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ARTICLE

# Screening for minor changes in the distal part of the human dystrophin gene in Greek DMD/BMD patients

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**The distal part of the human dystrophin gene is characterised by particular features and seems to play an important functional role. Additionally in recent years several data have implicated minor mutations in this gene region in some patients with mental retardation (MR). In order to screen for pathogenic mutations at the distal part of the human dystrophin gene we have used single-strand conformation analysis of products amplified by polymerase chain reaction (PCR-SSCA) in 35 unrelated male Greek DMD/BMD patients with no detectable deletions. Seven patients also had severe mental retardation. Direct sequencing of samples demonstrating a shift of SSCA mobility revealed six different and pathogenic minor changes, five in DMD and one in a BMD patient. Four of the mutations were found in DMD patients with severe MR. Three of these mutations were localised in exon 66, which presents an interesting similarity with part of the 3' end of the genome of eastern equine encephalomyelitis virus (EEEV). The present data from Greek DMD/BMD patients give further information about the phenotypic effects consequent on mutations in exons at the distal part of the human dystrophin gene.**

**Keywords: dystrophin gene; distal part; point mutations; mental retardation**

## Introduction

The dystrophin gene is an enormous gene which spreads over more than 2300 Kb, consisting of 79 exons[1,2] and encodes the protein dystrophin (427 kDa) localised in the inner surface of the sarcolemma of muscle fibres.[3,4] Defects of the gene cause the allelic neuromuscular disorders Duchenne and Becker muscular dystrophies. Two-thirds of gene mutations responsible for the disease are the result of large deletions which are clustered in two hot spot regions of the gene and are easily detected by a multiplex polymerase chain reaction system (multiplex PCR).[5,6] The remaining third of the mutations is thought to be caused mainly by minor changes that are difficult to detect by routine analysis, due to the enormous gene size.[7]

The dystrophin gene is subject to complex transcriptional and m-RNA processing control.[8] In particular the 3' end of the gene, which has a remarkable similarity to the corresponding region of the autosomal encoded dystrophin-related protein (DRP),[9] reveals a pattern of alternative transcripts, both in normal individuals and in patients with deleterious mutations at the region.[10,11] Additionally, Dp71 (coded by

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exons 63–79), Dp116 (exons 56–79) and Dp140 (exons 51–79) are dystrophin isoforms transcribed from alternative promoters in or near the 3' end of the dystrophin gene and are localised mainly in the nervous system.[12–14] Recent data have shown that some DMD cases with MR (observed in 30% of DMD patients), are associated with point mutations that disrupt the translational reading frame in the C-terminal region and lead to loss of function concerning the expression of Dp116 and/or Dp71 proteins.[15,11]

The significant functional role of the carboxy terminus domain is evident from biochemical studies which have demonstrated that dystrophin is closely associated, through its cysteine-rich and C-terminal domains, with the sarcolemmal membrane in a large glycoprotein complex.[16] This is thought to offer linkage between the extracellular matrix and the cytoskeleton and/or stability of the plasma membrane during muscle contraction and relaxation.[17]

In order to study the complex role of the 3' end coding region of the dystrophin gene in 35 Greek DMD/BMD patients with no detectable deletions, we screened for pathogenic mutations at this region. Six different minor changes were revealed, consisting of three microdeletions, two nonsense mutations and one donor splice site mutation. In two cases RT–PCR analysis demonstrated exon skipping, thus confirming the pathological effect of the respective mutations. Four of the mutations were found in DMD patients with MR; furthermore, three of them were found in exon 66.

## Materials and Methods

### *Patients*

Thirty-five unrelated Greek patients were selected (31 DMD and four BMD) for point mutation screening, in whom DMD deletions had been previously excluded using multiplex PCR.[6] Seven of the DMD patients had mental and/or language impairment. Diagnosis was based on clinical examination, family history, elevated serum creatine kinase activity and progression of the disease. In 15 cases the diagnosis was confirmed by dystrophin examination of muscle biopsy with immunofluorescence analysis. In the remaining 20 cases (8 familial and 12 sporadic) dystrophin analysis of muscle biopsy was not performed, either because the test was not available in Greece at the time of diagnosis, or because of family refusal.

