Partial gene duplication in Duchenne and Becker muscular dystrophies

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SUMMARY Duchenne and Becker muscular dystrophies (DMD and BMD) are progressive muscle wasting disorders with an X linked recessive mode of inheritance. We have surveyed 120 unrelated patients with DMD or BMD for gene duplications using a series of genomic probes from within the DMD/BMD gene locus. In three patients, two with DMD and one with BMD, a duplicated region within the DMD/BMD locus has been shown by Southern blot analysis and transmission densitometry. In two cases a new restriction fragment spanning the duplication junction has been visualised, indicating that the duplications are tandemly arranged. Mendelian inheritance of the duplication has been shown in two families by following the segregation of the duplication junction fragment. The three duplication cases have been analysed with a cDNA probe isolated from the *DXS206* region of the DMD/BMD locus and the duplication of a specific set of exons has been found in two cases. This study shows that all three duplications are internal to the gene and confirms that such a duplication can result in a genetic disorder through the disruption of exon organisation.

The gene responsible for both Duchenne muscular dystrophy (DMD) and the milder Becker muscular dystrophy (BMD) has been located at band Xp21 on the short arm of the X chromosome by linkage analysis,[1][2] by the identification of balanced X;autosomal translocations in affected females,[3] and by the detection of cytologically visible deletions of Xp21 in patients with a complex phenotype including DMD.[4][5] Cloned sequences from the DMD/BMD locus have been obtained by enrichment for sequences from within the deleted region of one of the deletion patients[6] and by cloning of the translocation junction from a t(X;21) translocation patient.[7] The two cloned regions, *DXS164* and *DXS206* respectively, have provided a series of probes (pERT87 and XJ series respectively) that detect a variety of deletions of a few kb (kilobase pairs) to a few hundred kb in about 10% of DMD and BMD patients.[8-10] Recently, expressed sequences have been identified in both *DXS164* and *DXS206* regions and used to isolate the complementary DNA (cDNA).[11-13] The cDNA clones can detect deletions in over 50% of DMD patients[13] and can be used to identify deletion and duplication of specific exons in DMD and BMD patients.

Given the high frequency of deletions and the possibility that deletion as well as duplication may result from unequal crossing over, duplications might be expected in some DMD and BMD patients. Indeed, there has been one report of an increased hybridisation intensity in a DMD patient, indicating a duplication of a part of the pERT87 (*DXS164*) region.[14] More recently den Dunnen *et al*[15] have also reported a duplication detected by field inversion gel electrophoresis in a DMD patient. We describe here the more detailed analysis of three patients, two with DMD and one with BMD, who have a duplication within the DMD/BMD locus. This study has been presented in abstract form to the 38th Annual Meeting of the American Society of Human Genetics.[16]

Materials and methods

PATIENTS
All three boys found to have duplications have been followed for several years in the Muscular Dystrophy Clinic of The Hospital for Sick Children and have been diagnosed as DMD or BMD on the basis of grossly raised serum creatine kinase activity, pseudohypertrophy of the calf muscles, electromyographic abnormalities characteristic of myopathy,

and muscle biopsy findings consistent with muscular dystrophy. Case 1 (Dup 1) is an adopted boy diagnosed as having DMD. He is reported to be an isolated case in his family, but his natural parents have not been available for study. He became wheelchair bound at nine years of age. His intelligence is superior; an IQ measurement when he was seven years old was 128. Case 2 (Dup 2) is an isolated case of DMD. His mother's serum creatine kinase (CK) activity is in the normal range, but as his sister has a CK level triple the normal upper limit for her age, the mother is classified as a presumptive carrier. The patient is a slow learner and has been treated for primary hypothyroidism. He became wheelchair bound at 11 years of age. Case 3 (Dup 3) is an isolated case of BMD, but his mother has a raised CK level and thus is a presumptive carrier. The parents are second cousins. The sister's CK activity is within the normal range. The patient's intelligence is slightly below average. He is still ambulant at 13 years of age.

