

Multiple mutation in an extended Duchenne muscular dystrophy family

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Abstract

We have investigated an extended pedigree with three cousins affected by Duchenne muscular dystrophy with apparent transmission through the male line. However, molecular studies have shown that one boy has a de novo duplication, another has a deletion, and the molecular mutation has yet to be defined in the third boy. All three X chromosomes in the affected boys appear to have a different origin. We speculate on the mechanisms by which the Duchenne locus may be particularly prone to mutation in this family and the possible involvement of transposons is discussed. Whatever the mechanism involved, the occurrence of three different mutations in one pedigree is a rare event.

Duchenne muscular dystrophy (DMD) is an X linked recessive neuromuscular disorder with an incidence of 1 in 3000 male live births. The DMD gene has been localised to Xp21. The disease is associated with a high mutation rate with one-third of cases resulting from new mutations. Affected boys usually die by the age of 20 and the disease is transmitted through non-manifesting carrier females.

The purpose of this report is to present data on a DMD family (fig 1) with three affected cousins. This pedigree came to our attention when the sister (IV.3) of one of the affected boys came for counselling regarding her carrier status. Usually the finding of three affected boys within the same pedigree would suggest the inheritance of a common mutation

through carrier females. However, this pedigree is not consistent with the normal female carrier mode of inheritance but may be interpreted as transmission through unaffected males. An alternative explanation is that all three cases have arisen through independent mutations; the chances of this occurring must be very small. We therefore present this as an unusual pedigree. Molecular studies have been carried out to identify the nature of the mutation within the DMD locus in each affected boy and to provide information on the mode of inheritance of the DMD phenotype in this family.

Methods

CLINICAL ASPECTS

The proband (IV.4), now aged 10, complained of pains in his legs, particularly after exercise, from the age of 5 years. It was also noted that he had difficulty in climbing stairs, in rising from the ground, and in running. His school teachers noticed some slowness in learning. Examination at 5½ years showed moderate weakness of the pectoral and quadriceps muscles, large calves, and a positive Gower's sign. Ankle reflexes were depressed. The creatine kinase level was 25 711 IU/l. A muscle biopsy showed early dystrophic process with necrosis, regeneration, hyaline fibres, and a minor degree of fibre type grouping. Electromyography showed an occasional myopathic unit in the quadriceps. The diagnosis of Duchenne muscular dystrophy was made, although it was realised that he was relatively mildly affected. He is now at a school for physically handicapped children. His mother (III.6) is an English nurse and his father (III.5) a Malaysian Sikh who is also a nurse. There are two further boys (IV.1 and IV.2) with the clinical picture of Duchenne muscular dystrophy in the father's family. One boy (IV.2), aged 13, is severely affected; his parents are both Malaysian. His cousin IV.1, of similar age, is also severely affected; his mother is Malaysian and his father is Swiss.

MOLECULAR STUDIES

RFLP analysis and cDNA screening

Blood from family members was collected and lymphoblastoid cell lines established. DNA was not available from I.1, I.2, II.2, II.3, or III.2. DNA extraction, restriction digests, agarose gel electrophoresis, and Southern blotting onto Nylon filters (Hybond N, Amersham) were carried out by standard methods.¹ Probes were labelled with ³²P by random primed hexanucleotide synthesis.² Table 1 lists

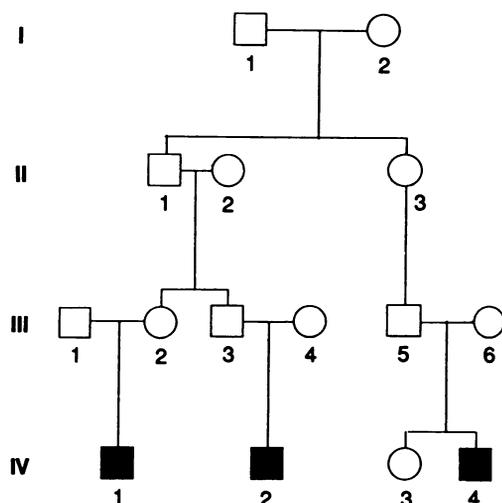


Figure 1 Family pedigree.