### *PCR-SSCA*

Exons 60–79, including intron boundaries, were amplified from genomic DNA using primers designed from sequence data in the EMBL/GenBank databases (primer sequence available on request). All amplifications were carried out using 100 ng of genomic DNA, 10 pmol of each primer, 0.5 U Taq polymerase (Promega Corporation, Madison WI, USA), 4.5 mM $MgCl_2$ and 0.2 mM each of dNTP. When a radioactive reaction was prepared instead of 0.2 mM of dCTP, 0.1 μl radioactive ($a^{32}P$) was used. The cycling conditions included an initial step of 96°C for 4 min, followed by 30 cycles of 93°C for 1 min, 58°C for 1 min, and 72°C for 3 min, with a final extension of 72°C for 5 min.

Following amplification, 5 μl of PCR reaction were diluted with 5 μl of 0.1% SDS/1 mM EDTA and 5 μl of 98% formamide dye. Diluted products were denatured by heating at 100°C for 5 min and immediately transferred to ice. Subsequently 5–10 μl of each PCR mixture were electrophorised through 0.5X nondenaturing vertical Hydrolink-MDE (FMC Bioproducts, Rockland, ME, USA) gel in TBE 0.6X. Electrophoresis was carried out at 4W for 18–23 h at –4°C. For band visualisation, alternatively with silver staining,[18] autoradiography was used.

### *RT-PCR*

It has been demonstrated that certain genes, including the dystrophin gene, produce ectopic transcripts (also called illegitimate), in non-specific tissues such as lymphocytes.[19] From two patients (probands D286 and D320) total RNA was extracted from peripheral blood lymphocytes (PBL) and reverse-transcribed using the method described by Roberts *et al*.[20] According to this protocol for the first round of PCR, primers 10a and 10b were used in both cases to amplify exons 67–77 (Reaction 10). The primer pairs used in the nested PCR were selected to cover the appropriate exons indicated by the PCR-SSCA: patient D286 who showed by PCR-SSCA a band shift in exon 70, was analysed by amplification of the region between exons 67 and 71 (primers 10c and 10f), and patient D320 with a band shift in exon 74 was analysed by amplification of the region between exons 67 and 77 (primers 10c and 10d).

### *Direct Sequencing*

PCR products were purified using the Qiagen purification kit (Cat. No 28104, Qiagen GmbH, Hilden, Germany). In samples with multiple PCR bands the appropriate band was eluted from 1.6% agarose gel according to the Qiagen gel extraction kit (Cat. No 28704, Qiagen GmbH, Hilden, Germany). Purified templates were directly sequenced with the dideoxy chain termination cycle sequencing kit (RPN 2440, Amersham, UK), using the primers as for PCR-SSCA and RT-PCR, except for the cDNA of patient D320 in which an inner primer (N1 primer nucleotides 10263–81) was used. Primers were labelled with Texas Red Labelling kit (RPN 2441, Amersham). Sequencing reactions were analysed on a Vistra automated DNA Sequencer (Vistra 725, Molecular Dynamics/Amersham Life Science, UK).

### *Haplotype Analysis*

In cases where DNA from other family members was available, haplotype analysis was performed to confirm segregation of mutations in the family. The mutations were traced by SSCA in other family individuals and the results were confirmed by linkage analysis data using previously described polymorphic markers along the dystrophin gene.[18]

## Results

PCR-SSCA of 35 Greek DMD/BMD patients displayed six band shifts. Direct sequencing revealed five different minor changes in the coding region and one splice site mutation (Figure 1). The clinical data of the patients with pathogenic mutations are shown in Table 1 and a summary of the molecular findings is presented in Table 2.

### *Minor Changes and One Polymorphism in Exon 66*

Three pathogenic mutations were found in exon 66 in three unrelated DMD patients with severe mental retardation (Figure 1A). All were familial cases with two or more affected individuals.

The mutation in patient D363 is a C to T substitution at nucleotide 9776 which creates a nonsense mutation predicting a premature termination of translation, resulting in truncated dystrophin. Mutation analysis of the family by SSCA showed that the mutation was present not only in the patient but also in his two younger brothers, aged 3 and 2 respectively, who had elevated serum kinase activity. Two second-degree relatives with DMD phenotype and cognitive impairment died at ages 15 and 16 respectively from severe cardiac failure. DNA analysis from these patients was not available. Haplotype analysis in the family using polymorphic markers revealed a recombinant event in the patient between intron 39 and 44. This analysis showed that the patient and his two brothers with elevated kinase activity, share the same dystrophin haplotype only after intron 44, where the point mutation was also detected.

