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Transcription of the Dystrophin Gene in Normal Tissues and in Skeletal Muscle of a Family with X-Linked Dilated Cardiomyopathy

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Summary

We recently described a family where a deletion of the dystrophin gene was associated with a severe dilated cardiomyopathy without skeletal muscle weakness. The deletion removed the muscle promoter region and the first muscle exon, but not the brain or Purkinje-cell promoters. Dystrophin was detected immunocytochemically in the skeletal muscle from this family, despite the fact that the deletion eliminated the transcriptional start site of the muscle isoform. In order to determine which promoter was driving dystrophin transcription in skeletal muscle of these individuals, we first evaluated the expression of the exon 1 of muscle, brain, and Purkinje-cell isoforms in normal human skeletal and cardiac muscles and in mouse brain and cerebellum. Our data indicate that, with the exception of minimal expression of the brain isoform, only the muscle isoform is significantly transcribed in skeletal muscle, whereas both the exon 1 muscle and brain isoforms are highly expressed in cardiac muscle. In contrast to what is observed in normal muscle, the skeletal muscle of our patients showed expression of both the brain and the Purkinje-cell isoforms. The overexpression, in skeletal muscle, of these two isoforms thus appears to be of crucial importance in preventing a myopathy in these affected males. The reason for the severe cardiomyopathy remains speculative, in the absence of dystrophin data on their heart. However, we have found in the 5′ end of intron 1, a region deleted in our cases, regulatory sequences that might be of importance for dystrophin expression in various tissues. It is also possible that the deletion present in this family affects specifically one of the two dystrophin actin-binding domains.

Introduction

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are both due to mutations that affect the production of the cytoskeletal protein dystrophin. Numerous dystrophin isoforms driven by various promoters have been described (for review, see Ahn and Kunkel 1993), but only three—namely, the muscle type, the brain type, and Purkinje-cell-type—are the result of transcription of a full-length (14 kb) dystrophin cDNA. A specific promoter, linked to one unique first exon, drives transcription for each of these three isoforms in a rather tissue-specific way. In particular, the muscle isoform was found to be mainly active in skeletal, cardiac, and smooth muscle (Chelly et al. 1990; Bies et al. 1992), although several studies have indicated that it was also active, to a lesser extent, in the CNS (Nudel et al. 1988, 1989; Feener at al. 1989; Chelly et al. 1990). Although initial in vitro data obtained in cultured neuronal and glial cells have indicated that its expression in these cell types was confined to glial cells (Barnea et al. 1990; Chelly et al. 1990), more recently, in situ studies have shown that the muscle isoform is present in vivo in cortical and hippocampal neurons. (Górecki et al. 1992). The brain isoform was initially found to be highly specific to neurons (Chelly et al. 1990), while subsequent studies have indicated that it might also be expressed in the cardiac (Bies et al. 1992) and skeletal muscle (Boyce et al. 1991; Geng et al. 1991), although its expression in skeletal muscle is still controversial (Tamura et al. 1993). Since the majority of these expression studies were performed using PCR analysis without quantitation of the amplified products, the possibility that some of these results were due to "illegitimate transcription" (i.e., low-level transcription of tissue-specific genes in noncognate tissues), rather than true expression, has been raised (Chelly et al. 1990). The recently described Purkinje-cell isoform is transcribed in the cerebellar Purkinje neurons (Górecki et al. 1992), but the pattern of its expression outside the CNS has not been characterized yet.

We recently described a family in which a deletion removing the entire muscle promoter region and the first muscle exon, but not the brain and Purkinje-cell promoters and first introns, was associated with a severe dilated cardiomyopathy (Muntoni et al. 1993). In this family it was unequivocally demonstrated that a mutation in the dystrophin gene could give rise to a "pure" cardiac phenotype, without any symptoms suggestive of skeletal muscle

