

## Original articles

# Screening of deletions in the dystrophin gene with the cDNA probes Cf23a, Cf56a, and Cf115

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### Abstract

We have analysed 38 DMD patients from 34 families and 30 BMD patients from 12 families using the cDNA probes Cf23a and Cf56a, which map near the centre of the dystrophin gene, and Cf115, which is close to the 3' end of this gene. Together, probes Cf23a and Cf56a detected deletions in 50% of the DMD families and 33% of the BMD families. Probe Cf115 detected a deletion in only one DMD patient, which has not been reported before in severe X linked myopathy. Most of the DMD deletions could be detected with Cf56a while all four BMD deletions were detected with Cf23a. The pattern of deletions could not be used to predict the precise clinical course of the disease and no correlation was found between the severity of the disease and the extent of the gene deletion. A higher frequency of deletions was observed in sporadic (73%) compared with familial DMD (28%) and BMD cases (33%). This result, if confirmed in a larger sample, would have important implications for genetic counselling.

Duchenne muscular dystrophy (DMD) is one of the

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most frequent and distressing X linked diseases of childhood with an incidence of 1:3500 male births. The first symptoms become manifest at 3 to 5 years of age with affected boys becoming wheelchair bound before 13 years of age, and death occurring before the third decade of life.<sup>1</sup> The allelic disorder, Becker muscular dystrophy (BMD), has a much milder clinical course and affected patients remain ambulant until 16 years of age or later.<sup>2</sup> Some patients who become wheelchair bound between 13 and 16 years of age have been classified as outliers.<sup>3</sup> Until an effective treatment is found to cure or arrest the progression of the disease, prevention of new cases through identification of carriers and prenatal diagnosis is fundamental.

Cytogenetic observations of females suffering from DMD who had balanced X;autosome translocations,<sup>4-6</sup> together with modern molecular technology, led to the localisation of the DMD gene to Xp21.<sup>7</sup>

DNA investigations of a large number of cases have shown that about 10% of DMD/BMD patients have deletions detectable with the genomic probes pERT87,<sup>8</sup> XJ1.1,<sup>10</sup> and HIP25,<sup>11</sup> <sup>12</sup> which has been confirmed with cDNA probes.<sup>13</sup> <sup>14</sup> The cDNA probes Cf23a and Cf56a (probes 7 and 8 from Koenig *et al*<sup>14</sup>), which are contiguous and located near the centre of the gene, have been reported to detect approximately 50% of deletions in DMD/BMD patients.<sup>13-16</sup>

No deletions near the 3' end of the gene have been identified in affected patients with a clinical phenotype typical of Duchenne or Becker muscular dystrophies. The only case, detected with the dystrophin cDNA 11-14, was reported by Darras and Francke<sup>17</sup> in a patient with a mild, non-progressive myopathy and glycerol kinase deficiency.

According to Hoffman *et al*,<sup>18</sup> the clinical course of the disease is related to quantitative (in DMD and intermediate dystrophy) or qualitative alterations (in BMD) in the muscle protein dystrophin. However, no clear correlation between the extent or site of deletions

in the dystrophin gene and the clinical phenotype has been found, and the spectrum of clinical variability from the most severe DMD to the most benign BMD is not yet understood.<sup>12</sup> Monaco *et al*<sup>19</sup> proposed the frameshift model to account for the differences in clinical severity between Duchenne and Becker muscular dystrophies. Malhotra *et al*<sup>20</sup> observed, however, that in some patients the reading frame was altered and yet the clinical evolution was benign.

Therefore, further studies in screening for deletions across the DMD/BMD gene in a large sample of well characterised affected patients would serve two purposes: first, in aiding prenatal diagnosis and genetic counselling and, secondly, to understand the molecular mechanism(s) by which mutations in the same gene result in a wide range of clinical variability.

The present study was undertaken with the following aims: (1) to estimate the frequency of deletions of Brazilian DMD and BMD patients using the cDNA probes Cf23a, Cf56a, and an additional cDNA probe Cf115; the latter is contained within cDNA 11–14 reported by Koenig *et al*<sup>14</sup>; and (2) to compare the frequency of deletions in isolated and inherited DMD cases.