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the genomic probes used in RFLP analysis of the DMD locus. Hybridisation and stringency washes were carried out using standard protocols.¹ cDNA probes were provided by Dr K E Davies and analysis with these probes on *Pst*I digested DNA was performed as described by Forrest *et al.*³

Pulsed field gel electrophoresis

Pulsed field gel electrophoresis methods used were those described by Anand.⁴ Agarose plugs, containing high molecular weight DNA, were prepared from blood lymphocytes by mixing 3.0×10^6 cells in phosphate buffered saline with an equal volume of 1% low gelling temperature agarose (LGT, Sea Plaque, FMC Bioproducts). Plugs were then treated with 1% lauryl sarcosine and 1 mg/ml protease type XXV (Sigma) at 50°C for 35 to 40 hours. Protease was removed by extensive washing with Tris/EDTA buffer. Blood was not available from subjects I.1, I.2, II.2, II.3, or II.4 for preparation of agarose plugs. Chromosomes were prepared from *Saccharomyces cerevisiae* (X2180-1B) and run on gels as size markers.⁴ Agarose plugs were digested with the rare cutting enzyme *Sfi* (New England Biolabs).

DNA was fractionated using the 'Waltzer' apparatus⁵ on 1.5% agarose (Sigma type II) gels in $0.5 \times$ TAE (0.02 mol/l Tris acetate, 0.01 mol/l EDTA) buffer. Gels were run for 32 hours at 150 V with a pulse time of 60 seconds, buffer temperature 19°C. After electrophoresis, gels were dephosphorylated (0.2 mol/l NaOH), denatured (0.25 N HCl), and DNA was transferred to Genescreen Plus nylon membrane (Du Pont) according to the manufacturer's instructions. Filters were hybridised at 65°C in 0.5 mol/l sodium phosphate, pH 7.5, 7% SDS,

1% BSA buffer. Probes used (table 2) were labelled with ³²P.

Results

RFLP analysis was undertaken to check that there was no unusual pattern of inheritance of the X chromosome in the region Xp21. Probe and enzyme combinations are given in table 1. The RFLP results shown in fig 2 confirm the normal pattern of inheritance with all three affected boys having received their respective maternal Xp21 chromosome regions.

In approximately 60% of Duchenne and Becker cases the molecular basis of the mutation giving rise to the disease phenotype has been shown to be the result of the deletion of sequences within the DMD gene.^{3,6} The majority of the deletions lie within an area defined by the two contiguous probes Cf56a and Cf56b and the genomic clone P20 lies within an intron between two exons encompassed by Cf56b. DNA from IV.2 failed to hybridise with the clone P20 although all other probes gave positive signals. Neither of his two affected cousins were deleted for any probe used. Further analysis using the cDNA probes Cf56a and Cf56b was undertaken to determine the extent of the deletion in subject IV.2 and to search for possible deletions in the other two affected boys (IV.1 and IV.4). No deletions were detected in any family members with Cf56a. Fig 3 shows the hybridisation pattern seen with Cf56b. Subject IV.2 has a deletion of the 3.8 kb fragment represented by exon F, which is consistent with the observed P20 deletion, since this sequence lies in the adjacent intron. The 15 kb fragment representing fragment C is also absent although an additional band of 20 kb is present. This is thought to be a rare *Pst*I polymorphism which has been reported elsewhere.⁷ This same polymorphism is seen in the boy's mother (III.4).

Fig 4 gives the locations of the *Sfi*I cutting sites across the DMD gene. Four intragenic probes were hybridised to *Sfi*I digested DNA. Table 2 lists the probes hybridised to the family members. XJ1.1 maps to an 690 kb and 840 kb partial digestion fragment. The adjacent probe pERT87-1 maps to a single 250 kb fragment. The most distal probes used, J-Bir and P20, both map to the same 500 kb fragment and also detect a 620 kb partial digestion fragment. In all family members tested, XJ1.1 identified the 840 kb and 690 kb bands; however, patient IV.4 alone had an additional 230 kb band. Fig 5 shows an autoradiograph where XJ1.1 identifies three bands in patient IV.4 and only two in his sister IV.3. The absence of the additional band in the patient's parents indicates that IV.4 has a de novo duplication within the XJ1.1 region. Patient IV.2 showed the normal 250 kb signal with pERT87-1 for the adjacent *DXS164* locus, as did IV.4 and his sister IV.3. The third affected boy, IV.1, was not tested with this probe. Normal hybridisation signals were also observed for all family members probed with J-Bir. Although patient IV.2 was probed with P20 instead of J-Bir, this probe detects the

Table 1 Genomic probes used for RFLP analysis.