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involvement. The finding of an X-linked dilated cardiomyopathy (XLDCM) secondary to a deletion removing the entire muscle promoter region and the first muscle exon was surprising: the lack of the transcriptional start site should impair transcription in the skeletal muscle. In addition, the deletion of the first muscle exon produces a frameshift. For these two reasons alone the affected males should have been affected by DMD. However, the absence of clinical skeletal muscle involvement, along with the finding of a relatively strong immunoreactivity by using a panel of anti-dystrophin antibodies in the skeletal muscle of the proband, was a clear indication that an in-frame message was produced. Since the entire muscle promoter region was missing in these affected males, there had to be another promoter able to drive transcription in their skeletal muscle. In addition, the discrepancy between the skeletal and cardiac muscle involvement in the XLDCM family had still to be explained. Preliminary data suggested that at least part of the transcription detected in the skeletal muscle of this family was due to the brain isoform (Muntoni 1994). However, in order to have a better characterization of the transcription pattern in this family, we first had to characterize in detail the expression of the muscle, brain, and Purkinje-cell isoforms in normal skeletal and cardiac muscle and in cerebral cortex and cerebellum. We then studied dystrophin expression in the skeletal muscle of two affected brothers from the family with XLDCM.

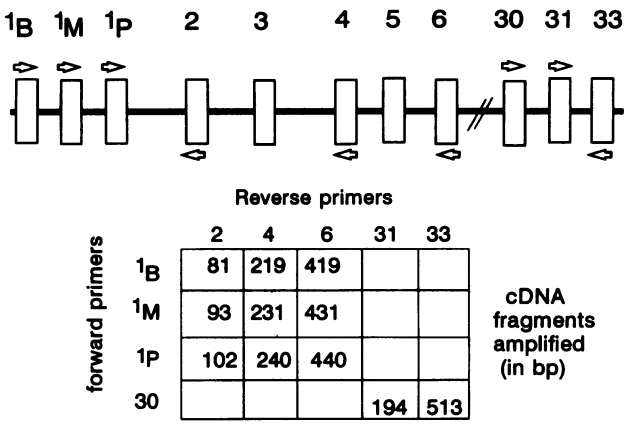

Figure 1 Schematic representation of part of dystrophin cDNA. Boxes represents exons, and the number above each box indicates the exon number; 1_B = first brain exon; 1_M = first muscle exon; and 1_P = first Purkinje-cell exon. The arrows below the boxes indicate the orientation of the various primers used. Primers sequences (5′–3′) were as follows: B1, AAAACAGCTGGCATGGAAGATG; M1, ATGCTTTGGTGGGAAGAAGTAGAG; P1, CAGCCTCCGCAGAATTTGAAATG; M2, CTTAGAAAATTGTGCATTTACCCA; M4, ACATTGTTCAGGGCATGAACTCT; M6, ACCCAGCTCAGGAGAATCTTTTCA; M30, GAGGCTGTAGGAGGCAAAAGTTG; M31, ATCCAATCTGATTTGACAAGTCAT; and M33, GACGGAAAATCCCAAAGAACTTGA. All PCR primers were derived from the human cDNA sequence, with the exception of the human brain promoter sequence (i.e., B1) (Boyce et al. 1991) and human Purkinje-cell promoter sequence (i.e., P1) (Górecki et al. 1992). In the bottom part of the figure, the molecular size of the amplified regions is indicated.

Patients and Methods

Patients

The family with XLDCM has been reported elsewhere (Muntoni et al. 1993). The transcription studies reported in the present paper were performed on the skeletal muscle from two affected brothers (II-1 and II-6; Muntoni et al. 1993). One boy with DMD (with an out-of-frame deletion removing exon 44) and two with BMD and an in-frame deletion in the central dystrophin gene region (exons 45–48 and 37–44, respectively) were also studied.

