; NTP, nucleotide triphosphate.

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