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Alternative splicing of dystrophin mRNA complicates carrier determination: report of a DMD family

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Abstract

Carrier determination is important for genetic counselling in DMD/BMD families. The detection of altered PCR amplified dystrophin mRNA fragments owing to deletions, insertions, or point mutations has increased the possibilities of carrier determination. However, problems may occur because of alternative splicing events. Here we present a family with a DMD patient characterised by a deletion of exons 45 to 54. At the mRNA level we detected a corresponding altered fragment which served for carrier determination. The mother and the sister of the patient showed the same altered dystrophin mRNA fragment as the patient and are therefore carriers. In the mother two additional altered dystrophin mRNA fragments were detectable, obviously resulting from alternative splicing in the normal allele. The grandmother and two other related females of the patient possess only the normal mRNA fragment. In a further female we detected an altered fragment owing to an mRNA deletion of exon 44. This fragment is created either by alternative splicing or a new mutation. Therefore, the carrier status of this female is still ambiguous indicating problems in carrier determination by the method of dystrophin mRNA analysis.

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Carrier determination is important for genetic counselling of families with Duchenne or Becker muscular dystrophy (DMD/BMD) and includes biochemical, physical, and molecular analyses. DNA based methods involve linkage analysis and mutation detecting techniques like gene dosage estimation or junction fragment detection.[1–6] The introduction of DNA analysis has resulted in substantial progress in carrier determination, but misdiagnoses owing to crossover events and misinterpretations of gene dosage data cannot be neglected. For deletions, the detection of a junction fragment is a rare event requiring the technically difficult pulsed field gel electrophoresis. The analysis of dystrophin mRNA transcripts from peripheral blood lymphocytes[7 8] has increased the possibilities for carrier determination.

Using a system consisting of reverse transcriptase and 10 sets of overlapping nested PCR reactions it is possible to amplify every region of the 11 kb dystrophin coding sequence.[9 10] Reactions bridging deletions or duplications result in altered PCR products.[11–14] Here we report a DMD family in which carrier determination using this method was complicated by the presence of alternative splicing events.

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Material and methods

REVERSE TRANSCRIPTION AND NESTED PCR

Total RNA was prepared from peripheral blood lymphocytes[15] and about 500 ng of total lymphocyte RNA were transcribed using the primers DMD N7b and DMD 8b. Nested PCR was performed as described by Roberts *et al*[10]: 10 µl of the PCR assay was electrophoresed in a 2% agarose gel.

PRIMER SEQUENCES

Set 7

DMD N7a	TCATAGCAAGAAGACAGCAGC
DMD N7b	CTCGTTGATATCCTCAAGGTC
DMD N7c	GTGGAAAGGGTGAAGCTACAG
DMD N7d	ACTTGATCAAGCAGAGAAAGC

Set 8 (10)

DMD 8a	CTAGAAATGCCATCTTCCTTG
DMD 8b	CTCAGGAGGCAGCTCTCTGG
DMD 8c	CTGCTCTGGCAGATTTCAAC
DMD 8d	GGGCTCCTGGTAGAGTTTCTC

DIRECT SEQUENCING OF PCR PRODUCTS

Nested PCR products were purified from 2% agarose gels using USBioclean MP (USB) and sequenced with fmol DNA Sequencing System (Promega) using $^{32}P\,\gamma$ ATP labelled primer DMD N7c.

Results

Fig 1 shows the pedigree of the DMD family requesting genetic counselling. A deletion of exon 45 to 54 inclusive was identified in patient III·5 by Southern transfer/hybridisation and PCR. A junction fragment to be used for carrier determination was not detectable. Therefore we used primer sets 7 and 8 covering exons 43 to 51 and 51 to 58 respectively for dystrophin mRNA analysis.