SOUTHERN BLOT ANALYSIS

DNA was extracted from lymphoblast cell lines or leucocytes of patients with DMD or BMD, their family members, and normal subjects by the methods previously described.[7] DNA concentrations were determined using a spectrophotometer (Gilford), as well as by monitoring the intensity of ethidium bromide staining on a test gel. A 5 μg sample of DNA was digested with the appropriate restriction enzyme, loaded onto a 0·8% agarose gel, and separated by electrophoresis. The XJ probes and the cDNA clone were isolated from the *DXS206* region of the DMD/BMD locus in our laboratory.[7 12] The other genomic probes were generous gifts from L M Kunkel, G J B Van Ommen, and J-L Mandel and the isolation of these probes has been described elsewhere.[6 17-19] The probes were labelled with ^{32}P-dCTP using the random oligonucleotide priming reaction.[20]

TRANSMISSION DENSITOMETRY SCANNING

The autoradiographic film was scanned by transmission densitometry using a Jocye Loebl Chromoscan 3. The area under each peak (peak value) represents the intensity of each hybridisation band. The peak area was divided by the total peak area of all bands in that sample lane to obtain the relative peak value, that is, the relative band intensity. Owing to the fact that the bands with double intensity in the duplication cases increase the total peak area and therefore alter the relative peak value, a normalisation factor is required. The bands showing single intensity in both a normal male as well as a duplication case were selected and the ratio of mean peak values of these bands for the normal male and the duplication case provides the appropriate normalisation factor. All the relative peak values from the duplication case were then multiplied by this factor. Multiple exposure of the autoradiograph film was used to ensure the appropriate linear intensity response of all the hybridisation bands.
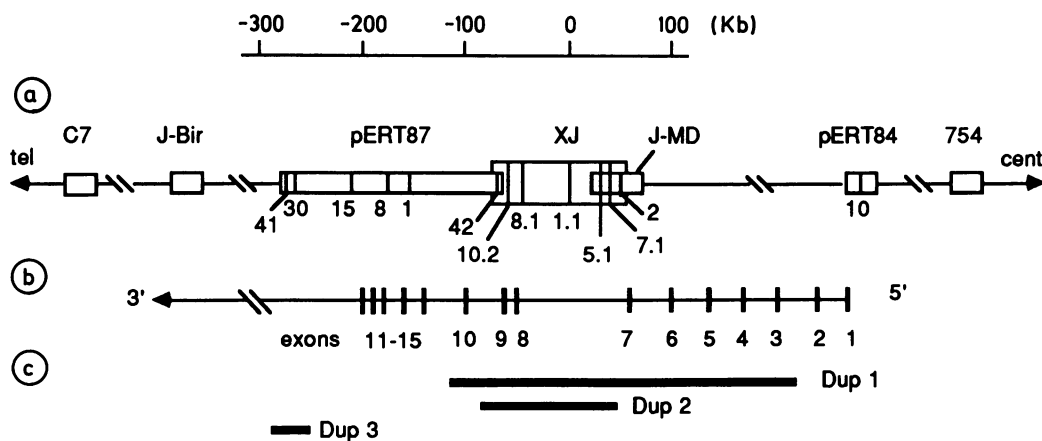

FIG 1   *(a) A schematic map of the DMD/BMD gene locus. The blocks represent regions of cloned DNA described in the text. The numbers below the blocks represent the positions of genomic subclones. (b) The line represents the region known to contain expressed sequences of the DMD/BMD gene. The vertical bars show the relative position of exons detected with the cDNA clone described in the text. The exons are numbered from the 5' end of this cDNA. Exons 7 to 15 have been mapped precisely within the pERT87, XJ, and J-MD genomic clones. Exons 1 to 6 have not been precisely mapped and are arbitrarily spaced at equal intervals. (c) The three black bars show the extent of the duplicated regions in the three duplication patients.*

