Identification of a new DMD gene deletion by ectopic transcript analysis

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Abstract

The detailed genetic analysis of the Duchenne/Becker muscular dystrophy gene is hindered by the large number of exons involved and their separation by huge introns. These problems can be overcome by the analysis of mRNA rather than genomic DNA and ectopic transcripts derived from peripheral blood lymphocytes provide a convenient source of material. Using reverse transcription and nested PCR, we show here a comprehensive strategy for the rapid and complete analysis of the coding sequences from complex genes and illustrate its potential by the identification of a hitherto undescribed single exon deletion.

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Duchenne muscular dystrophy (DMD) is an X linked recessive disorder affecting approximately 1 in 3500 newborn males.[1] In contrast to the mild allelic Becker muscular dystrophy (BMD), the Duchenne type is progressive and usually results in death during the second decade of life. The cDNA of the gene responsible for DMD/BMD has been cloned[2] and sequenced.[3] The gene product has been named 'dystrophin' and is encoded by an mRNA of 14 kb derived from at least 74 exons spread over 2300 kb of the X chromosome.[4]

The extraordinary size of the gene causes extreme difficulties in molecular analysis. Although some 60% of DMD patients exhibit deletions, detection of these requires Southern blots and hybridisation with six different radiolabelled cDNA clones.[5] Alternatively, 98% of the deletions may be detected after amplification of 19 different genomic fragments.[6-8] Since both procedures are difficult to perform for quantitative evaluation, and since only 13% of deletions result in a readily detectable junction fragment,[9] pulsed field gel electrophoresis is occasionally used for carrier detection in female relatives of Duchenne patients with deletions. This laborious procedure must also be applied to the detection of duplications, exhibited by another 6% of patients.[10]

The remaining one-third of patients are thought to carry mutations in promoter elements or point mutations and microdeletions either in the coding sequence or in splice site consensus sequences. Until recently, these mutations could not be detected, leaving the families to be counselled with rather unsatisfactory estimates of risk. Although a number of intragenic RFLPs are known and have been used for segregation analysis,[11] indirect approaches are complicated by an intragenic recombination rate of up to 12%[12] and the occurrence of new mutations in one-third of isolated cases.[13] The use of flanking RFLP markers or the recently reported SSCP analysis of deleted regions[14] might prove helpful in a fraction of cases, but are limited to informative allele constellations.

Bulman *et al*[15] reported the first point mutation in the DMD gene. Western blot analysis of biopsied muscle material indicated a truncated protein. Subsequent cDNA sequence analysis in the implicated region of specifically expressed muscle transcripts showed a base substitution, creating a stop codon within exon 26. RNA analysis, in principle, should be much more straightforward for the analysis of the DMD gene since it reduces the size of the substrate to be studied to a coding region of approximately 11 kb. Both procedures used by the above authors, however, required a muscle biopsy to obtain expressing tissue.

We and others have previously shown that 'ectopic' or 'illegitimate' transcription[16,17] in peripheral blood lymphocytes can be exploited to circumvent muscle biopsies for RNA studies and that pathological DMD/BMD transcripts resulting from a genomic deletion can be detected in both hemizygous patients and heterozygous carriers.[18,19] Roberts *et al*[20] have already shown that this procedure can be used for a comprehensive analysis of the complete coding region without the use of radioactive substrates. However, all such studies to date were aimed at deletions already characterised at the genomic level. We report here a new DMD gene mutation, identified exclusively by means of ectopic lymphocyte cDNA analysis in a patient with no apparent deletion after extended multiplex DNA amplification.

Case report

The patient is of Polish origin. His early development was normal, and he was able to walk at 16 months. His family history was unremarkable. Progressive weakness, frequent falls, and the inability to climb stairs led to a clinical examination at the age of 4 years. He showed all the clinical symptoms of muscular dystrophy. His CK was raised to 13526 U/l (normal up to 50 U/l). The muscle biopsy showed the typical dystrophic changes of DMD with negative staining for dystrophin using the two antibodies NCL-Dysl and NCL-Dys2 (Novocastra Laboratories). No genomic deletion was detected after multiplex amplification of 19 fragments, performed

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according to Chamberlain *et al*[6,7] and Beggs *et al*[8] with minor modifications.