Methods

Reverse transcription and PCR.—Total RNA (Chomczynski and Sacchi 1987) was isolated from frozen skeletal muscle biopsies of patients who had a needle muscle biopsy as part of the diagnostic procedure. Frozen right-ventricular-wall cardiac muscle was obtained from individuals who underwent cardiac transplantation for coronary artery disease. Mouse heart, cerebral cortex, and cerebellum were also studied. The cDNA synthesis was performed using random hexanucleotide primers, following the procedure described elsewhere (Muntoni and Strong 1989; Sherratt et al. 1993). PCR was performed essentially as described by Saiki et al. (1988), in a reaction volume of 25 μl containing the cDNA template (2 μg) and oligonucleotide primers designed to amplify the brain, the muscle, and the Purkinje-cell isoforms, using either an exon 2 reverse primer, an exon 4 reverse primer, or an exon 6 reverse primers. Exons 30–31 or 31–33 were coamplified in the same tube, as discussed below. In figure 1 a schematic representation of the various regions amplified, together with the molecular weight of the amplified product, is shown. PCR reactions (25 μl) utilized 0.5 units *Taq* polymerase, 0.25 M each primer, and 200 m each dNTP, in 10 mM Tris-HCL (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. Amplification conditions were 94°C denaturation (30 s), 58°C annealing (60 s), and 72°C extension (120 s), for 35 cycles. Ten microliters of the reaction products were analyzed on 3% agarose gels containing 0.2 g ethidium bromide/ml, prior to photography. Radioactive PCR (see below) was performed as described above but included 25 μCi of α^{32}P-dCTP and was for 22 cycles only.

Expression of the three dystrophin isoforms.—In order to have a semiquantitative estimate of the transcription derived from the various promoters in the tissues studied, coamplification of both one of the dystrophin promoters and a more 3′ region of dystrophin cDNA was performed in the same tube. The primer combination that gave the more reproducible result in terms of linear amplification ratio between the two products was exons 30-31 coampli-

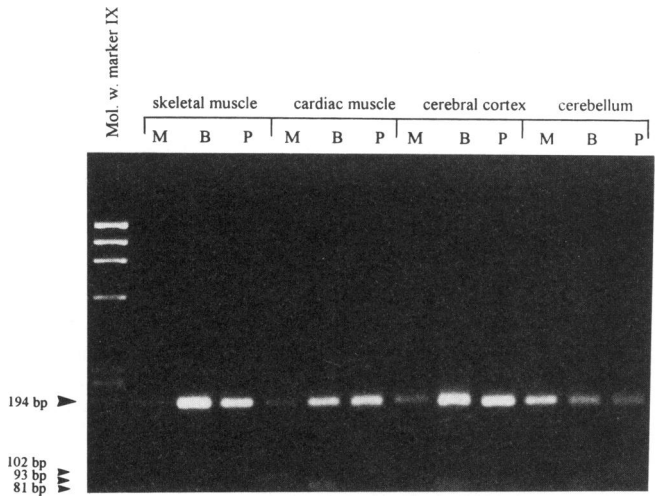

Figure 2 Amplification of the cDNA from normal skeletal and cardiac muscle and from cerebral cortex and cerebellum, using the three promoter-specific forward primers and a reverse primer located in exon 2. The muscle isoform (M) was amplified as a 93-bp fragment, the brain isoform (B) as an 81-bp fragment, and the Purkinje-cell isoform (P) as a 102-bp fragment. Exons 30–31 were coamplified in each reaction tube ("194 bp" arrow). Molecular-weight marker IX was obtained from Boehringer. The agarose gel is stained with ethidium bromide.

fication with exon 4 as reverse primer to amplify the three isoforms. Coamplification of exons 30-33 with exon 6 as a reverse primer to amplify the three isoforms was also satisfactory. The radioactive amplification was interrupted after 22 cycles, in the exponential phase of the procedure. A 10-μl portion of the reaction mixture was electrophoresed in a 6% nondenaturing polyacrylamide gel. The gel was then autoradiographed. The ratio of the band corresponding to the amplified promoter versus that of the control region was established in a panel of control tissues (three human normal skeletal muscles, two human hearts, and cerebral cortexes and cerebellum from three mice) after a scanning with a computing densitometer (Vidas system; Kontron Electronics). The same procedure was then applied to the patients' cDNA.

Results

Normal Human Skeletal Muscle

Amplification of the cDNA from normal skeletal muscle, using the three isoform-specific forward primers and a reverse primer located in exon 2, is shown in figure 2. The muscle isoform was apparently the only one transcribed in the normal skeletal muscle. When radiolabeled PCR was performed, and the reverse primer located in exon 4 used, a faint but consistent signal corresponding to the brain isoform was also detected (fig. 3, *top left panel*). No product corresponding to the Purkinje-cell isoform was seen in the skeletal muscle after amplification performed in the exponential phase. The results of densitometric analysis regarding the ratio of the amplified products corresponding to the three isoforms and to the internal control region obtained from three normal adult human skeletal muscles are shown in table 1.