	Code	Probe	Enzyme	RFLPs	
DMD locus		E,c	P20	<i>Eco</i> RV	7.0/7.5
		B,b	J-Bir	<i>Bam</i> HI	5.0/21
		B,b	87.15	<i>Bam</i> HI	2.3,7.1/9.4
		X,x	87.15	<i>Xmn</i> I	1.2,1.6/2.8
		T,t	87.15	<i>Taq</i> I	3.1/3.3
		B,b	87.8	<i>Bst</i> XI	2.2,4.4/6.6
		X,x	87.1	<i>Xmn</i> I	7.5/8.7
		B,b	87.1	<i>Bst</i> NI	0.6,2.5/3.1
		T,t	XJ1.1	<i>Taq</i> I	3.3/3.8
		T,t	XJ2.3	<i>Taq</i> I	6.4/7.8
		P,p	754	<i>Pst</i> I	9.0/11.0

Table 2 Pulsed field gel electrophoresis studies. Probes hybridised to *Sfi*I digested DNA. N denotes that the probe detected the normal fragment size(s) and actual fragment sizes are given in the text. DEL denotes deletion of probe.

Pedigree No	XJ1.1	J-Bir	P20	pERT87-1
II.1	N	N	—	—
III.1	N	N	—	—
III.2	N	N	—	—
III.3	N	—	—	—
III.5	N	N	—	—
III.6	N	N	—	—
IV.1	N	N	—	—
IV.2	N	—	DEL	N
IV.3	N	—	—	N
IV.4	N + 230 kb	N	—	N

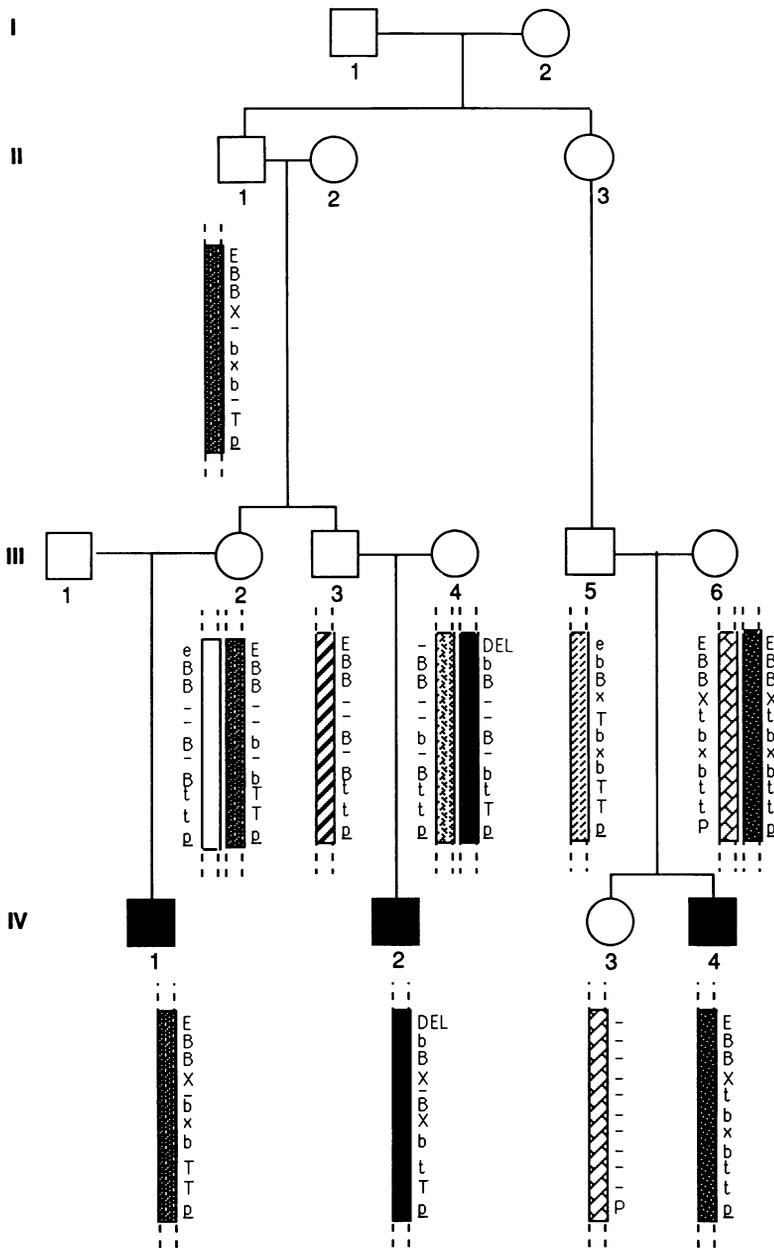


Figure 2 RFLP analysis of pedigree. Probe and enzyme combinations used are given in table 1. DEL denotes deletion of probe.