Results

IDENTIFICATION OF GENE DUPLICATION

Initially, three patients were identified as having a partial gene duplication because some genomic probes from within the gene detected a hybridisa- tion intensity corresponding to two copies per cell, whereas probes flanking the gene (754 and C7) detected single copy intensity. The map of the DMD/BMD locus, the location of the probes used, and the extent of the duplicated regions are shown in fig 1 and the table. For gene dosage analysis, a
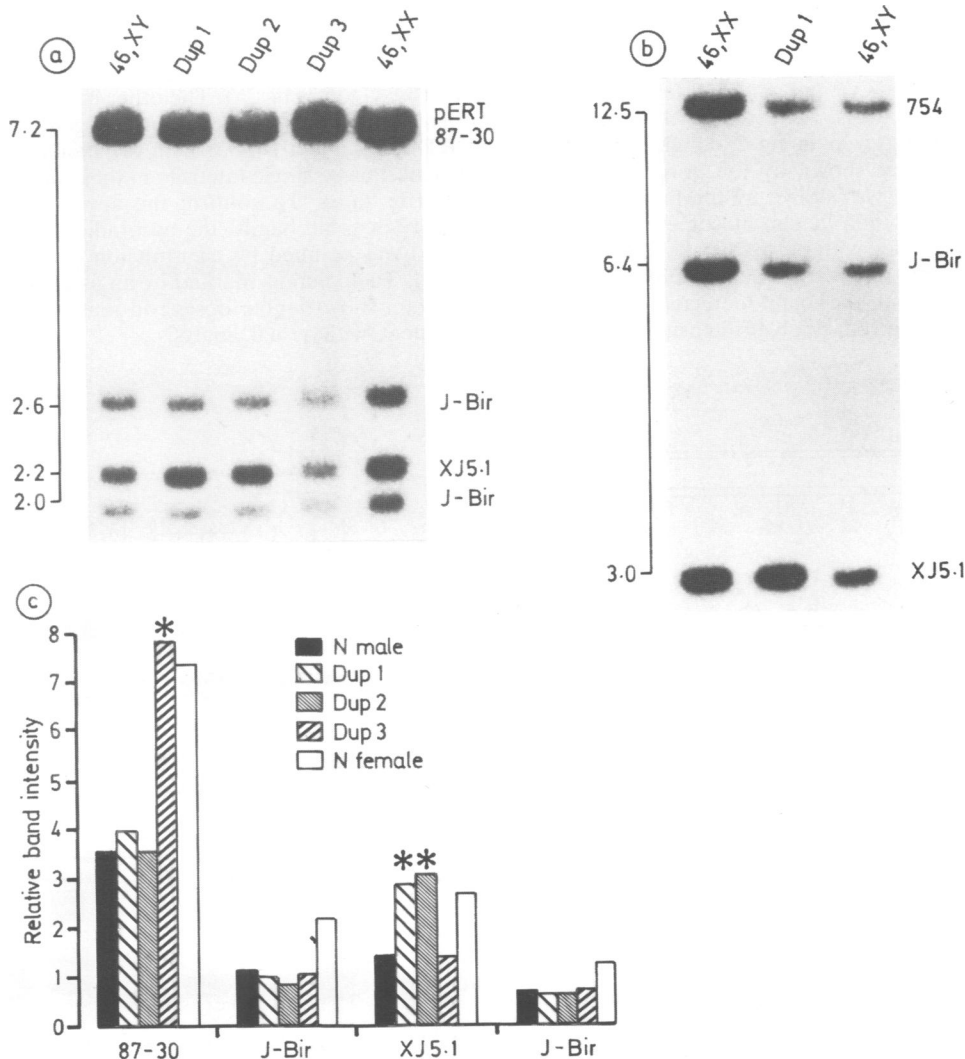
FIG 2   *Southern blot analysis on the duplication patients and normal subjects. Sample lanes contain DNA from a normal male (46,XY), a normal female (46,XX), and three male duplication patients (Dup 1, Dup 2, and Dup 3). The DNA was digested with EcoRI (a) or XbaI (b), separated by electrophoresis on agarose gels, and blotted onto Hybond-N filter. Filters were hybridised with a mixture of three radiolabelled probes, (a) pERT87-30, J-Bir, and XJ5.1 or (b) 754, J-Bir, and XJ5.1. The probes are indicated at the right side of each autoradiograph. The size of the hybridisation bands (kb) is shown on the left side of the autoradiograph. (c) The autoradiograph from (a) was scanned by transmission densitometry. The relative band intensity (Materials and methods) is represented by the columns on this histogram. The columns marked by an asterisk show double intensity of the represented bands. The probes used are shown on the bottom (87-30= pERT87-30).*

