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Duchenne and Becker muscular dystrophy mutations: analysis using 2.6 kb of muscle cDNA from the 5′ end of the gene

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SUMMARY

We have isolated overlapping human fetal muscle cDNAs encompassing 2.6kb which are localised very close to the 5'end of the Duchenne muscular dystrophy (DMD) gene. Using DNA from patients with deletions of previously reported genomic probes, we have mapped the exons across the region. Investigation of deletions in both DMD and Becker muscular dystrophy (BMD) patients shows the deletions to be present in 10% of cases and heterogeneous.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder which affects 1 in 3000 males (for review see reference 1). Affected boys are wheelchair bound by the age of twelve and usually die in their late teens. Becker muscular dystrophy (BMD) shows a much milder clinical course and affected males develop muscle weakness much later. Some of them have a normal life span. These diseases have been mapped to Xp21 on the short arm of the human X chromosome by the cytogenetic analysis of affected females with balanced translocations involving this part of the X chromosome[2], by family studies[3,4], and by the demonstration of the deletion of DNA markers in this region in both DMD and BMD patients[5,6].

A 14kb mRNA transcript has been identified in human fetal muscle by the isolation of the fetal cDNA and hybridisation on Northern blots[7,8]. More recently, a similar transcript has been identified by Forrest et al.[9] in fetal muscle and by Burghes et al.[10] in adult muscle. The DMD gene is one of the largest gene sequences so far identified.

We have previously reported a conserved sequence (HIP25) from the 5' region of the DMD locus which is not part of the cDNA sequence transcribed from the Xp21 region[11]. Genomic walking from this locus and screening of cDNA libraries did not reveal any exons of the DMD gene over 50kb of DNA. We decided, therefore, to explore the exons of the DMD gene in this region by walking in cDNA libraries starting from an adult muscle cDNA which we

have previously reported[12]. The adult cDNA clone used, Ca1B, is 0.9kb in length and extends from pERT87-30 to pERT87-8. The data presented here describe the characterisation of 2.6kb of fetal muscle cDNA extending 5' from Ca1B, and its analysis in DMD and BMD patients. This work both complements and extends the work reported by Koenig et al.[8] and Burghes et al.[10]. A detailed study of the precise breakpoints in DMD and BMD patients should lead to a better understanding of the differences between these two disorders at the molecular level. Our data do not indicate an increase in deletion frequency near the 5' end of the gene as seen in regions towards the 3' end[8,9].

EXPERIMENTAL METHODS

Southern blot analysis

DNA was prepared from whole blood or EBV lines essentially as described in Davies et al.[3] using the Triton method of Kunkel et al.[13]. Southern blotting was carried out according to standard procedures[14] onto Hybond N membrane (Amersham). The cDNA was labelled using the oligonucleotide labelling method of Feinberg and Vogelstein[15]. Blots were hybridised as described previously[3], washed to 3xSSC at 65°C and exposed on film for 2-5 days.

Library screening

Oligo dT and randomly primed human fetal muscle cDNA libraries in λgt.11[9] were screened with the cDNA clones of Cross et al.[12] by the method of Benton and Davis[16]. The positive recombinant phage were secondary and tertiary screened before being subcloned into the EcoRl site of pUC 13 (Pharmacia).

RESULTS AND DISCUSSION

Both oligo dT and randomly primed human fetal muscle cDNA libraries were screened with the Ca1B adult cDNA subclone of Cross et al.[12]. Positive clones were then screened with a second cDNA molecule, Ca1A, which is contiguous with Ca1B but extends towards the 3'end of the gene. A negative signal indicated that clones extended in the 5' direction. Three overlapping cDNA clones were isolated which extend from Ca1B towards the 5'end of the gene covering 2.6kb of cDNA. Phage DNA was extracted and analysed by restriction enzyme digestion (EcoR I) to determine which clones contained the largest inserts. Two clones were quite short, Cf14 and Cf16 (500 and 600bp respectively) and one clone, Cf27, contained a 2.2kb fragment.