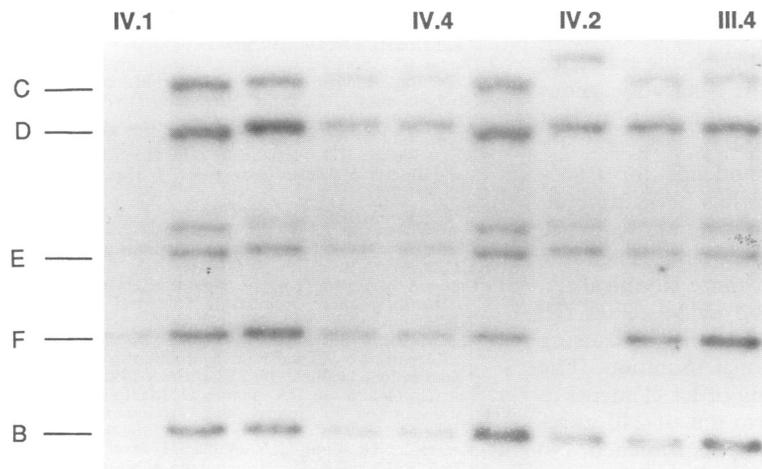


Figure 3 Autoradiograph of PstI digested DNA probed with Cf56b. Contiguous exons are labelled B to F. An extra band found in all DNA tracks between exons D and E has been observed in a limited number of other gel runs with different DNA samples. We are unsure of the significance of this and unfortunately there was insufficient DNA to repeat this hybridisation.

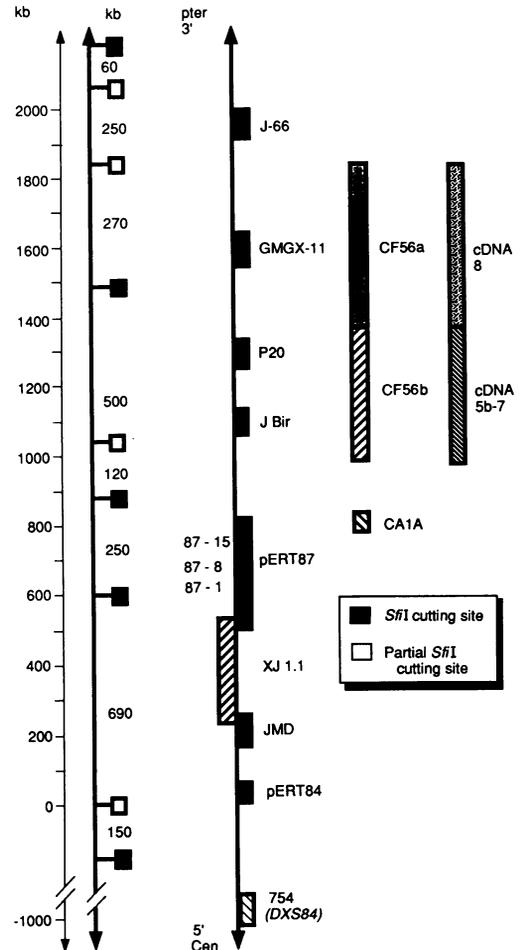


Figure 4 Relative location of genomic cDNA probes and SfiI sites at the DMD locus. Numbers show distance in kb between adjacent sites.

same SfiI fragment as J-Bir. The result was a complete P20 deletion in patient IV.2 and this is consistent with the Cf56b deletion detected in this patient, as this probe maps to the same SfiI fragment as P20. Unfortunately, blood was not available from the patient's mother (III.4) for pulsed field studies.

The normal hybridisation signals for patient IV.1 with the probes J-Bir and XJ1.1 indicate that whatever the mutation, it is different from that of his two cousins.