The mutation of patient D165 is a microdeletion of 2A in position 9806–9 from a 4A run which generates a stop codon (TAA), eight codons downstream. The patient (Table 1) is 4 years old and does not yet speak. Imaging of his brain with magnetic resonance imaging (MRI) demonstrated a mild asymmetry of the lateral ventricles and areas of increased signal were seen in the periventricular white matter frontally and occipitally. From family history it is known that two maternal aunts gave birth to affected boys both of whom were wheelchair bound at the age of 8. One of the cousins died at age 15 and the others is now 11 years old. Segregation of the SSCA band pattern in the family (the three affected boys, their mothers and their grandmother) is consistent with haplotype analysis using polymorphic markers.

The mutation in patient D171 is a microdeletion of 8 bp in position 9827 which generates a stop codon (TGA) 21 codons downstream. The patient is 5 years old and his family history showed that two of his maternal uncles became wheelchair bound at the age of 9 and died at the age of 15. Genetic analysis of the family was not available.

Beside the three mutations identified in exon 66, a polymorphism was also detected in six of the 35 patients (Figure 1A). This polymorphism is a C to T transition at position +15, in intron 66 and was also observed in 15 out of 100 Greek unrelated normal males (15%).

### *Identification of a Nonsense Mutation in Exon 62*

The shift mobility which was revealed by SSCA in patient D074 at exon 62 (Figure 1A) showed after direct sequencing of PCR amplified products, a substitution at nucleotide 9391 (G to A) generating a stop codon (TGA). This stop codon is predicted to cause premature termination of the translation reading frame (Figure 1B). Mutation detection by SSCA on the family, showed that the mutation is also carried by the patient's mother. Furthermore, haplotype analysis with polymorphic markers showed that the proband's X chromosome was of grandpaternal origin.

### *Characterisation of a Splice Site Mutation in Intron 70*

Direct sequencing of the band shift from SSCA in patient D286 (Figure 1A) revealed a donor splice site mutation (G to C) at position +1, intron 70 (Figure 1B). This mutation has previously been described at DNA level, in a sporadic DMD patient with some degree of mental handicap.[21] Our patient is a familial case and has severe psychomotor delay and language impairment. From family history it is known that a maternal uncle was wheelchair bound at the age of 12 and died at the age of 19. The mutation in our case was also confirmed by RT-PCR analysis that revealed three frameshift products in the region of exons 67–71, demonstrating activation of alternative splicing. In order to find the transcriptional effect of this donor splice site mutation, dystrophin mRNA was obtained from peripheral blood lymphocytes. RT-PCR analysis was performed between nucleotides 9974–10483 (approximately 67–71 exons) and the amplification showed three different and shorter fragments (about 250 bp, 370 bp and 410 bp respectively), instead of the one product (507 bp) expected (Figure 2). Sequencing