Materials and methods

RNA was isolated from peripheral blood lymphocytes and reverse transcribed as described elsewhere,[18] using eight cDNA primers pooled in two sets of four 'odd' and four 'even' oligonucleotides (50 ng each) in a total volume of 30 µl. A total of 7 µl of the cDNA products was used for first round PCRs with eight oligonucleotide pairs spanning the whole coding region as overlapping fragments. Cycling conditions were: 30 × one minute at 92°C, 45 seconds at 53°C, three minutes at 72°C, final extension seven minutes, with 50 ng of each oligonucleotide, 0·2 mmol/l dNTPs, and 2·5 units *Taq*-DNA-polymerase (Amersham, UK) in a total volume of 50 µl, according to the manufacturer's recommendation, in a thermocycler type 60 (Biomed, FRG).

Second round PCR was performed with 1 µl of the first round product and nested primers as described above. A total of 5 µl of second round PCR products was electrophoresed in 1·2% agarose and visualised by ethidium bromide staining. Direct sequencing of PCR products was performed according to Zielenski *et al*[21] All oligonucleotides were synthesised automatically (392 DNA/RNA synthesiser, Applied Biosystems, USA) and purified on Nensorb columns (DuPont, FRG). Sequences and usage of the different oligonucleotides are summarised in the table.

Genomic DNA was isolated by the method of Miller *et al*[22] and amplified with 50 pmol of primers 21F (5'GATGAAGTCAACCGGCTATC, identical to the first bases of exon 21) and 21R (5'GTCTGTAGCTCTTTCTCTC, two nucleotides upstream of the 3' end of exon 21) in 30 cycles (one minute at 92°C, one minute at 48°C, 90 seconds at 72°C, final extension seven minutes at 72°C) as described above using exon 55 as parallel control (55F: 5'GGCTGCTTTGGAAGAAACTC, 55R: 5'TTACGGGTAGCATCCTGTAGGA, 10 pmol each).

Results

Reverse transcription of peripheral blood lymphocyte RNA and two stage PCR amplification showed a truncated fragment in the DMD patient indicating the loss of approximately 200 bp between DMD gene exons 20 to 23 (fragment 3.2, fig 1). Direct sequencing of this fragment showed that exon 21 was absent in its entirety and that exon 20 was directly spliced to exon 22 (fig 2). Exon 21 is not included in routinely performed 'multiplex' genomic amplifications. Its absence, therefore, could have been caused either by a genomic deletion or by the mutation of a splice site junction leading to exon skipping. In order to distinguish between these two possibilities, exon 21

Sequences of the oligonucleotides used for cDNA synthesis and PCR. The nomenclature refers to the cDNA sequence position (according to Koenig et al[3]) of the first nucleotide. Usage for cDNA synthesis and PCRs (see also fig 1) is indicated. Oligonucleotides homologous to the non-coding strand are marked (R).

Primer sequence (5'-3)		cDNA	1st PCR	2nd PCR
30	ACTCAGATCTGGGAGGCAATTA		1	1·1
996 (R)	TCTTTAGTCACTTTAGGTGGCC			1·1
857	GATGTTGATACCACCTATCCAG			1·2
1409 (R)	GTAGAATATTACCAACCCGGCCC			1·2
1437	CCTGTTCCAATCAGCTTAC	'Odd'	1	
1367	TGTAAAACGACGGCCAGTATGGATTTGACAGCCC		2	2·1
1916 (R)	GGCACTGTTCTTCAGTAAGACG			2·1
1809	AGGTATTGGGAGATCGATGG			2·2
2311 (R)	CTCTTGAGCATGCTTTACCAG			2·2
2850	GATAGCCGGTTGACTTCATC	'Even'	2	
2225	CAGCCATCACTAACACAGAC		3	3·1
2850 (R)	GATAGCCGGTTGACTTCATC			3·1
2766	TGAAAATCCAACCCACCACC			3·2
3486 (R)	AGTCTGCACTGTTTCAGCTGC			3·2
3416	TTTCTGAAGGAGGAATGGCC			3·3
4119 (R)	TCAGAGTTTCCTCAGCTCCG			3·3
4167	GGGTTATCCTCTGAATGTCG	'Odd'	3	
4033	GGAGAAAGCAAACAAGTGGC		4	4·1
4983 (R)	ATATCTGTAGCTGCCAGCC			4·1
5283	GTGATGTGGTCCACATTCTGG	'Even'	4	
4918	GAAATTGTCCCGTAAGATGCG		5	5·1
5283 (R)	GTGATGTGGTCCACATTCTGG			5·1
5233	GGAATACCAGAAACACATGG			5·2
5744 (R)	CCTCTCTCTTTCTCTCATCTG			5·2
5619	GGGTGAATCTGAAAGAGGAAG			5·3
6579 (R)	TCAGCTTCTGTTAGCCACTG			5·3
6742	GGCATCTGTTTTTGAGGATTGC	'Odd'	5	
6477	AAATGTACAAGGACCGACAAGG		6	6·1
6742 (R)	GGCATCTGTTTTTGAGGATTGC			6·1
6604	TCCTGAGAATTGGGAACATGCT			6·2
7562 (R)	TAGTAACCACAGGTTGTGTCAC			6·2
7496	GGACTGACCACTATTGGAG			6·3
8344 (R)	GGCAGTTGTTTCAGCTTCTGTA			6·3
8370	TTACGGGTAGCATCCTGTAGGA	'Even'	6	
8251	GGCTGCTTTGGAAGAAACTC		7	7·1
9084 (R)	GATCCCTTGATCACCTCAGCTTGG			7·1
8903	AATGTCACTCGGCTTCTACGAAA			7·2
9785 (R)	TCCCTGTTCGTCCCGTATC			7·2
9830	TACACAGGGAAATGATGCCAG	'Odd'	7	
9706	CAATTTGGTCAACGTCCCTC		8	8·1
10600 (R)	GCTCTCATTAGGAGAGATGC			8·1
10451	TTCTGGCCAGTAGATTCTGC			8·2
11321 (R)	GATACTAAGGACTCCATCGC			8·2
11380	CATGCGGGAATCAGGAGTTG	'Even'	8	