## Materials and methods

### SUBJECTS

A total of 38 patients belonging to 34 DMD families and 30 patients from 12 BMD families was included in the present investigation. All Duchenne cases had a clinical evolution compatible with severe DMD, with the exception of one isolated case, who was still ambulant at the age of 13. All Becker patients had a mild clinical course and at least one older affected relative (through maternal lines) with a benign evolution. Among the DMD patients, 11 were familial cases, 22 were isolated cases, and one boy was an adopted child. All the families were ascertained at the Centro de Miopatias at the University of São Paulo, Brazil. Diagnosis was confirmed through clinical examination and evolution, family history, grossly raised serum creatine kinase (CK) and pyruvate kinase (PK), and typical muscle biopsy or electromyography or both.

All DNA analyses were performed at the Nuffield Department of Clinical Medicine, University of Oxford, England. DNA extraction was performed according to the method of Kunkel *et al*<sup>21</sup> and DNA was digested with the restriction enzyme *Pst*I.

DNA was electrophoresed in 0.8% agarose and blotted to nylon membranes (Hybond N, Amersham) according to the method of Southern.<sup>22</sup> The blots were hybridised with the cDNAs Cf23a, Cf56a, and a 1.2 kb *Hind*II fragment from Cf115.<sup>13 15</sup> These cDNAs were labelled with <sup>32</sup>P by hexanucleotide primed synthesis.<sup>23</sup> The filters were washed three

times in standard saline citrate at 65°C and auto-radiographed for one to five days at -70°C.

## Results

The patterns and the frequency of all deletions detected with Cf23a and Cf56a in DMD and BMD patients are shown in fig 1. The band patterns seen with these probes are shown in fig 2.

### DMD PATIENTS

A total of 17 deletions was detected with two of the three cDNA probes studied among 34 families with DMD patients: 44% (15/34) with Cf56a and 21% (7/34) with Cf23a. Five of the seven deletions detected with Cf23a extended to the sequence seen with Cf56a.

The above deletions were observed among 13 of the 22 isolated cases, three of the 11 familial cases, and the adopted boy. However, in seven of the former group, one or more females at risk for the DMD gene had high serum CK and PK activities, suggesting that the gene had been inherited. If such cases are analysed together with the familial ones, then the frequency of deletions among the inherited cases is 28% (5/18), compared with 73% (11/15) among the isolated cases. This difference is statistically significant ( $Z=3.88$ ;  $p<0.001$ ).

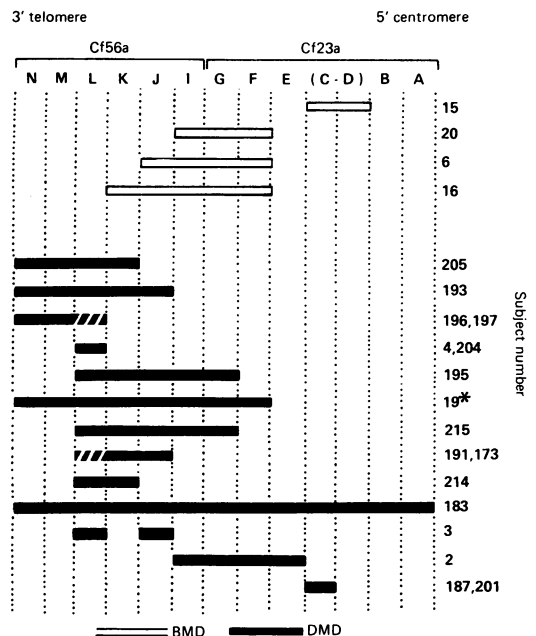


Figure 1 Patterns and frequencies of deletions seen with the cDNA probes Cf23a and Cf56a in DMD/BMD patients. Exon H, which has been reported previously,<sup>24</sup> does not exist. The hatched bars indicate uncertainty regarding the extent of a deletion. \*Outlier.

Only one DMD patient had a deletion in the region detected by Cf115. The DNA of this patient was further digested with *Hind*III and *Bam*HI and hybridised with Cf77a.<sup>15</sup> This cDNA hybridises with sequences proximal to the 1.2 kb *Hind*III fragment of Cf115. This analysis showed that the 5' end point of this patient's deletion starts in Cf77a and extends as far as the 3' end of Cf115, which contains the translation stop signal. The precise distal endpoint is still under investigation.