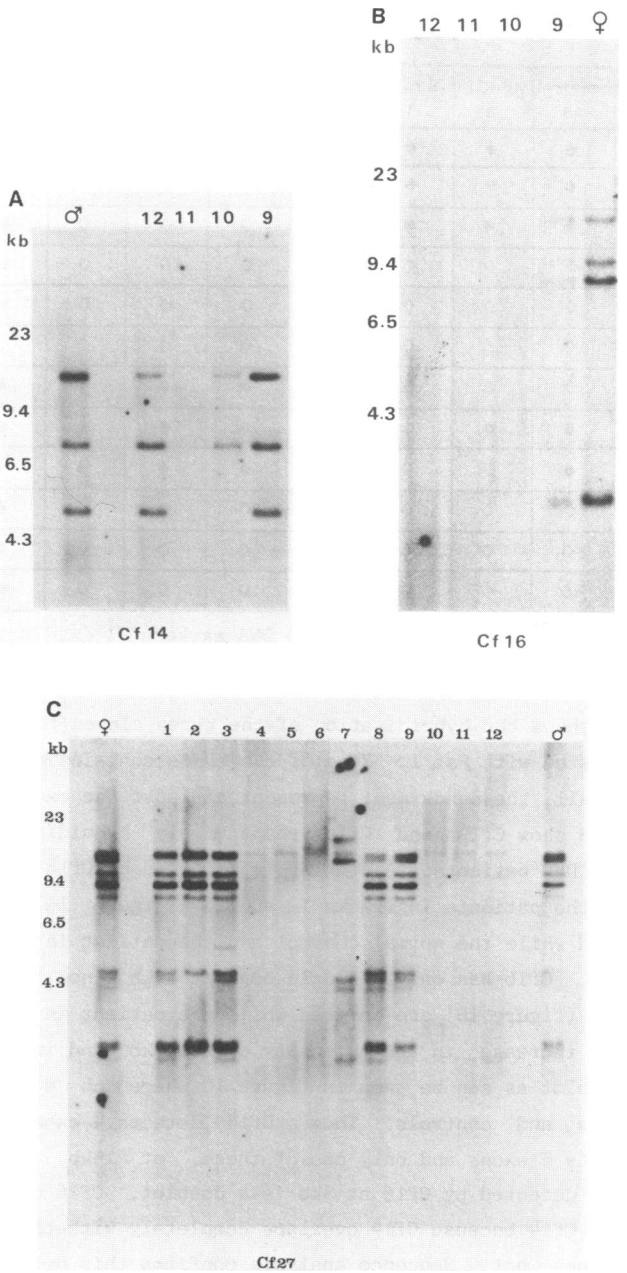

Figure 1

Southern blot analysis of overlapping cDNA clones. Genomic DNA was digested with Pst I and hybridised to A: Cf14; B: Cf16; C: Cf27. The track numbers are the same as the patient numbers given in Table I.

Table 1

	← pter									cen →
	← 30	15	pERT 8	87 1	42 →	XJ1.1	HIP25	JMD	J-47	pERT84
1	◆	◆	◆	◆	◆	◇	◇	◇	◆	◆
2	◆	◆	◆	◆	◆	◇	◇	◇	◆	◆
3	◆	◆	◆	◆	◆	◇	◇	◇	◆	◆
4	◇	◇	◇	◇	◇	◇	◇	◇	◆	◆
5	◇	◇	◇	◇	◇	◇	◇	◇	◆	◆
6	◇	◇	◇	◇	◇	◇	◇	◇	◇	◆
7	◆	◇	◇	◇	◇	◆	◆	◆	◆	◆
8	◆	◆	◆	◇	◆	◆	◆	◆	◆	◆
9	◆	◆	◇	◇	◆	◆	◆	◆	◆	◆
10	◆	◇	◇	◇	◇	◇	◇	◇	◆	◆
11	◇	◇	◇	◇	◇	◇	◇	◇	◆	◆
12	◆	◇	◇	◇	◇	◇	◇	◇	◆	◆

Deletions in patients 1-12 in relation to DNA markers (◇ , deleted; ◆ , present).