TABLE   *Hybridisation intensity with genomic probes.*

	C7	J-Bir	pERT87						XJ					J-MD 2	pERT 84–10	754
			41	30	15	8	1	42	10·2	8·1	1·1	5·1	7·1			
Dup 1 (DMD)	+	+	+	+	+	+	+	++	++	++	++	++	++	++	+	+
Dup 2 (DMD)	+	+	+	+	+	+	+	++	++	++	++	+J	+	+	+	+
Dup 3 (BMD)	+	+	++	+J	+	+	+	+	+	+	+	+	+	+	+	+

Probes are described in the text and their map locations are shown in fig 1. Intensity of hybridisation: ++, double; +, single; J, junction fragment.

normal male (46,XY) and a normal female (46,XX) were used as controls. An example of the results of such analysis is given in fig 2. Single intensity of hybridisation was shown by the normal male and double intensity was shown by the normal female, proportional to their X chromosome complement. The band detected with probe XJ5.1 showed double intensity in two duplication males (Dup 1 and Dup 2, fig 2a,b) and the band detected with probe pERT87–30 showed double intensity in another

male case (Dup 3, fig 2a). The other bands detected either with probes from within the gene (XJ5.1, pERT87–30, and J-Bir) or with the flanking probe (754) all showed single intensity in these duplication cases (fig 2a,b). To confirm the apparent double intensity of some bands, the autoradiographic film (fig 2a) was scanned by transmission densitometry (fig 2c). The columns marked by an asterisk on the histogram show double dosage in male patients in agreement with visual analysis.
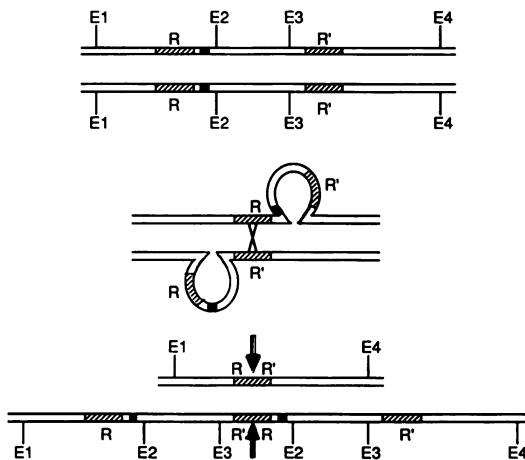

FIG 3   *A simplified schematic diagram of the generation of a tandem gene duplication by unequal but homologous crossing over. Unequal crossing over between the repeats, R and R', results in two new chromatids, one with a deletion and the other with a tandem duplication of the sequence between R and R'. The junctions of the duplication and deletion are indicated by the arrows. The letters E1 to E4 are hypothetical restriction enzyme recognition sites and the black boxes show the position of the probe which could be used for detecting the junction fragment. The fragment between E3 and E2 on the bottom shows the junction fragment of the duplication that would be detected with the probe. The size of this fragment is different from the size of the normal fragment between E1 and E2. Although it is recognised that meiotic recombination occurs at the four strand stage of meiosis, the diagram here is simplified to show only the two recombinant strands.*
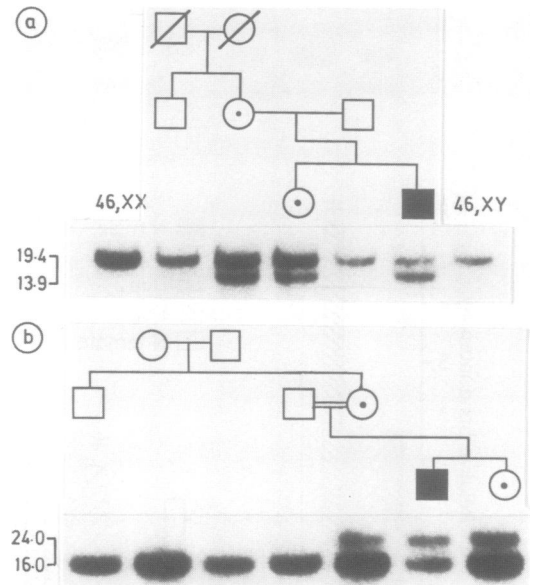