Two of our families with DMD patients were of particular interest since the affected patients were related through paternal lines (fig 3). In the first

family (fig 3a), two different deletions were found in the affected patients using Cf56a. Patient IV.1 had a complex deletion which removed exons J and L, but not exon K, whereas his affected relative, patient IV.3, was deleted for exons E through to I. The patients' mothers and other female relatives had normal serum enzyme levels, which suggests two separate de novo DMD mutations in this family. In the second family (fig 3b), two affected brothers belonged to the same sibship (their mother and two sisters had grossly raised serum enzyme levels) while their affected DMD cousin was an isolated case (his mother and two sisters had normal enzyme levels). No

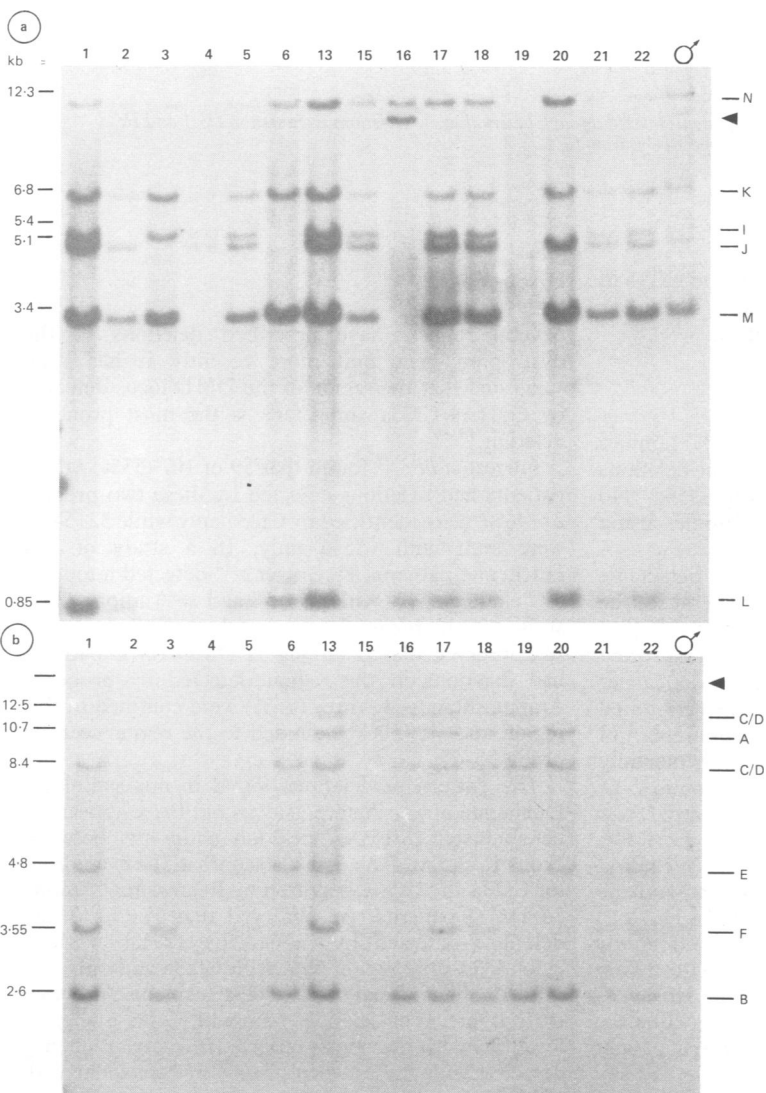


Figure 2 Band patterns with Cf56a (a) and Cf23a (b). Lanes 1-4, 14, and 15 are DMD patients, all other tracks are BMD patients. The arrow indicates RFLP. The letters on the right correspond to the order of the exons of Cf56a and Cf23a, respectively, from the 5' to the 3' end.