Figure 1 shows the hybridisation of the three clones to human genomic DNA samples digested with Pst I. None of the cDNAs contains a Pst I site and therefore all the bands must represent at least one exon of the gene. Figures 1A and 1B show Cf14 and Cf16 respectively hybridised to normal individuals and DMD patients. A doublet is detected by Cf14 at 15kb. This is evident from the patients in tracks 12 and 10 (figure 1A) who have a single-dose band while the normal control and the patient in track 9 have a double-dose band. Cf16 has only a single band at 15kb since the patients in tracks 12 and 10 (figure 1B) are deleted while the patient in track 9 has the band. The increase in the intensity of the 9kb band in track 9 is an artefact of the blot as can be seen in figure 1C where no dosage is seen between patients and controls. Thus, Cf14 detects 4 exons of the gene, while Cf16 detects 5 exons and only one of these, at 15kb, is common to both. The band detected by Cf16 at 3kb is a doublet. Cf16 extends more 5' in the gene than Cf14 because Cf14 overlaps completely with the Ca1B cDNA, whereas Cf16 does not. Sequence analysis confirms this orientation since Cf16 extends the open reading frame of Ca1B cDNA[12]. Ordering of the exons in the cDNAs can be deduced from deletions in particular patients. For example, the 5kb band detected by Cf14 is absent in the patient in track 10

Figure 2
Restriction map of Cf27. The four fragments used for mapping the cDNA are labelled F1-F4. B = Bgl II; E = EcoR I; H = Hinc II.

who is deleted from pERT87-30 towards the centromere but is present in the patient in track 12 whose deletion begins around pERT87-15. Thus, this exon must map between pERT87-30 and pERT87-15. Both patients are deleted for one of the 15kb bands which must, therefore, correspond to the most centromeric exon. This exon must lie distal to pERT87-8 since the patient in track 9 is deleted at this locus but not deleted for either of the 15kb bands.

Figure 1C shows the more complex pattern obtained with the much longer cDNA clone, Cf27. This cDNA covers the whole of Cf16 and extends over one exon of Cf14 (band at 15kb). Cf27 detects at least fifteen exons, some of them being doublets or triplets. Most patients in tracks 1-12 have deletions of at least two exons of the gene. Their deletions with respect to other DNA markers in the region are given in Table I. The DMD patients in tracks 8 and 9 are not deleted for any Pst I bands when compared to the normal controls. These patients are deleted for pERT87-1 (track 8) and pERT87-1 and 87-8 (track 9). Thus, we detect no exons between these two DNA markers. Interestingly, the BMD patient in track 2 is deleted for two extra Pst I bands (at 5kb and 2.5kb) than the DMD patients in tracks 1 and 3 whose deletions lie in the same area of the gene. Presumably the exon deletions in the BMD case leave the protein in phase whereas the DMD deletions do not. Alternatively, the deletion in the BMD patient leaves the protein structure less disrupted than in the case of the DMD deletions.

Because of the complexity of the exon pattern, precise ordering of the exons was achieved by constructing a restriction map of Cf27 (figure 2), and hybridising smaller subclones from within the 2.2kb cDNA to DNA samples digested with Pst I. Figure 3 shows the pattern obtained with the various fragments of Cf27. Figure 3A shows the hybridisation of fragment 1 (the most 3' fragment) to Pst 1 digested DNA. This fragment hybridises to bands of 15kb and 2.8kb. Figure 3B shows the result of hybridising fragment 2 to the same blot; it detects a band of 3kb and a faintly hydridising band at 14.5kb which can not be seen with the complete Cf27 cDNA; fragment 3 detects bands of 14.5kb, 10kb, 9.5kb (which is a doublet), 9kb, 5.2kb, and 4.5kb