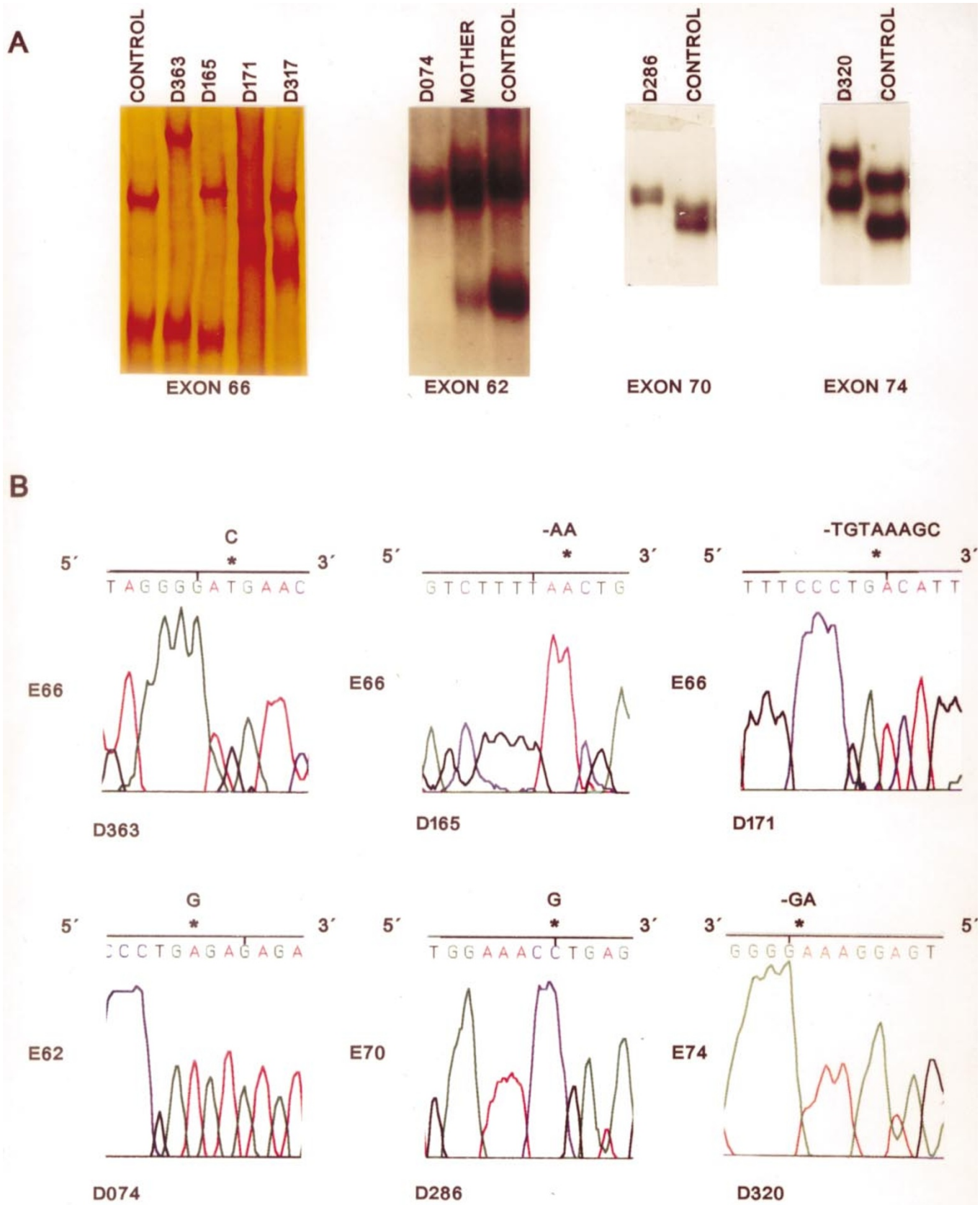


**Figure 1** **A)** *SSCA of exon 66 (silver staining) and exons 62, 70, 74 (autoradiography) of the dystrophin gene in probands D363, D165, D171, D074 (and his mother), D286 and D320 in comparison with normal controls. The mobility shift of proband D317 was found to be a polymorphism since it was also present in normal controls.* **B)** *Part of direct sequencing of exons 66, 62, 70, 74 (including intron boundaries) of patients demonstrating a band shift in SSCA. Sequencing revealed six pathogenic mutations: three microdeletions (D165, D171, and D320), one donor splice site mutation (D286), and two substitutions creating stop codons (D074 and D363). Asterisks demonstrate the site of nucleotide change.*

**Table 1** Clinical data of patients with pathogenic minor changes

| *Patient No* | *Age at diagnosis (years)* | *Famillial (F)/ Sporadic (S)* | *Diagnosis* | *CK level (IU/L)* | *Muscle biopsy* | *Mental retardation*[a] |
|---|---|---|---|---|---|---|
| D074 | 6 | S | DMD | 11 000 | no | ++ |
| D165 | 4 | F | DMD | 8 000 | yes | +++ |
| D171 | 5 | F | DMD | 11 560 | no | +++ |
| D286 | 8 | F | DMD | 7 000 | no | +++ |
| D320 | 13 | S | BMD | 7 600 | no | – |
| D363 | 5 | F | DMD | 10 000 | yes | +++ |

[a]Level of MR: +++ (severe), ++ (moderate), – (absence).