PCR products resulting from set 7 amplifications are summarised in fig 2. For the DMD patient III·5, who is deleted for exon 51, the target sequence of primers N7b,d, no PCR product is visible. The females I·2, II·2, II·4, III·2, III·6, and III·8 show a normal full sized fragment of 1271 bp. In the mother (II·2) of the patient two additional fragments of 797 bp and 611 bp occur. By sequencing we identified

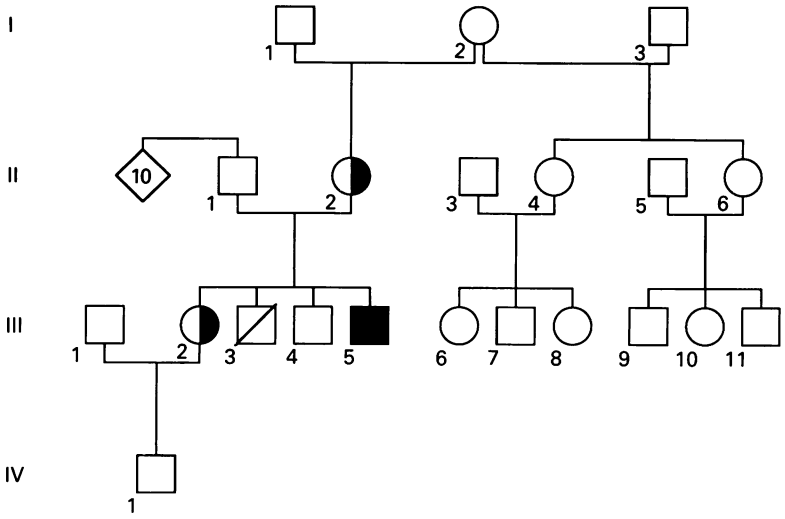

Figure 1 Pedigree of the DMD family requesting genetic counselling. Genomic DNA of patient III·5 was analysed by Southern transfer/hybridisation with cDNA probes cf56a,b and by PCR covering exons 41 to 55. A DNA deletion of exons 45 to 54 was detectable. Family members I·2, II·2, II·4, III·2, III·5, III·6, and III·8 were analysed by nested amplification of reverse transcribed mRNA (RT-PCR).

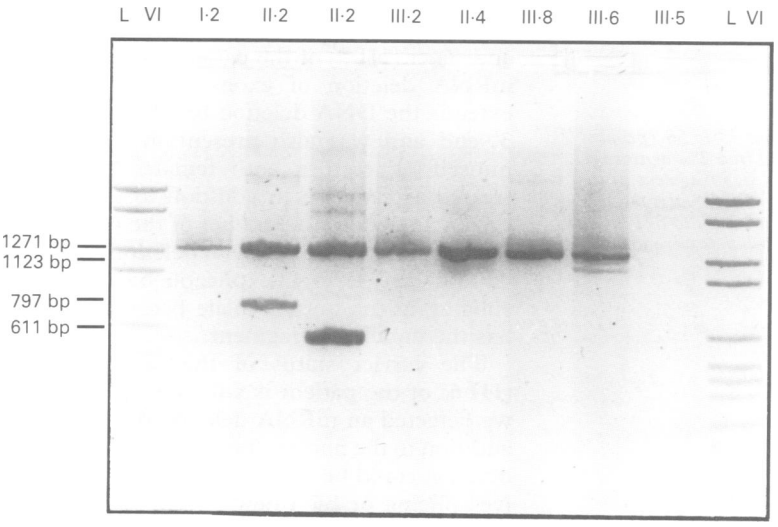

Figure 2 Products of nested RT-PCR spanning exons 43 to 51 (set 7, see also diagram in fig 7) shown in an ethidium bromide stained 2% agarose gel. For the DMD patient III·5 a signal is missing owing to the deletion of exons 45 to 54. The mother (II·2) has two altered fragments of 797 bp and 611 bp in addition to the full sized 1271 bp product. Female III·6 shows a faint additional band of 1123 bp.

an mRNA deletion of exons 45 to 47 for the 797 bp fragment (fig 3) and an mRNA deletion of exons 45 to 48 for the 611 bp fragment. The female III·6 also shows an altered fragment of 1123 bp. The sequencing data show an mRNA deletion of exon 44 (fig 4).