Discussion

Mutations within the DMD locus have been shown in two of the three affected boys in this pedigree. Patient IV.4 has a duplication and patient IV.2 a deletion within the dystrophin gene. As yet, no mutation has been defined in the third affected boy, IV.1. The Duchenne phenotype is therefore the result of a different mutational event in each affected boy.

The XJ1.1 duplication was not detected in either parent of IV.4. The usual conclusion would therefore be that this duplication has arisen as a de novo event. In a study of 120 unrelated DMD and BMD boys, a frequency of 7.5% for duplications was reported.⁸ However, the possibility that the boy's mother has germline or somatic cell mosaicism for the

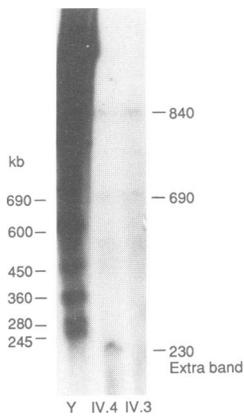


Figure 5
Autoradiograph of *Sfi*I digested, *Xj1.1* probed DNA after pulsed field gel electrophoresis, showing the normal fragment pattern for IV.3 and the presence of an additional band for patient IV.4. Yeast chromosomes (Y) were used as size markers and fragment sizes are given in kb.

duplication cannot be ruled out. In a recent study, Bakker *et al*⁹ estimated that germline mosaics could account for at least 14% of apparent new mutations. Similarly, we are uncertain of the origin of the deletion of P20 and exon F seen in patient IV.2. Whatever the molecular basis for the disease in patient IV.1, this boy does not possess either of the mutations found in his two cousins. The entire dystrophin gene has not yet been probed and we cannot as yet exclude the finding of a detectable mutation. However in 40% of cases no observable deletion is detected.⁷

It is intriguing that three different mutations in the DMD gene have occurred in this family. The three affected boys are linked by a common ancestral couple (I.1 and I.2 in the pedigree); intervening relatives have been both male and female and there has been no known consanguinity.

An interpretation based on the conventional means of female transmission is that each mutation has occurred independently. Given the fact that the affected boy (IV.4) is a new mutation, what is the chance that two further mutations occurred in his relatives of generations II, III, and IV? These relatives number approximately 50 of whom half are likely to be females, providing a total of 75 X chromosomes. Therefore, the chance of two further mutations is the mutation rate,¹⁰ multiplied by 75, and squared. This approximately equals $(10^{-4} \times 75)^2 = 1$ in 16 000. Patient IV.4 has a de novo duplication and patient IV.2 a deletion with no blood available from his mother for pulsed field analysis to determine the origin. The absence of a mutation in patient IV.1 also leaves the origin of mutation in this patient unknown. However, this still poses the problem of three affected boys, all with different mutations in the same pedigree, a very rare occurrence by whatever mechanism proposed.

It is possible that the Duchenne locus in this family is particularly prone to mutation and it is interesting to speculate on the possible involvement of transposons in disrupting the dystrophin gene within this pedigree. This suggestion was also made by Zatz *et al*¹¹ in their recent report of four Brazilian families, in which Duchenne muscular dystrophy recurred in the paternal lineage. Although the existence of transposable elements in eukaryotic genes has been well documented, the best studied being the P elements in *Drosophila* and Ty elements in yeasts, the evidence for transposons in human genomes has been indirect. However, the transposition of an alu sequence into a target gene in a lung carcinoma cell line has been reported.¹² Even more significantly, Kazazian *et al*¹³ provided evidence for the involvement of retrotransposons in causing human disease by insertional mutation. The authors reported the finding of L1 elements in the factor VIII gene in two out of 240 unrelated male haemophilia patients screened. No abnormalities were detected in the gene in either parent suggesting that the mutations

had occurred de novo. It was suggested that the movement of such sequences in the genome represents a fundamentally different method of mutation producing human disease. One notable feature of transposons in *Drosophila* is that they cause mutations when two strains are mixed, hence the term hybrid dysgenesis. It is interesting to note that two of the three mutations have occurred in the offspring of racially mixed couples. We do not know whether the Brazilian families contained any mixed marriages.¹¹

Molecular studies have served to highlight the difficulties in genetic counselling in this pedigree. Although IV.3 does not possess the duplication found in her affected brother, the unusual nature of this pedigree has led us to offer her prenatal diagnosis involving an extensive screen for mutations in the dystrophin gene of her male fetus.

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