**Table 2** Minor changes in the distal part of the dystrophin gene in Greek DMD/BMD patients

| *Patient No* | *Exon*[a] | *Nucleotide change*[b,c] | *Name*[c] | *Mutation* | *Expected translational effect* |
|---|---|---|---|---|---|
| D074 | 62 | G9391A | W3061X | nonsense | truncation |
| D171 | 66 | deletion of TGTAAAGC between 9827 and 9834 | 9827del8 | frameshift | truncation (stop codon 21 codons downstream) |
| D363 | 66 | C9776T | R3190X | nonsense | truncation |
| D165 | 66 | deletion of AA between 9806 and 9807 | 9806delAA | frameshift | truncation (stop codon 8 codons downstream) |
| D286 | 70 | G---> C at +1 intron 70 | 10431+1G-->C | splice site | three truncated products[d] |
| D320 | 74 | deletion of GA between 10716 and 10717 | 10716delGA | frameshift | altered size product[d] (deletion of 110 amino acids) |

[a]According to Roberts *et al*[2]; [b]Nucleotide and amino acid numbering according to Koenig *et al*[3]; [c]Nomenclature according to Beaudet and Tsui[22]; [d]Results from RT-PCR study of PBL transcripts.

of the 250-bp band revealed complete loss of exon 68 and a 101-bp deletion in exon 70 (nucleotides 10330–10401), as a cryptic splice site was used (dinucleotide GT at 10331–2 of exon 70). The 370-bp fragment of the patient revealed a product with total loss of exon 70. The product of 410 bp demonstrated 101 bp deletion in exon 70, the same as was previously described for the 250-bp band. All fragments had lost the altered donor splice site of intron 70, and frameshifts are expected in the translational reading frame.

### *A Microdeletion in Exon 74*

In exon 74, SSCA demonstrated a mobility shift in patient D320 (Figure 1A). Direct sequencing showed a GA microdeletion in the coding area (Figure 1B). RT-PCR analysis was performed for the region between nucleotides 9974 and 11297 (exons 67–77) and showed only one shorter product (about 1000 bp) instead of the expected normal 1325 bp (Figure 2B). Direct sequencing of the product revealed the complete loss of exons 71–74 (Figure 2B). Although mutation at genomic DNA level creates a frameshift in the reading frame, at cDNA level the reading frame is restored, lacking only the coding sequences of skipping exons (110 aminoacids).

### *Distribution of Mutations*

Figure 3 presents the minor mutations identified in this study compared with those obtained from two databases for the corresponding region (January 1998). The sites of the two databases are http://www.dmd.nl[23] and http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html.[24]

## Discussion

We used PCR-SSCA of exons 60–79 of the dystrophin C-terminal domain to screen for minor changes in 35 Greek DMD/BMD patients. Four out of seven patients with severe MR had minor changes at the 3' end of the dystrophin gene. It is interesting that although this gene region represents only 25% of the entire coding region of the gene in our study there is a cluster of minor changes in patients with MR. These findings support previous screening data[15] as well as reports of isolated cases[11,25,26] that implicate mutations in the distal part of the human dystrophin gene of some