Normal Human Cardiac Muscle

The rate of transcription of the muscle isoform in normal human heart was similar to that obtained in the skeletal muscle (fig. 3, *top left panel,* and table 1). In addition, the brain isoform was found to be also significantly expressed in the heart, although the level of its amplification was lower than that detected in brain (fig. 3, *top left panel,* and table 1). The Purkinje-cell isoform was not amplified in the cardiac muscle.

CNS

The brain isoform was found to be highly expressed in mouse cerebral cortex. A faint band, corresponding to the muscle isoform, was also detected (fig. 3, *bottom left panel*). We could not detect any signal corresponding to the Purkinje-cell isoform in the cerebral cortex (table 1).

As far as the mouse cerebellum was concerned, no transcription of the muscle isoform was detected (table 1). The rate of amplification of the brain isoform was very similar to that detected in the cerebral cortex; the Purkinje-cell isoform was also significantly transcribed in this tissue (fig. 3, *bottom left panel,* and table 1).

XLDCM Family

As expected, no transcription derived from the deleted muscle isoform was detected in the XLDCM family. A strong signal was, however, found when either the brain or Purkinje-cell isoform was amplified (fig. 3, *top right panel*). When the ratio of these two isoforms versus the internal control region was evaluated, levels of amplification were found to be very similar to those detected in the organs where they are maximally active (e.g., cerebral cortex and cerebellum) (table 1). Virtually identical results were obtained by studying the brain and Purkinje-cell isoforms transcription from the two affected brothers (not shown).

Patients with DMD or BMD

No difference in transcription of either the brain or the Purkinje-cell isoform was found in these patients, compared with normal muscle; a clear signal from the control exons (30–31) was detected in all three patients (not shown). In the BMD patient with a deletion of exons 45–48, the ratio of the control exons versus the muscle isoform was suggestive of a mild up-regulation of the muscle isoform (fig. 3, *right-hand panel*).

Discussion

The recent finding that a deletion of the muscle promoter and first muscle exon was responsible for a dilated cardiomyopathy in one family raised several important

