Screening for mutations in the muscle promoter region and for exonic deletions in a series of 115 DMD and BMD patients

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Abstract

Mutations in the muscle promoter region and exonic deletions were screened in a series of 115 unrelated DMD and BMD patients from north-east Italy. No gross mutations of the promoter region were found. In three cases in which dystrophin of normal size was expressed at low levels, the analysis of DNA sequences of the promoter region failed to detect abnormalities. The majority of deletions in coding sequences, detected by cDNA probes, occur in the deletion hot spot identified by the probe P20. Intrafamilial variability in the severity of the disease is reported and discussed.

Several studies have reported that the great majority of cases affected by Duchenne or Becker muscular dystrophy are the result of deletions in the dystrophin gene, mapping in Xp21. Most deletions occur in two regions, one of which was first identified by pERT probes¹ and the second by the probe P20.²

No correlation was found between the extent or the position of the deletion and the clinical phenotype, whereas strong correlation exists with the status of the reading frame in the mRNA resulting from deletion.³

Immunohistochemistry and immunoblotting showed that 85% of DMD/BMD patients with unequivocal labelling on blots had a protein of abnormal size, while 15% had a dystrophin of normal size but of reduced amount,^{4,5} suggesting the possibility of regulatory mutations. Studies on the regulation and expression of the dystrophin gene led to identification of different promoters, one of which is specific for muscle.⁶⁻⁹

The present study deals with the molecular analysis of dystrophin gene mutations in a series of 115 unrelated Italian DMD/BMD patients, with special reference to deletions in the muscle promoter region or in coding sequences or both.

Materials and methods

Blood samples from unrelated patients with DMD or BMD were mostly referred to the laboratory by the Centre for Neuromuscular Disorders in the Neurology Department, University of Padua, where the patients were diagnosed. The clinical diagnosis was established by muscle histology and clinical assessment. DMD cases were usually differentiated

from BMD by the age at which the patients became chairbound, as suggested by Emery,¹⁰ or, in younger patients without a family history of the disease, by the results of dystrophin testing. Immunohistochemistry was performed in the laboratory of the same Neurology Department, while immunoblots were done in the laboratory of Dr E Hoffmann, School of Medicine, University of Pittsburgh. Subjects whose muscle specimens proved to be dystrophin negative (less than 3% compared with normal subjects) were considered to be affected by DMD.

DNA was extracted¹¹ from peripheral blood samples obtained in evacuated test tubes coated with K-EDTA (Vacurette, Greiner) and digested with *Hind*III (Amersham) according to the manufacturer's recommended conditions. Samples were run in 0.9 agarose (Seakem) by reverse field electrophoresis (Hoefer Pulse Controller PC750) in order to improve the resolution of the fragment separation. Agarose gels were blotted onto Hybond-N+ (Amersham) membranes. cDNA probes, kindly supplied by Dr L Kunkel, were labelled by random priming (Random priming kit, Promega) with ³²P-dATP (Amersham). Filters were hybridised at 68°C in 7% SDS, 0.5 mol/l disodium phosphate, 1 mmol/l EDTA, with the addition of 400 µg/ml sonicated and denatured salmon sperm DNA.

After hybridisation, filters were washed four times (20 minutes each) at 68°C in 1% SDS, 50 mmol/l disodium phosphate, 1 mmol/l EDTA. Films (Kodak, X-OMAT S) were exposed with intensifying screens for one to six days at -80°C.

For reprobing, filters were usually stripped twice in 0.1 × SSC, 0.5% SDS at 95°C for 15 minutes and once for five minutes in 0.1 × SSC at 95°C.

Primers flanking the sequence of the muscular promoter (Fwd CCCAAGCTTGCAAGTAC-TATGTCCACTGTGC; Rev CCCGTCGAC-CACAACTAAACGTTATGCCACAG) were designed directly from the sequence reported by Klamut *et al*¹² (underlined sequences, containing *Hind*III and *Sal*I sites respectively, were added for cloning). The sequence between these two primers corresponds to the segment between -217 and +330 and includes the 150 bp segment recognised to be essential for dystrophin gene expression in the muscle cells.¹²

Polymerase chain reaction was performed on a MJResearch Programmable Thermal Controller, using 12.5 pmol/l of each primer,

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Received 3 April 1991.
Revised version accepted
6 June 1991.

2.5 units of *Taq* polymerase (Cetus Co), and 125 ng genomic DNA per 25 µl reaction.

Samples were incubated for one minute at 95°C, followed by one minute at 65°C for annealing, and one minute at 72°C for elongation. The amplification cycle was repeated 35 times and the reaction ended with five minutes of elongation at 72°C. Amplification products were run on 1.5% agarose or 5% acrylamide gel and subsequently stained by ethidium bromide solution.

Muscle promoters from controls and from patients affected with BMD, amplified by PCR and purified on low melt agarose, were individually cloned in pUC19 using the restriction sites added to the primer sequence.