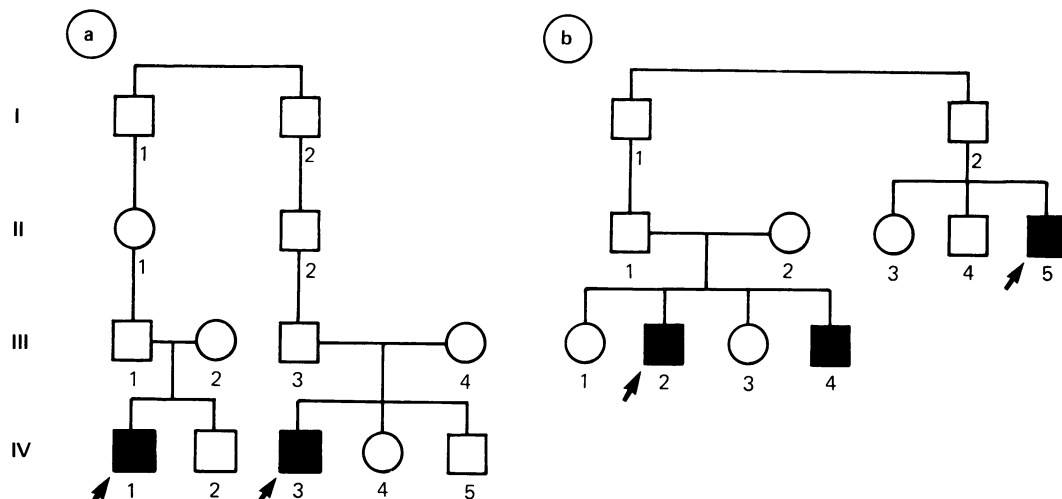


Figure 3 Pedigrees of two families with DMD patients related through paternal lines. Deletions were seen in IV.1 and IV.3 (family a), which correspond to subjects 3 and 2, respectively, from fig 1.

deletion was detected with Cf23a, Cf56a, and Cf115 in the two affected brothers from the same sibship or with probe Cf56a in their affected cousin.

#### BMD PATIENTS

Deletions were detected in four of 12 (33%) families with BMD patients using Cf23a; three of the deletions extended to the region detected with Cf56a. No deletion was observed in our BMD families using Cf115.

Two of the four BMD families with detectable deletions were of particular interest because the patients were discordant for mental capacity. In the first family, the affected grandfather had five unaffected and seven affected grandsons through his six carrier daughters. Five of the BMD patients also exhibited abnormal behaviour (diagnosed as schizophrenia); of the remaining two, one, aged 38 years, was mentally normal and the youngest, aged 4, was too young to be psychologically evaluated. No mental disorder was observed among the five unaffected subjects. DNA was analysed in five of the BMD patients (including the only one with no mental disorder) and no differences in the pattern of deletion (subject 16, fig 1) were observed among them. In the second family (subject 6, fig 1), two affected brothers with a very mild myopathy exhibited the same deletion. However, the older brother is of normal intelligence while the younger is mentally retarded. No other BMD patient among the 30 studied in the present sample had any mental disorder.

#### Discussion

##### DMD PATIENTS

Several reports have described deletions in the dystrophin gene and more recently it has been suggested that the region of the DMD locus detected by cDNAs Cf23a and Cf56a is the most prone to deletion.<sup>13-16</sup>

Forrest *et al*<sup>15, 25</sup> found that 59 of 107 (55%) DMD patients had deletions detected by these two probes: 27 (46%) were identified by Cf23a only while 32 (54%) were seen with Cf56a only. In a study of 104 Duchenne patients, Koenig *et al*<sup>14</sup> detected a total of 53 deletions: 51% with probe 8 and 36% among these started in the region detected with probe 7. Darras *et al*<sup>16</sup> observed that 12 among 32 DMD/BMD patients had deletions in the region detected by probe 8. Among these, only three (25%) were confined to this region and six (50%) extended to the region seen by probe 5b-7.

The pattern of deletions found in our sample of Duchenne boys is heterogeneous but there appears to be a cluster (8/17) of deletion endpoints between exons K and M. The data also confirm the exon order for Cf23a and Cf56a, reported by Read *et al*.<sup>24</sup> Among 34 DMD patients, we observed that 17 (50%) had deletions detected by Cf56a: five (29%) of them extended to the region seen with Cf23a and only two (12%) were confined to the Cf23a segment. Although the proportion of deletions seen with Cf23a is slightly lower than that previously reported, the total frequency detected with these two probes is in agreement with published data.<sup>13-16</sup> Our observations suggest that the

DMD deletions are confined mainly to Cf56a, in accordance with Forrest *et al.*,<sup>25</sup> who pointed out that the screening of DMD patients should begin with the Cf56a probe.

Our finding of a deletion detected with the 3' DMD cDNA Cf115 in a boy with DMD is apparently new. This patient has a clinical evolution typical of severe DMD and has two affected maternal uncles who died at 13 and 15 years of age. He has no glycerol kinase deficiency or other associated X linked disorder. Only one deletion in the 3' end of the dystrophin gene has been reported so far, in a patient with a very mild non-progressive myopathy and glycerol kinase deficiency.<sup>17</sup> According to Darras *et al.*,<sup>16, 17</sup> deletions near the 3' end of the gene would be expected to cause a milder and non-progressive myopathy and should not be found in DMD/BMD patients, since the cDNA used for detection of this region of the gene is mostly untranslated sequence. Therefore, the region that is missing in our patient seems to be fundamental for the expression of the DMD phenotype.