FIG 4   *Identification of duplication junction fragments and the inheritance of the duplication. (a) Sample lanes contain DNA from a normal male (46,XY), a normal female (46,XX), and five members of the family of case 2. DNA was digested with HindIII and hybridised with the probe XJ5.1. The duplication junction fragment is seen as a restriction fragment of 13·9 kb. (b) Sample lanes contain DNA from seven members of the family of case 3. DNA was digested with PstI and hybridised with the probe pERT87-30. The duplication junction is seen as a restriction fragment of 24 kb. ⊙ Carrier. ■ Affected male.*

DUPLICATION JUNCTIONS AND INHERITANCE OF THE DUPLICATION

The tandem organisation of intergenic and intragenic duplications has been described in a few human genes.[17] [21–25] The tandem organisation results in the creation of a duplication junction flanked by two segments of DNA which are normally separated in the genome. This duplication junction can be detected as a restriction fragment of altered size on Southern blot analysis, by using a restriction enzyme with recognition sites on both sides of the junction and a DNA probe that is close to the junction (fig 3). All three duplications were therefore examined for possible altered size restriction
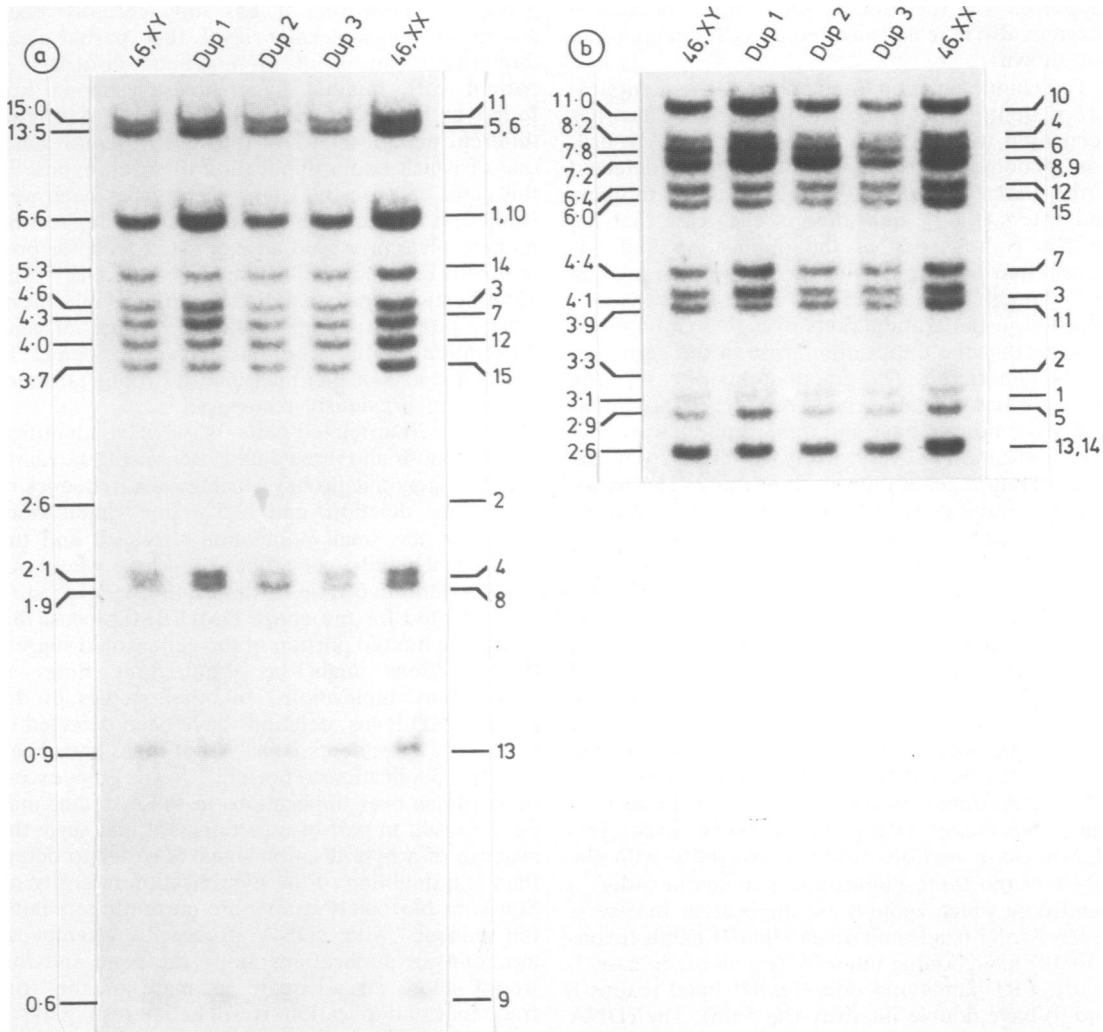