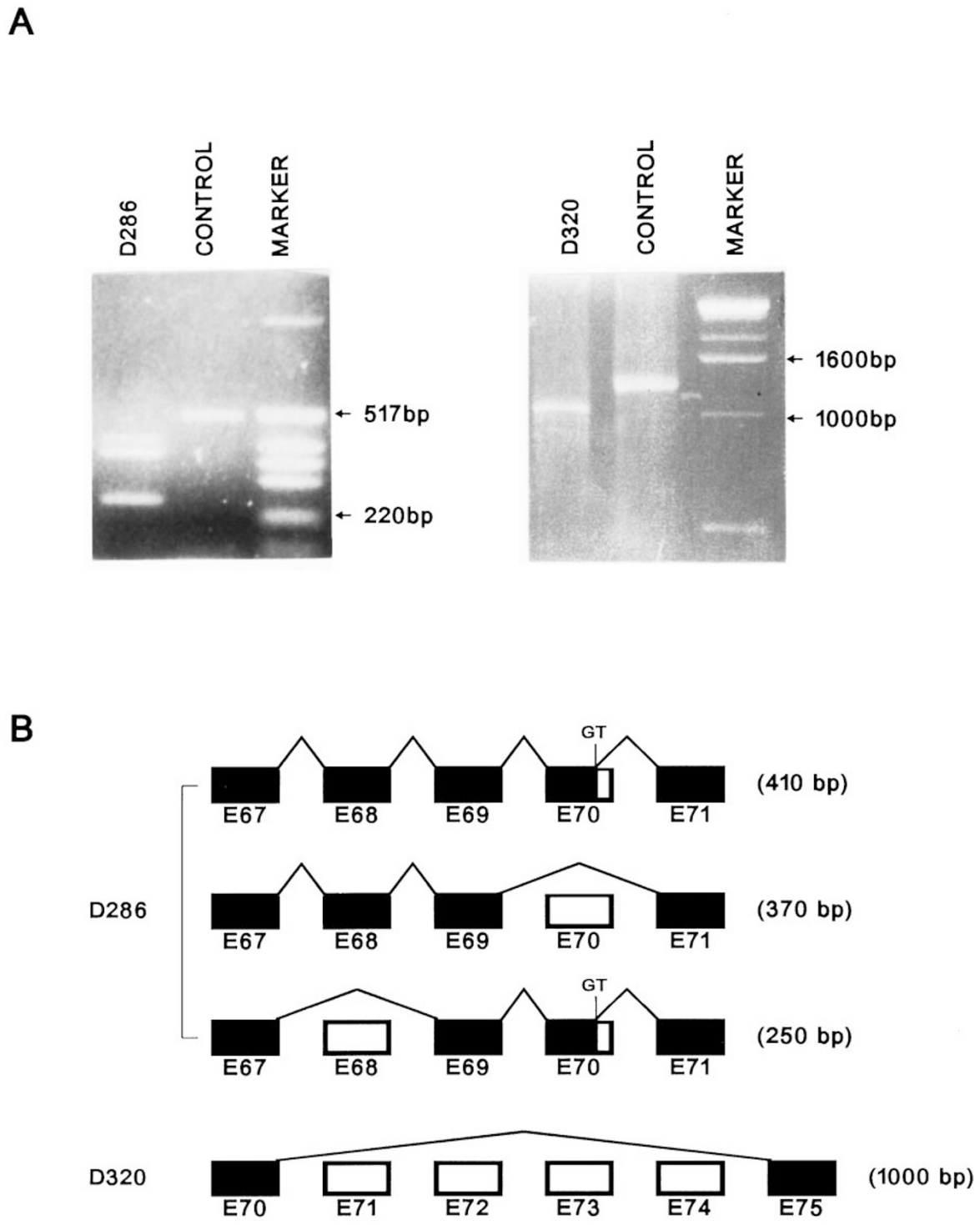


**Figure 2** **A)** *2% agarose gel electrophoresis of products of nested RT-PCR from patient D286 (left), spanning nucleotides 9973–10480 and from patient D320 (right) spanning nucleotides 10213–10891. The probands' products displayed shorter bands than the normal controls indicating exon(s) loss. Marker 1 kb molecular weight marker (BRL, USA), bp: base pairs.* **B)** *Schematic presentation of the splicing effect on mRNA transcripts from the donor splice site mutation of patient D286 and from the microdeletion of patient D320. Solid boxes: regions that are retained in cDNA; blank boxes: regions that are missing from mRNA transcript; GT cryptic splice site*

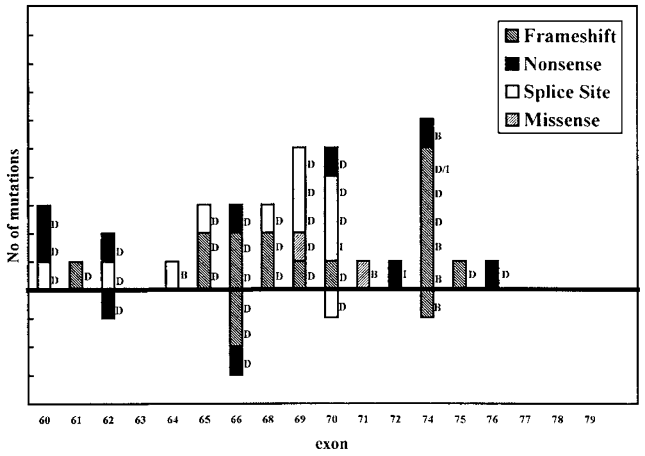


**Figure 3** *Distribution of minor changes detected in this study at the distal part of the dystrophin gene (beneath the horizontal line) compared with the small lesions of the corresponding area obtained from two databases (above the horizontal line). D: Duchenne muscular dystrophy; I: Intermediate muscular dystrophy; B: Becker muscular dystrophy.*