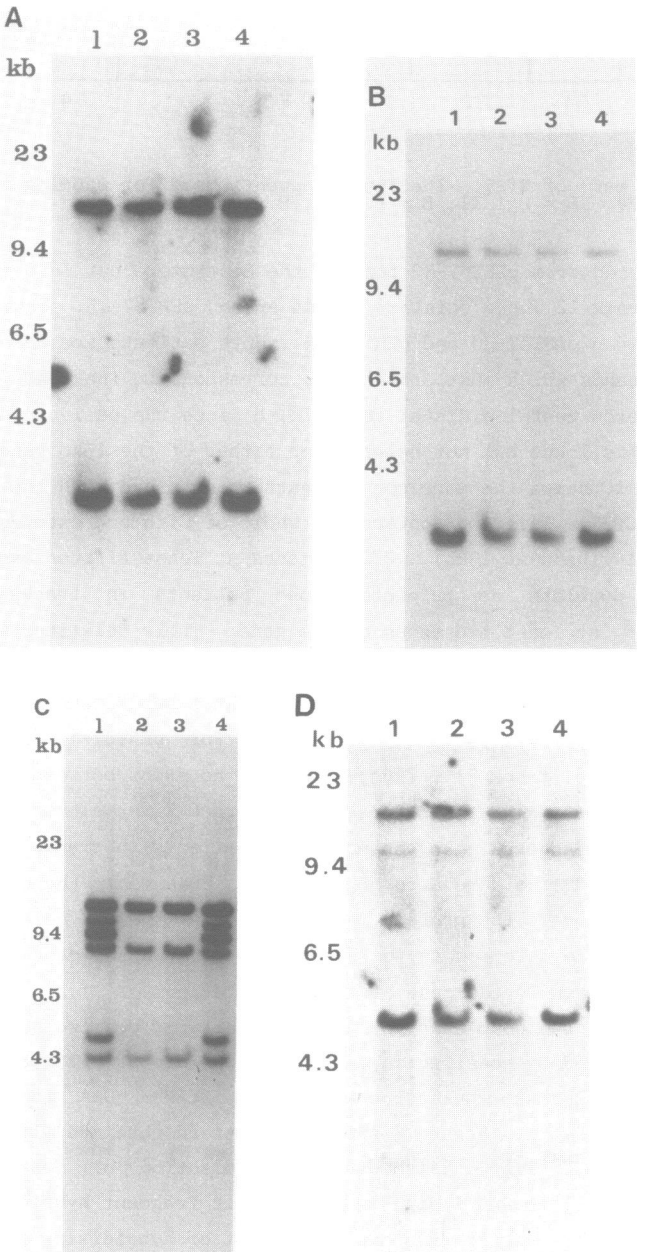

Figure 3
Southern blot analysis of Pst I digested genomic DNA hybridised to A: fragment 1; B: fragment 2; C: fragment 3; D: fragment 4. Tracks 1 and 4 are female controls; tracks 2 and 3 are DMD patients.

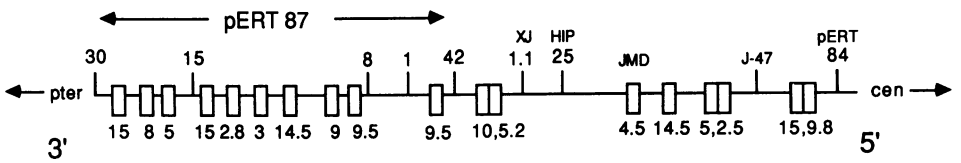

Figure 4
Schematic representation of the order of cDNA fragments in relation to previously mapped genomic markers[18]. The sizes of the Pst I fragments detected by the cDNAs are indicated below the exons which are denoted by open squares.

(figure 3C). The most 5' fragment (fragment 4), hybridises to Pst 1 bands of 15kb, 9.8kb, 5.0kb, and 2.5kb (figure 3D). The bands hybridising at 14.5kb and 4.5kb (fragment 3) were assigned to the centromeric side of XJ1.1 because of their hybridisation to the derivative X chromosome of the X;21 translocation (data not shown; XJ1.1 is the junction fragment of this translocation[17]). Combining these data with the deletion of previously described DNA markers (Table 1) it is possible to construct a map of the exons across this region (figure 4). The order of genomic markers has been described previously (for review see reference 18). Our results extend the exon map reported by Burghes et al.[10] since we detect an extra exon between J-47 and pERT84 and four more exons in the region between pERT87-8 and pERT87-30. The ordering of JMD distal to the exon seen as a Pst 1 band at 14.5kb was derived from the mapping of a chromosome jump clone (Kenwrick et al unpublished). We were not able to unequivocally assign the 4.5kb band with respect to JMD since our patient deletions did not separate them.