FIG 5   *Southern blot analysis with cDNA probe. Sample lanes contain DNA from a normal male (46,XY), a normal female (46,XX), and the three duplication patients (Dup 1, Dup 2, and Dup 3). DNA was digested with EcoRI (a) or HindIII (b) and blots were hybridised with the cDNA probe described in the text. In Dup 1, seven EcoRI fragments and seven HindIII fragments (exons 3 to 10) show double intensity. (The EcoRI fragment containing exon 1 comigrates with the fragment containing exon 10 and is visible only in patients from whom exon 10 is deleted.) In Dup 2, two EcoRI fragments and one HindIII fragment (exons 8 and 9) show double intensity. The exon numbers are shown on the right side of the autoradiographs and the size of the fragments containing exons is shown on the left. The EcoRI and HindIII fragments containing exon 2 were seen on longer exposure autoradiographs but are not reproduced well in this figure.*

fragments, and such a fragment was found in two of the duplications. A new *Hind*III restriction fragment of 13·9 kb was detected in case 2 (fig 4a) and a new *Pst*I fragment of 24 kb was detected in case 3 (fig 4b). No suitable probe was available to detect the duplication junction in case 1. The interpretation of the altered size fragment as a duplication junction and not a rare polymorphic variant was supported by the finding that other restriction enzymes also revealed an altered size fragment (data not shown).

The unique junction fragment revealed in duplication patients 2 and 3 provided a marker which has been used to trace the inheritance of the duplication. In both families the junction fragment detected in the affected boy was also found in their mothers and sisters (fig 4), indicating in each case that the mother is a carrier of the duplication and has transmitted the duplication chromosome to her son and daughter. The junction fragment was not detected in the grandparents of case 3 (fig 4b), indicating that the duplication arose in the germ line of this generation. The grandparents of case 2 (fig 2a) were not available; however, the grandmother had seven normal boys and this would indicate that the duplication is very likely to arise from the grandparental generation in this family. The hybridisation intensity seen in these duplication carriers (fig 4) further confirms their carrier status.

DUPLICATION OF EXONS WITHIN THE GENE

Recently a cDNA clone has been isolated from the DXS206 region of the DMD/BMD locus.[12] This cDNA clone identifies 13 bands with both *Eco*RI and *Hind*III digested human genomic DNA, and these contain a minimum of 15 exons of the gene (fig 1). The order and map position of exons within the gene has been determined by probing a panel of DNA from translocation and deletion patients with the cDNA clone[12] (unpublished observations). This cDNA clone was also used to hybridise with the DNA of the three duplication patients in order to determine which exon(s) are duplicated. In case 1, seven *Eco*RI bands and seven *Hind*III bands (exons 3 to 10) have double intensity (fig 5a,b). In case 2, two *Eco*RI bands and one *Hind*III band (exons 8 and 9) have double intensity (fig 5a,b). The cDNA clone used does not encompass the region that is duplicated in case 3. The exon assignment within the pERT87 region previously reported by Monaco *et al*[11] indicates, however, that there is at least one exon located within this duplicated region. These results were further confirmed by transmission densitometry scanning. All the bands showing double intensity on the autoradiograph films also showed double dosage with the transmission densi-

tometry scanning (data not shown). The duplication of specific exons but not others confirms that all three duplications are internal to the gene.

Discussion

Gene duplication is known to be an important mechanism in evolution for the generation of new genes.[21-24] However, it has only recently been shown at the molecular level that partial gene duplication can result in a genetic disorder. A patient with familial hypercholesterolaemia was found to be a compound heterozygote with two different mutant alleles of the LDL receptor gene, one of which had a duplication of seven exons. In this case, because the LDL receptor has been well studied, it was possible to show that the duplication mutant allele produced an elongated receptor protein with reduced binding capacity for LDL.[25] The identification and analysis of three duplications among our 120 patients indicate that partial gene duplication is the essential molecular defect in Duchenne and Becker muscular dystrophy far more often than previously recognised.