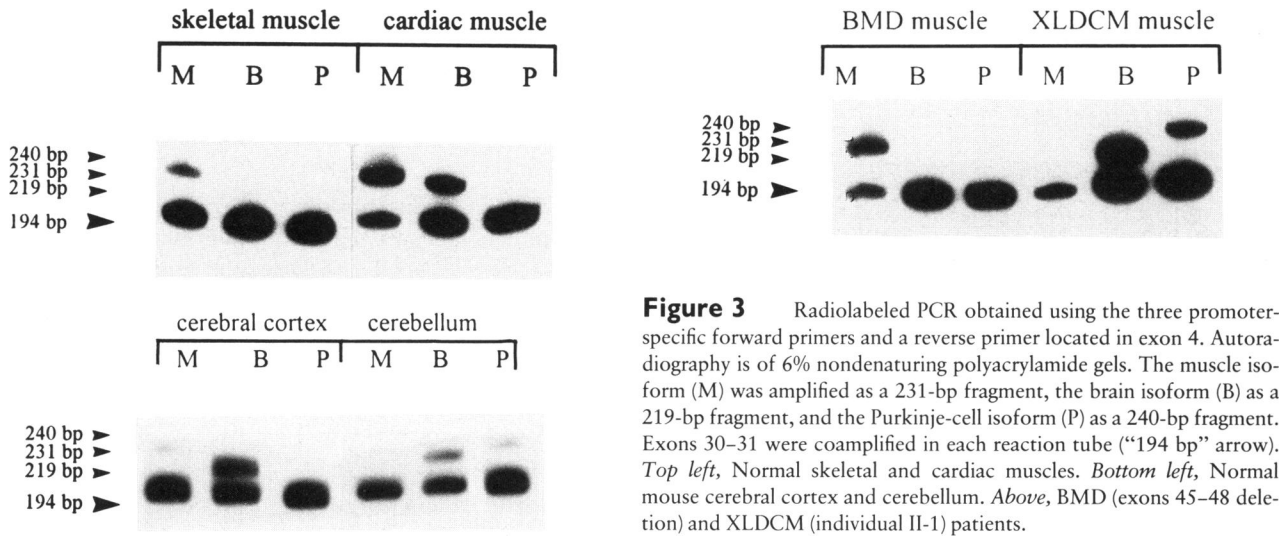

Figure 3 Radiolabeled PCR obtained using the three promoter-specific forward primers and a reverse primer located in exon 4. Autoradiography is of 6% nondenaturing polyacrylamide gels. The muscle isoform (M) was amplified as a 231-bp fragment, the brain isoform (B) as a 219-bp fragment, and the Purkinje-cell isoform (P) as a 240-bp fragment. Exons 30–31 were coamplified in each reaction tube ("194 bp" arrow). *Top left,* Normal skeletal and cardiac muscles. *Bottom left,* Normal mouse cerebral cortex and cerebellum. *Above,* BMD (exons 45–48 deletion) and XLDCM (individual II-1) patients.

questions. First, why do these patients escape a severe myopathy? Second, why do they develop a severe cardiomyopathy?

A detailed characterization of the transcription of the various isoforms of dystrophin in a variety of tissues was essential for answering the first question. As far as the transcription of the muscle isoform is concerned, our results confirm what has already been described by the observation of other authors: that high levels are found in normal skeletal and cardiac muscles (Chelly et al. 1990; Bies et al. 1992) but only trace amounts are in the cerebral cortex (Barnea et al. 1990; Chelly et al. 1990) and none are in the cerebellum. Moreover, we found relatively high levels of transcription of the brain isoform, not only in the cerebral cortex and cerebellum but also in the cardiac muscle, as recently reported by Bies et al. (1992). Regarding this latter finding, both the high levels of the amplified product detected by us in ventricular tissue and the study by Bies et al., which found no transcription of this promoter in mRNA isolated from cardiac Purkinje conduction fibers but which did find it in mRNA from the whole heart (Bies et al. 1992), suggest that the brain isoform is coexpressed with the muscle isoform in the cardiomyocytes. We also detected low levels of the brain isoform in the normal skeletal muscle, similar to what had been found by others (Boyce at al. 1991; Geng et al. 1991). There is no information on which cells express the brain isoform in the skeletal muscle; this might result from a low level of expression either (*a*) in all skeletal muscle fibers or in a proportion of them with a specialized function, such as the intrafusal fibers, or (*b*) in the smooth muscle cells contained in the intramuscular blood vessels. If the brain isoform is not expressed evenly in all muscle fibers, sampling differences between distinct areas of muscles studied might explain some of the discrepancies found in the literature regarding its detection (Chelly et al. 1990; Boyce et al. 1991; Geng et al. 1991; Tamura et al. 1993).

We found transcription of the Purkinje-cell isoform to be confined to the cerebellum, confirming the recent data of Górecki et al. (1992), who found expression of this isoform exclusively in the cerebellar Purkinje-cells, using in situ hybridization. We found no transcription of this iso-

Table 1

Summary of Densitometric Scanning Results

Tissue	Muscle Isoform/Exons 30–31	Brain Isoform/Exons 30–31	Purkinje-Cell Isoform/Exons 30–31
Human skeletal muscle	28.3 (3.3)/71.8 (1.8)	4.3 (1.8)/94.8 (9.2)	0/100
Human cardiac muscle	38 (14.5)/65 (15.3)	22.2 (4.2)/70.2 (7.5)	0/100
Mouse cerebral cortex	6.6 (5.4)/88 (12.3)	35.5 (6.6)/66 (5.3)	.3 (0.3)/98.9 (6.9)
Mouse cerebellum	.3 (0.4)/99.5 (.7)	41.2 (8.2)/62.7 (6.9)	10.4 (2.2)/89.2 (4.6)
XLDCM skeletal muscle	0/100	40.3 (5.5)/58.6 (5.6)	11.7 (2.6)/87 (4.5)

NOTE.—Each value represents the mean of the optical density of the peaks derived from at least three separate experiments. These values are expressed as percentage of the peak density of the various dystrophin isoforms (amplified using an exon 4 reverse primer), versus the exon 30–31 peak. Values in parentheses are SDs. Regarding the XLDCM muscle, the experiments obtained in the two affected brothers were pooled and analyzed together.

form in normal skeletal muscle, normal heart, and cerebral cortex.