By use of primers 8b,d and 7a,b, which bridge the DNA deletion, a normal full sized PCR product of 2460 bp is expected (fig 5). All females show a normal full sized fragment. In addition, the mother (II·2) and sister (III·2) of the patient have a smaller fragment of 681 bp. The same fragment is also detectable in the DMD patient. By sequencing we observed an mRNA deletion of exons 45 to 55 (fig 6), which includes one more exon than in the DNA deletion. In addition to this fragment the patient has two other PCR products of 871 bp and 723 bp. Sequencing data indicate that they

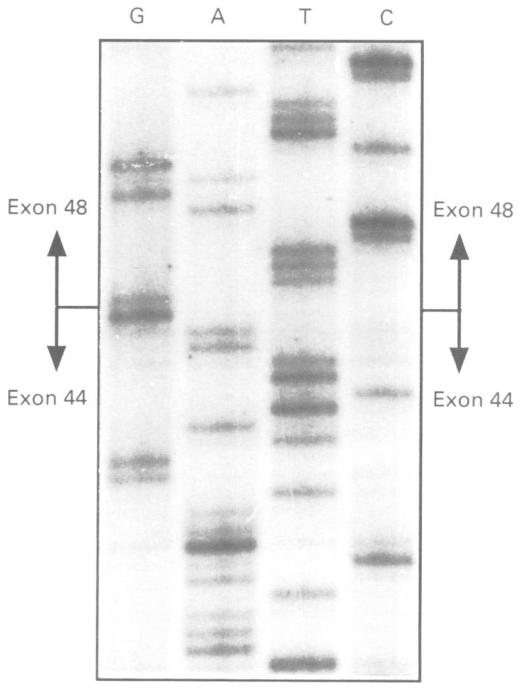

Figure 3 Direct sequence analysis of the 611 bp fragment of female II·2 amplified with primer set 7. The sequence data indicate splicing of exon 44 into exon 48. Therefore exons 45 to 47 are missing from the mRNA transcript.

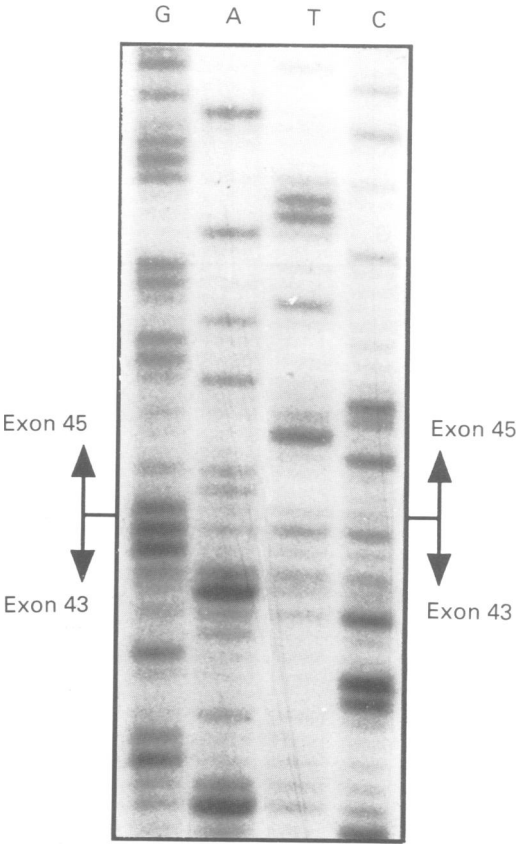

Figure 4 Direct sequence analysis of the 1123 bp band of female III·6 amplified with primer set 7. Splicing of exon 43 into exon 45 indicates that exon 44 is missing from the mRNA transcript.