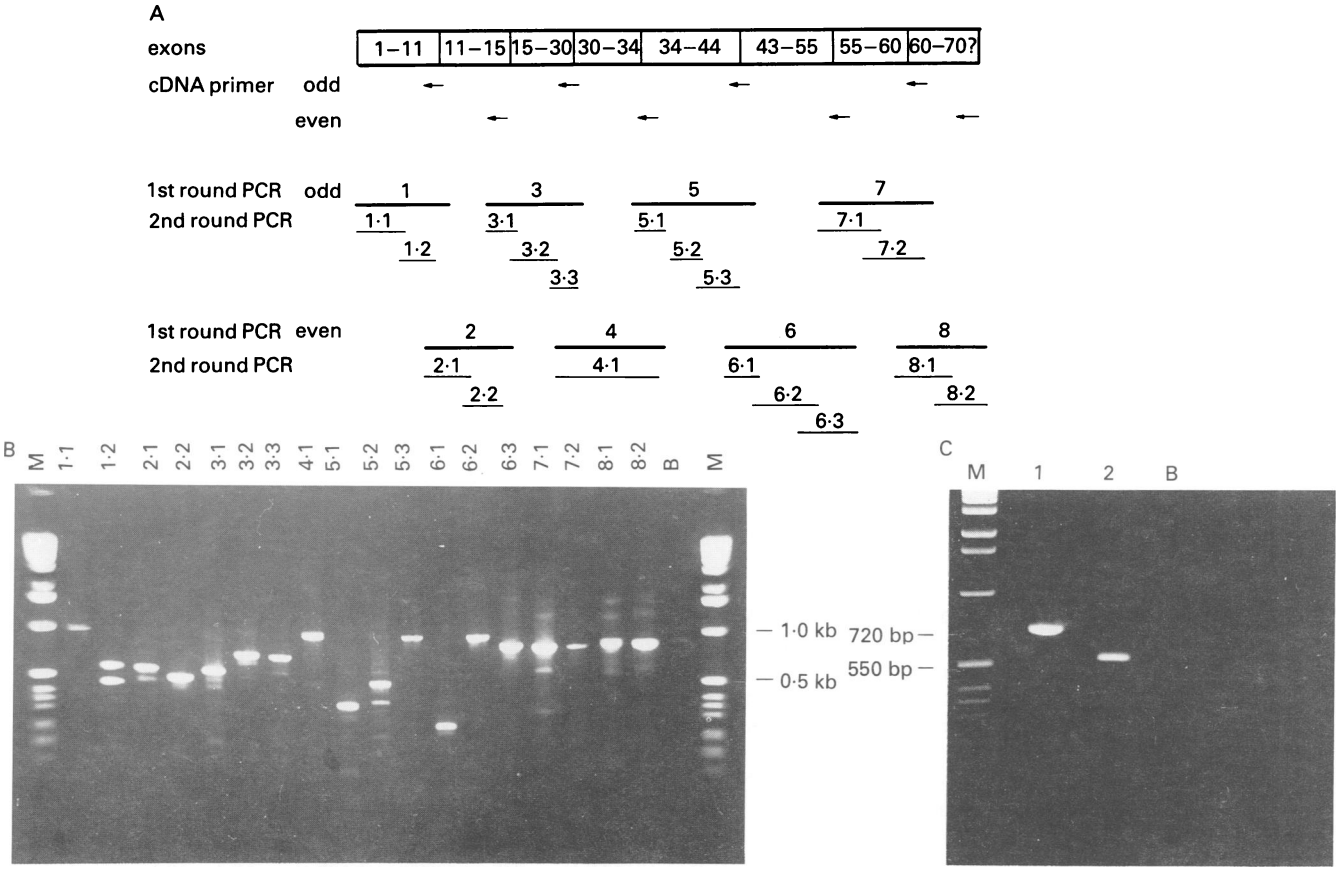

Figure 1 Two stage cDNA amplification of the DMD gene transcript using peripheral blood lymphocyte RNA. (A) Diagram of reverse transcription and amplification strategy. (B) The 18 overlapping fragments of a healthy control after second round PCR and electrophoresis. B blank (no RNA included), M size standard (1 kb ladder, BRL, USA). The doublet band in lane 1·2 is the result of skipping of exon 9 as already observed by Roberts et al.[19] This was confirmed by DNA sequencing (data not shown). (C) Aberrant fragment 3·2 (see A). Lane 1 healthy control, lane 2 DMD patient.

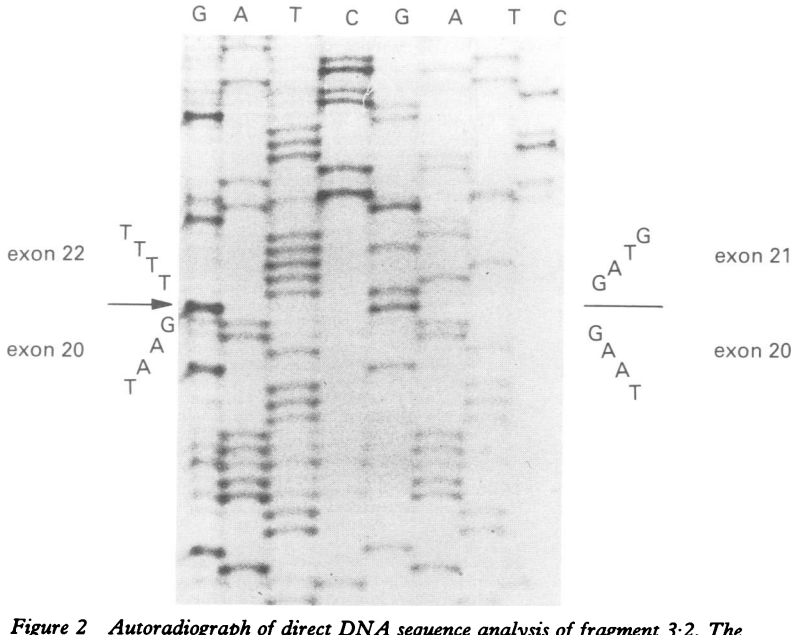

Figure 2 Autoradiograph of direct DNA sequence analysis of fragment 3·2. The patient's cDNA (left) shows direct joining of exons 20 and 22 (arrow) as compared to the exon 20 to 21 junction in a healthy control (right).

specific amplification was performed on genomic DNA (fig 3). This indicated the absence of exon 21 in the patient's genomic DNA. Another 11 DMD patients without an apparent deletion in 'multiplex' amplifications were tested simultaneously, but no other deletion of exon 21 was detected.

Discussion

The limited detection rate of DMD gene mutations does not only complicate family studies, but also drastically diminishes the value of negative results in differential diagnosis. Nevertheless, the screening for deletions alone is extremely laborious and time consuming, since either multiple hybridisations or numerous genomic DNA amplifications have to be performed. The latter concentrate on the two deletion 'hot spots' at the 5′ end and in the middle of the DMD gene. Furthermore, not all exon boundaries are known.