When we studied the pattern of dystrophin transcription in the skeletal muscle of two members of the XLDCM family, we found that both the brain and the Purkinje cell isoforms were present in high abundance. Our analysis suggests that levels of expression of these two isoforms were as high as those detected in the tissues in which they are maximally active, namely, the cerebral cortex and the cerebellum, respectively. These data on the transcription of brain and Purkinje-cell isoforms in skeletal muscle of the XLDCM patients do not support the hypothesis that a fourth, cryptic promoter is activated by mutations affecting the muscle promoter region of dystrophin.

Deletion of the first muscle exon has rarely been reported before in DMD/BMD. The phenotype observed in such patients can vary from a mild phenotype (Beggs et al. 1991; Boyce et al. 1991; Yoshida et al. 1993; present study) to a severe DMD phenotype (Malhotra et al. 1988; Bulman et al. 1991). We have no explanation for the discrepancy between the clinical severity observed as a result of an apparently similar deletion. It should be noted, however, that the latter patients with DMD (Malhotra et al. 1988; Bulman et al. 1991) were not screened for the deletion of the brain or Purkinje-cells promoters, which might also have been affected. Indeed, one patient, reported more recently, with a deletion involving both the muscle and brain promoter had a severe phenotype (den Dunnen et al. 1991). Since this individual was believed to be within the spectrum of mild DMD (since he was alive at the age of 32 years), it would be interesting to see whether the Purkinje-cell promoter was able to drive some dystrophin transcription in his skeletal muscle. In the few patients with a deletion removing all three promoters (brain promoter-exon 18 [Rapaport et al. 1992] and brain promoter-exon 6 [Muntoni et al. 1994]), a typical, severe DMD phenotype is invariably observed.

In order to determine whether the up-regulation of the brain and Purkinje-cell isoforms was a nonspecific event occurring in other patients with Xp21 muscular dystrophies, we studied the pattern of dystrophin transcription in the skeletal muscle of two BMD patients and one DMD patient. The results observed in these individuals were very similar to those found in normal skeletal muscle: although one BMD (deleted for exons 45–48) apparently showed a mild up-regulation of the first exon muscle isoform, none showed up-regulation of the brain and Purkinje-cell isoforms. These findings indicate that the transcription in the skeletal muscle of the brain and Purkinje-cell isoforms is not a general finding in patients with Xp21 muscular dystrophy. One possible explanation for the specificity of the phenomenon observed in the XLDCM family is that the deletion removes negative regulatory elements that have already been described in the muscle promoter region (Gilgenkrantz et al. 1992). Alternatively, up-regulation of the

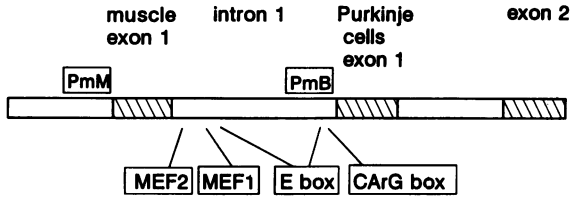

Figure 4 Schematic representation of various cis-acting DNA boxes present in the 5′ end of intron 1 and in the Purkinje-cell promoter. The explanation and references regarding the MEF2, MEF1, E box, and CArG box are in the Discussion.