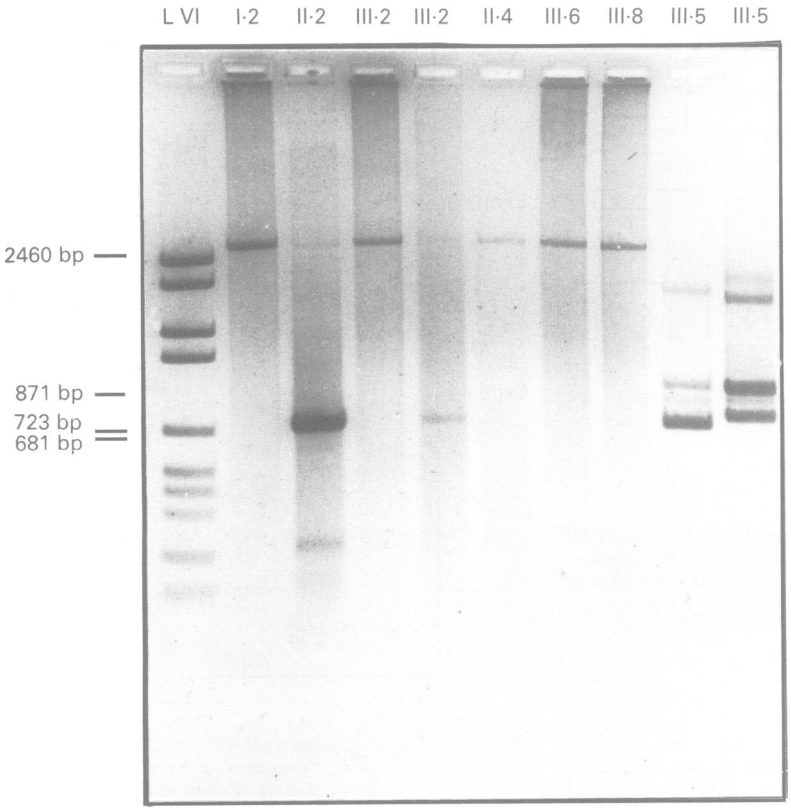

Figure 5 Summarised results of nested RT-PCR crossing exons 43 to 58 (primer combinations: N7a–8b; N7b–8). Products were electrophoresed in a 2% agarose gel. The mother II·2 and sister III·2 have the same 681 bp reduced size fragment as the DMD patient III·5. Therefore they were identified as carriers. All females tested show the normal fragment of 2460 bp. Patient III·5 has additional products of 871 bp and 723 bp representing alternative spliced transcripts. Some higher bands were identified as heteroduplex molecules.

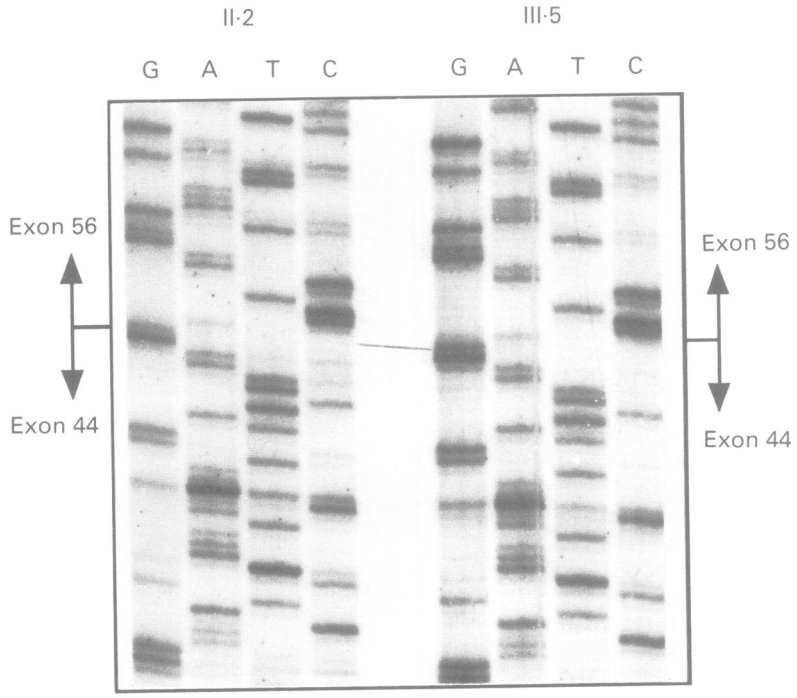

Figure 6 Direct sequence analysis of the 681 bp product of the mother II·2 and patient III·5. Data indicate the loss of exons 45 to 55 from the dystrophin mRNA transcript.