In our 120 unrelated patients, we have identified nine deletions and three duplications using currently available genomic probes which gave a frequency of 7·5% for deletion and 2·5% for duplication. Although the small population surveyed and the insufficient number of probes used here would not allow a definitive ratio of duplication *v* deletion to be estimated for the entire DMD/BMD locus, our study of a limited portion of the gene would suggest that deletions might be about three times as frequent as duplications. In other studies of the DMD/BMD locus, deletions have been detected in up to 50% of patients,[8-10 13 15] but there have been only two duplications reported.[14 15] The great excess of deletions over duplications in these studies may be the result in part of ascertainment bias since the absence of a hybridisation signal is easier to detect than is a doubling of the hybridisation intensity on Southern blot analysis. We are currently screening the patients with cDNA probes to attempt to ascertain all duplications along the gene and this would allow an adequate estimate of the true frequency of duplication. It will be interesting to see if the region recently reported to be associated with a high frequency of deletion[13 15 26] is also associated with an increased frequency of duplication.

The duplication of genes that occurs during evolution is thought to arise by two mechanisms, unequal but homologous crossing over,[23] as depicted in fig 3, and non-homologous crossing over.[27] In both cases, such an event, if reciprocal, should create two new chromatids, one with a deletion and

one with a duplication, so that in the absence of selection one might expect this mechanism to result in equal numbers of duplication and deletion. Even though we do not yet know what mechanisms are involved in the generation of duplications and deletions in DMD and BMD, the apparently higher frequency of deletions would suggest that there are some mechanisms that generate deletions without the generation of a concomitant duplication. Linkage analysis using a series of polymorphic probes on deletion patients with DMD would be consistent with this, since not all deletions correlated with a crossover event.[28] A model in which deletion could arise as a result of a recombination on a single chromatid has been proposed for the generation of some deletions in human β-like globin genes[29] and in familial hypercholesterolaemia.[30]

The identification of a unique junction fragment in patients carrying a duplication has facilitated carrier identification. Before DNA analysis, it was not certain that the mother of the boy with DMD (fig 4a) and the sister of the boy with BMD (fig 4b) were carriers since both had normal serum creatine kinase activity and the affected boy is the only patient in each of these families. The identification of a duplication junction fragment clearly shows their carrier status. Furthermore, since the unique junction fragment was not detected in the grandparents of case 3, this indicates that the duplication arose in the grandparental generation. We are currently performing RFLP analysis on this family, as well as cloning and sequencing the duplication junction point, in order to determine the origin and the molecular basis of the duplication event.

It is of interest that a duplication within the DMD/BMD locus can cause DMD in two boys and the milder BMD in another. The situation is similar for deletions, as there have been many DMD patients and at least four BMD patients reported.[8][31] All three duplication cases described here have at least one exon that is duplicated, but the relationship between the specific duplication and the severity of the phenotype is unknown. One possibility is that certain exons encode protein domains that have especially important functional roles and that duplication of these exons leads to an alteration of the structure and function of the protein. As an example, the duplication of seven exons which encode the ligand binding domain of the protein in the LDL receptor gene results in an elongated receptor with reduced binding capacity for LDL.[25] A second possibility is that the duplication of certain exons could result in a frame shift of the nucleotide sequence in the message and subsequently produce a dysfunctional protein. Nucleotide sequence analysis of human genes has revealed that

exons do not necessarily contain an integral number of triplet codons[22][23] and duplication or deletion of a segment of DNA containing such an exon would cause a frame shift mutation. The consequence of a frame shift duplication would be expected to be more severe in general than that of an in frame duplication. In our duplication cases perhaps an in frame duplication is responsible for the BMD mutation, whereas a frame shift duplication may be the mutation in the two DMD cases. Nucleotide sequence analysis of the duplicated exons or the analysis of the mRNA from these patients or both would test this hypothesis. A detailed molecular understanding of these duplications in relation to the disease severity may give important insights into the structure/function relationships within this large and highly mutable locus.

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