brain and Purkinje-cell isoforms in the XLDCM family could be secondary to the presence of cis-acting DNA boxes in their respective promoters that become functionally important under specific circumstances. In this respect it should be noted that the brain promoter contains a CArG-like sequence (Makover et al. 1991). The CArG box is a conserved DNA motif present in the regulatory regions of several muscle-specific genes (Walsh 1989), including the dystrophin muscle promoter (Klamut et al. 1990), and was also found to be the only sequence to be functionally essential in determining transactivation of the dystrophin muscle isoform (Gilgenkrantz et al. 1992). In order to establish whether the Purkinje-cell promoter also contained sequences that might be relevant for the expression of its isoform in the skeletal muscle of patients with XLDCM, we undertook an analysis of the published sequences and found that the region between nucleotides −228 and −218 displayed 80% homology to the CArG box consensus sequence described by Minty and Kedes (1986) (fig. 4) also found an "E" box between nucleotides −389 and −383 of the Purkinje-cell promoter (fig. 4). The "E" box is a helix-loop-helix factor-binding site, specifically recognized by the myogenic MyoD1 factor, which acts as a transcription factor by trans-activating many muscle-specific genes during differentiation (Weintrab et al. 1991). It is therefore possible that in the XLDCM family these DNA boxes are implicated in transcription of the brain and Purkinje-cell isoforms in the skeletal muscle. Further experiments are needed to show whether this latter hypothesis holds true.

As to the second question—i.e., why the family with XLDCM showed a severe cardiomyopathy—we have no final answer, primarily because of the nonavailability of cardiac muscle from the affected individuals. Nevertheless, the two most likely possibilities are (1) that dystrophin is produced in the heart but is not functional or (2) that there is a complete lack of its expression in the cardiac muscle. Regarding the first hypothesis, we have shown that the brain isoform is physiologically expressed in the normal heart; since this isoform was up-regulated in the skeletal muscle of the family with XLDCM, it is possible that the brain isoform is also expressed in their heart. If this were

the case, the implication would be that the brain isoform, although capable of rescuing the skeletal muscle from degeneration, is unable to do so in the cardiac muscle. Sequences present exclusively in the first muscle exon could therefore have an essential role for dystrophin function in the heart. In this respect it should be noted that two actin-binding domains have been described at the 5′ end of dystrophin, one around the first two muscle exons (nucleotides 10–32; Levine et al. 1990) and the other one in the region of exon 6 (residues 128–156; Levine et al. 1991). From the data on our XLDCM family one could hypothesize that the most N-terminal actin-binding domain is crucial for dystrophin function in the heart, while this is not the case for the other binding domain. However, severe cardiac dysfunction has been reported in only three of the patients with a deletion limited to the first muscle exon (Muntoni et al. 1993; Yoshida et al. 1993), not in the remaining six (Malhotra et al. 1988; Beggs et al. 1991; Boyce et al. 1991; Bulman et al. 1991).

Regarding the other possibility—that no dystrophin is produced in the heart of this XLDCM family—this would imply that their deletion encompasses regions essential for dystrophin expression in cardiac muscle. Since other patients who have a deletion of part of muscle exon 1 extending into intron 1, but who have an intact muscle promoter, have also been shown to have a severe dilated cardiomyopathy in absence of significant skeletal muscle involvement (Yoshida et al. 1993), it is unlikely that these putative regulatory sequences lie in the muscle promoter region, deleted only in the cases that we studied. Intron 1 has a very large size and already had been considered to have an important functional role (Klamut et al. 1990). Similarly to what we have done for the Purkinje cell promoter, we have also analyzed the published sequences of intron 1. We were able to find the presence of several muscle-specific regulatory elements: a sequence of 12 oligonucleotides with an 83% identity with the MEF2-binding site (myocyte-specific enhancer factor 2; Gosset et al. 1989) was located at positions 18–29 of this intron (fig. 4). Moreover, a region with 72% sequence homology to the MEF1-binding site (myocyte-specific enhancer factor 1, also believed to bind the myogenic MyoD1 factor; Buskin and Hauschka 1989) and an "E" box were found farther downstream (position 226–236 and 228–233, respectively; fig. 4). Both the sequence conservation of this intron and the presence of several muscle-specific regulatory regions might therefore suggest that intron 1 is implicated in the regulation of dystrophin expression.

In conclusion, our data, those of Yoshida et al. (1993), and the fact that linkage to the 5′ end of the gene was recently reported in two XLDCM families without an identifiable deletion (Towbin et al. 1993), all indicate that mutations in this region of the gene are most likely involved in determining a severe cardiac phenotype with little or no skeletal muscle involvement. Both the first muscle exon and the first intron are good candidate regions for searching for mutations in patients with XLDCM.

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