We have previously shown the diagnostic utility of ectopic lymphocyte RNA by the characterisation of a new point mutation in the F8 gene causing haemophilia A,[23] and the detection of pathological DMD gene transcripts in a female muscular dystrophy carrier.[18] Here we show that the DMD gene transcript, which is distributed over approximately 0·1% of the human genome, can be

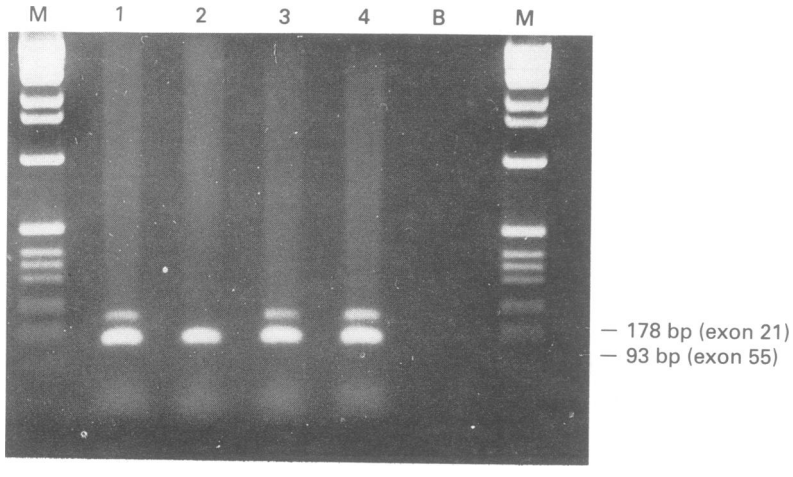

Figure 3 Genomic amplification of exon 21 and 55 (internal control). Lanes 1, 3, 4 healthy controls; lane 2 DMD patient; B blank (no genomic DNA), M size marker (1 kb ladder, BRL, USA).

reverse transcribed in only two separate reactions and subsequently amplified in 18 overlapping fragments. By these means we identified a deletion of exon 21 in the cDNA of a DMD patient, who exhibited no mutation in standard multiplex DNA amplification.

Analysis of the genomic DNA indicated that the 'skipping' of exon 21 resulted from a chromosomal deletion and direct sequence analysis confirmed that exon 20 was precisely spliced to exon 22. This juxtaposition results in a reading frame shift, which in the majority of cases causes the severe Duchenne phenotype according to the hypothesis of Monaco *et al.*[24] Here, an in frame termination signal (TGA) is created 14 codons downstream in exon 22. Chelly *et al*[25] showed that ectopic lymphocyte DMD transcripts reflect the mRNA structure found in expressing tissue and we have confirmed this observation for transcripts of the cystic fibrosis transmembrane conductance regulator (CFTR) gene.[26] According to these findings, a dystrophin protein truncated after exon 20 can be expected in our DMD patient. This is in agreement with the absence of detectable dystrophin in immunohistochemical analysis and confirms the diagnosis of a severe Duchenne type muscular dystrophy.

To our knowledge, a deletion of exon 21 alone has not been reported so far. The detection of such small deletions outside the deletional hot spots of the DMD gene at the genomic level would be extremely laborious. Following the above strategy, abnormal splicing patterns owing to mutated junction sequences[27] might be as readily detectable as the described deletion. Furthermore, the number and size of our second round PCR products renders them suitable substrates for point mutation screening. A number of different scanning procedures are at hand for this purpose. Plieth *et al*[28] have recently shown that the rapid single strand conformation polymorphism (SSCP) analysis detected all five CFTR mutations tested in a retrospective study. Roberts *et al*[29] recently reported seven point mutations in the dystrophin gene identified by chemical mismatch detection.

Thus, all kinds of mutations might be detectable in a single comprehensive procedure using readily accessible lymphocyte RNA. An increased detection rate for DMD mutations would also be a major advantage for differential diagnosis. However, the above procedures are in no way limited to the DMD gene. Slomski *et al*[30] have stressed the ubiquitous nature of ectopic transcripts by the rather extreme example of characterising transcripts of spermatid specific genes in lymphocytes of adult non-pregnant females. As peripheral blood can substitute for specifically expressing tissue, ectopic RNA studies are also suitable for rapid carrier detection in female relatives of DMD patients carrying a deletion. Since the family of our patient currently lives abroad, no such analysis was performed here. Such applications, however, have already been described elsewhere.[19 31]

Note added in proof

Since submission of this paper, we have identified a genomic deletion of exon 18 in another patient by the same means.

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