are produced from an mRNA deletion of exons 45 to 54 and 44 to 54 respectively.

A summary of all detectable RNA deletions compared to the DNA deletion is given in fig 7.

Discussion

The detection of altered PCR amplified dystrophin mRNA fragments owing to deletions, insertions, or point mutations has created new possibilities for carrier determination in DMD/BMD families. The DMD family presented in this paper underlines a problem in the application of this method for carrier determination because of alternative splicing events.

In the DMD patient of this family, we found an out frame DNA deletion of exons 45 to 54. The same deletion was also detectable at the RNA level. Alternative splicing events on the 5′ or 3′ end of the deletion, extending it by an exon, create two in frame RNA deletions of exons 45 to 55 and 44 to 54 respectively.

For the grandmother (I·2), aunt (II·4), and one cousin (III·8) of the patient, dystrophin mRNA analysis shows no altered fragment resulting from deletion. For the mother (II·2) and sister (III·2) of the patient we detected an mRNA deletion of exons 45 to 55 which extends the DNA deletion by one exon at the 3′ end and was also present in the patient himself. Therefore both females should be treated as carriers. In addition to the disease causing deletion, the mother of the patient has two further in frame mRNA deletions of exons 45 to 47 and 45 to 48 explicable by alternative splicing in the normal allele because she also has the undeleted fragment.

The carrier status of the second cousin (III·6) of the patient is still ambiguous. Here we detected an mRNA deletion of exon 44 in addition to the normal fragments. This mRNA deletion could be explained either by alternative splicing or by a new mutation. Although PCR product analyses of exon 44 at the DNA level (data not shown) indicate no strong gene dosage effect the latter possibility cannot be completely excluded.

This family illustrates a problem for the application of dystrophin mRNA analysis for DMD/BMD carrier diagnosis owing to various alternative splicing events and the possibility of new mutations.

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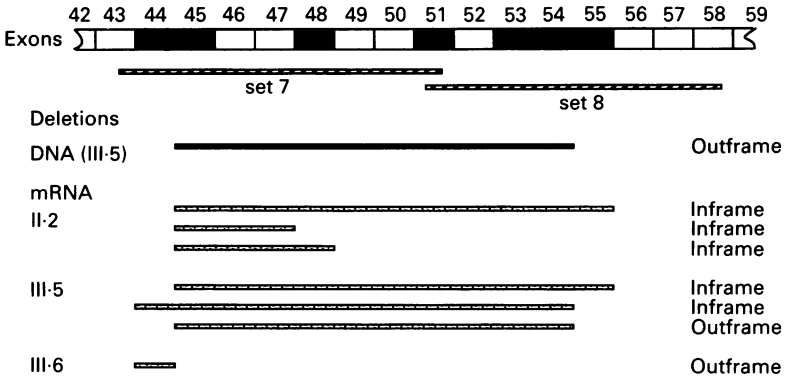

Figure 7 The diagram summarises all detectable DNA and mRNA deletions in the region of exons 43 to 58. Shaded boxes represent exons which were amplified from genomic DNA. Regions of dystrophin transcript which were amplified by RT-PCR are designated beneath the exon diagram (set 7 and set 8). Black bar represents the extent of DNA deletion identified with cDNA probes cf56a,b and PCR of genomic DNA. Shaded bars designate the extent of all detectable mRNA deletions in the family members tested in our study. Effects on the translational reading frame are indicated to the right of the diagram.

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