The deletion found in our DMD boy begins in the region detected by probe Cf77a and extends towards the 3' end of the gene, including the translation stop signal.

#### BMD PATIENTS

In a study of BMD patients, Forrest *et al.*<sup>15</sup> reported that 17/36 (47%) had deletions detected with probe Cf23a and that 16 of these 17 were also detected with Cf56a, but no new deletions were detected with Cf56a. Darras *et al.*<sup>16</sup> observed deletions in three BMD cases using probe 8 (equivalent of Cf56a), and these extended towards the 3' end. In our sample of 12 families with BMD, four (33%) had deletions detected with Cf23a, and three of these four extended to the sequence seen with Cf56a. The deletions observed in our BMD families are similar to those reported by Forrest *et al.*,<sup>13, 15</sup> suggesting that BMD mutations are particularly concentrated in the Cf23a region. However, more BMD families should be studied to determine if deletions extend to the Cf56a region.

An interesting finding was the two BMD families with affected patients discordant for mental capacity but apparently with the same pattern of DNA deletions. Intrafamilial variability of clinical evolution with apparently identical DNA deletions has been reported,<sup>13</sup> suggesting that other factors, such as genetic background, may interfere in the clinical phenotype.<sup>13, 20</sup> Furthermore, these results suggest that the mental disorder in our patients is not only related to abnormal dystrophin.

The deletions observed in our BMD patients were homogeneous to the extent that the 5' endpoints of three of the four patients were located in the same intron. The BMD patient deletions were not observed

in any DMD patient, with the exception of case 19 (fig 1) who is still ambulant at the age of 13 and might be an outlier or a case of BMD. Furthermore, no correlation was found between the clinical severity and the extent of the gene deletions, as reported previously by other authors.<sup>16, 17</sup> In order to predict if a patient has DMD or BMD as proposed by Read *et al.*,<sup>24</sup> these studies should be extended to a larger sample of patients, in particular those with a positive family history. This criterion would ensure that patients with a clinical picture similar to DMD but with autosomal recessive inheritance are not included in the analysis.

#### FREQUENCY OF DELETIONS DETECTED WITH CF23A AND CF56A IN FAMILIAL AND ISOLATED CASES

An interesting observation was the higher frequency of deletions detected in sporadic (73%) compared with inherited (28%) cases of DMD using the probes Cf23a and Cf56a. Although the sample size is small, a similar proportion of deletions was estimated for BMD, who are all familial cases. Kunkel *et al.*<sup>9</sup> reported a higher frequency of deletions in familial than in isolated cases for the pERT87 region; however, this difference was not statistically significant. Furthermore, no significant differences between sporadic and familial DMD cases were observed in the pERT87, XJ1.1, JBir, and HIP25 regions.<sup>12</sup> There is no such comparative study for the probes Cf23a and Cf56a in DMD and BMD families.

Our preliminary results, if confirmed in a larger sample, would support the previous hypothesis of two different mechanisms for the origin of DMD mutations.<sup>26, 27</sup> One, mainly occurring in the male germline, would correspond to the usual type of point mutation in the dystrophin locus, and therefore more frequently inherited through the maternal grandfather. The other, restricted to female meiosis, would result from unequal crossing over, as suggested recently by the reports on duplication of segments in the DMD gene.<sup>28, 29</sup> If deletions could arise more frequently in ova than in spermatozoa through different mechanisms such as unequal crossing over, it would explain the higher deletion frequency found in sporadic DMD patients than in familial DMD or BMD cases. The similar frequency of deletions between sporadic and familial cases observed in the 5' end of the gene<sup>9, 12</sup> could be a result of the smaller percentage of deletions detected in this region which would make it more difficult to test this effect. An alternative hypothesis is that deletions in the central region of the gene may be more deleterious and therefore more easily transmitted through the ovum.

If the deletions detected with Cf23a and Cf56a are confirmed to be more frequent in patients who present as new mutations, this will have important implications for genetic risk estimates, in particular in

mothers and sisters of isolated cases, owing to the difficulty of detecting deletions in female carriers.

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