We have previously shown that the breakpoint of the X;1 translocation in an affected female lies between HIP25 and pERT84[19]. Since the data presented here demonstrate that several exons of the DMD gene lie in this region, this translocation must separate exons of the gene. We attempted to determine whether the breakpoint of this patient actually lay within coding sequence or in an intron of the gene. Hybridisation of Cf27 to this patient's DNA digested with seven different restriction enzymes did not show a changed band. An example of the digests is shown in figure 5A and 5B. No deletion of exon material is seen in any of the digests although the deletion of a small exon cannot be excluded. The change band in the Bam H1 digest between the two samples is a restriction fragment length polymorphism. The breakpoint, therefore, probably lies in an intron as seen with the X;21 translocation female[10]. There does not appear to be any major secondary rearrangement at the site of translocation though deletion of a

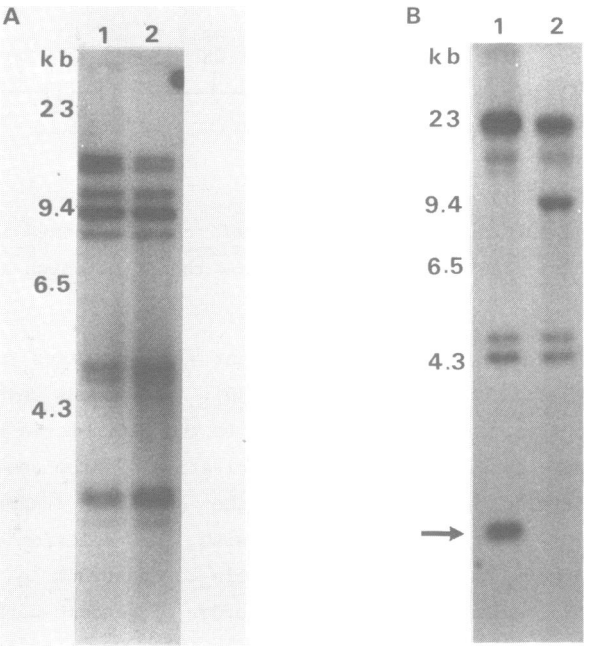

Figure 5
Southern blot analysis of an X;1 DMD translocation patient: a) Pst I digest of X;1 (track 1) and female control DNA (track 2); b) Bam HI digest of X;1 (track 1) and female control DNA (track 2). Arrow denotes RFLP.

small number of nucleotides as seen with the X;21 translocation[20] cannot be ruled out. The DMD phenotype has arisen presumably due to disruption of the gene by separating exons at the 5' end and preferential inactivation of the normal X chromosome.

Seventy-one DMD patients were tested for deletions of parts or all of this cDNA sequence. No additional deletions were found to those already ascertained through our analysis with HIP25. Thus, the deletion frequency in this region of the gene is about 10%. This is not dissimilar to the deletion frequency found in this region by Koenig et al.[8] (14.5%) but is in contrast to other regions of the gene[8,9]. We cannot, however, rule out the possibility of small deletions within introns, particularly within the 110 kb intron in the HIP 25 region[10]. The deletions in both DMD and BMD were highly heterogeneous with respect to both the number of exons deleted and the specific region deleted. Interestingly, almost all deletion endpoints occur in introns as seen from the fact that we detected only two altered bands in seventy-one patients using the cDNA probe Cf27. These deletions

may be the result of recombination events occurring between repetitive DNA sequences in introns such as those seen in the LDL receptor[21] and in the alpha globin locus[22].

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