DMD patients with MR. This could be the result of disruption of the C-terminal isoforms (Dp140, Dp116 and Dp71) which are mainly expressed in the nervous system and also interact with sarcolemmal proteins.[27] However, further data will be necessary in order to establish correlation of mutations located in the 3' end of the dystrophin gene with MR. In recent years several studies, most of them *in vitro* assays, have investigated and revealed critical domains in the interaction of dystrophin with other proteins.[28] Another approach to clarifying the role of distinct dystrophin domains is to study the effect of specific mutations of the dystrophin gene in relation to the clinical phenotype.[25]

The three different minor alterations that were found in exon 66 in three unrelated DMD patients are predicted to lead to premature termination of the protein. Furthermore, the pathogenic role of the detected mutations was supported by haplotype analysis available for two families (probands D165 and D171).

The incidence of three different minor changes in the same exon led us to search GenBank/EMBL/DDBJ/PDB databases using BLAST[29] for any similarity. The search showed that 36 bases of exon 66 (85 bp in length) present 80% similarity with the 26S genomic mRNA of eastern equine encephalomyelitis virus (EEEV). Two of the three mutations that were found in exon 66, both microdeletions, were detected in this part of the dystrophin sequence. It is interesting that the frameshift mutation of patient D165 generates a stop codon eight codons downstream and shows short motif matches of four aminoacids with the viral protein. The 26S virus mRNA codes the nonstructural protein for its capsid, using a different reading frame from dystrophin.[30] The virus is an RNA (plus-strand) which infects many organisms, including human, causing neuroradiographic abnormalities best visualised by magnetic resonance imaging (MRI).[31]

The small cluster of three alterations at exon 66 in our patients (Figure 3) may be due to the conditions of our technique or to the fact that this analysis was performed on Greek patients only. Previous studies in the Greek population have also described differences in deletion breakpoints and in recombination events in the STR 44–50 region of the dystrophin gene.[32,33] Moreover, the incidence of the polymorphism C to T at position +15 of intron 66, in Greek population, is found with a frequency of 15% which is greater than the 5% frequency reported by Lenk *et al*.[15]

The three frameshift products that were revealed by cDNA analysis in patient D286 demonstrate the usage of the alternative splicing mechanism. This property of the carboxy terminus of the gene is also evident in the case of BMD patient D320 in whom the DNA defect on exon 74 activates exon skipping and finally restores the frameshift mutation. It must be pointed out that even though our results are obtained from ectopic transcripts and not from muscle mRNA they are consistent with the phenotypic expression. Despite the speculation that the integrity of the C-terminal domain is critical for protein function[7] the BMD phenotype of patient D286 indicates that absence of exons 71–74 is not critical enough to cause severe phenotype. Our findings are compatible with the finding of several normal features in the muscle of an mdx mouse that bears a deletion of exons 71–74.[34] As Figure 3 shows, mutations at exon 74 are reported in cases with different phenotypes and some of them (BMD and IMD) were inconsistent with the nature of mutation. In one of these cases RNA study was available but failed to explain the mild phenotype.[35] Phenotypic variation was also observed in deletions of certain exons and according to Sheratt *et al* this may be due to both the different efficiency of alternative splicing and to the abundance of dystrophin positive fibres.[36]

The mutation in exon 62 of proband D074 is predicted to result in lack of the entire cysteine-rich region and carboxy terminus domain which is compatible with the DMD phenotype.[37] The absence of point mutation in the grandfather provides strong evidence that the mutation is responsible for the DMD phenotype. It is

remarkable that two other mutations that have been described in exon 62 are observed in DMD patients with MR or speech delay and accord with the moderate mental deficiency of our patient.[35]

To our knowledge, with the exception of the splice site mutation, the remaining five minor alterations that are reported here have not been described so far in the literature. Characterisation of these mutations provides more information about the role of distinct exons in the distal part of the dystrophin gene in relation to the phenotypic expression.

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