Plasmid DNA was directly sequenced on both strands using Sequenase 2.0 (USB) with two nested primers (Fwd CACTGTGCTA-TTCTGGTTTGG; Rev CTTTGTACTT-ACAACAGTCCTC).

Results

Deletion screening in 72 DMD and 43 BMD cases was performed by cDNA probes 1–2a, 2b–3, 4–5a, 5b–6, 7, 8, and 9–10. A representative Southern blot is shown in fig 1. Deletions were detected in 56.5% of the DMD/BMD patients. The percentage of deletions was 51% among DMD patients and 66% among BMD patients.

Deletion sizes ranged between a minimum of one exon (one DMD and three BMD cases) and a maximum of 43 exons. In one case (DMD) the entire gene sequence was deleted.

The extension and location of the deletions is shown in fig 2. Most deletions occur in the sequences identified by cDNAs 7 and 8, span-

ning exon 44 to exon 52. While the 5' deletion breakpoint in this region is variable in DMD cases, in 70% of BMD cases it lies between exons 44 and 45, confirming previous observations obtained by hybridisation with the P20 intragenic probe.^{13–15} In 57 cases (28 DMD and 29 BMD) the status of the reading frame resulting from deletion was established. Among DMD patients, 96% (27 out of 28) were out of frame and among BMD patients 96% (28 out of 29) were in frame.

Clinical variability among affected subjects in the same family was observed in several instances, as shown in fig 3. All 35 DMD patients whose DNA did not show deletions in the coding sequences and three BMD cases with reduced amounts of normal dystrophin were tested for deletions in the muscle promoter region by PCR amplification and subsequent gel electrophoresis (fig 4).

None of these 38 patients showed deletions in the segment that includes the muscle promoter. As expected, no promoter deletions were found among patients showing exonic deletions or showing a dystrophin altered in size.

The muscle promoter regions of the three BMD patients showing reduced levels of normal dystrophin (less than 50% of normal) were cloned in pUC19 and sequenced. Four independent clones were analysed for each patient and for two unrelated healthy male controls. The comparison of sequences showed minor variations in one control and in one BMD patient (deletion of a T in a series of seven in one, and in a series of eight in the other), but no significant differences were present (data not shown).

Discussion

The absence of gross mutations in the region of the muscle promoter is not surprising, because of the small size of the sequence involved and the presumed selective pressure for the conservation of consensus sequences. A deletion of the promoter region has only been described once so far.⁹ The rarity of this kind of mutation is confirmed by the present study, where no promoter deletions were found in a series of 115 unrelated patients.

None of the three BMD cases with reduced dystrophin of normal size showed mutations in the sequence including the muscle promoter, but the reduction of dystrophin molecules might be the result of point mutations in the coding sequence. Such mutations could make the protein more prone to rapid degradation. Alternatively, the observed reduction of dystrophin could be explained by mutation in still undefined additional regulation sites.

The large majority (86%) of 5' deletion breakpoints found in the present study map between the exons identified by cDNA probes 7 and 8. The occurrence of deletion breakpoints in two hotspots has been pointed out in several reports,^{3,16–18} but here only 11.5% of DMD deletions and 10% of BMD deletions did not include exons 40 to 55. These percentages are much lower than previously reported. The difference can hardly be attributed to the

Figure 1 *Hind*III digested DNA samples from DMD and BMD (3, 5, 13, 17, 21) patients probed with cDNA 8. Lanes marked C correspond to the DNA of healthy males. Track M is the DNA molecular weight marker (lambda DNA digested with *Eco*RI and *Hind*III). A complete deletion in lane 15 and partial deletions in lanes 4, 5, 8, 10, 13, 14, 16, 17, and 21 are shown.

Figure 2 Summary of 65 deletions found among 115 unrelated patients affected with DMD or BMD. From left to right: cDNA probes used in the study; exon numbers; corresponding HindIII fragments (in kb) detected by cDNA probes; extent of deletions among BMD patients; extent of deletions among DMD patients. Numbers below the bars indicate the number of patients carrying the same deletion. J indicates the presence of a junction fragment. The horizontal line shows the location of the intragenic probe P20 (see text for explanation).

size of the sample. Perhaps the homogeneity of the location of the 5' deletion breakpoints results from the geographical homogeneity of the sample. Persons who share common ancestors are likely also to share homologous non-coding sequences of DNA. Since deletions occur preferentially in specific introns, a differential deletion pattern could actually exist in different populations. In the present study, 50% of DMD and 73% of BMD cases were from one geographical area of 66 722 square km (north-east Italy), where there has been little migration and where the internal mobility is still quite low. The relationship between the location of deletions and clinical phenotypes confirmed the frameshift hypothesis,¹⁹ although in one DMD case (deletion 10→53) the correct frame was maintained.

A similar in frame deletion 10→53 was reported by Koenig *et al*³ in a DMD patient, whereas a deletion of comparable location and slightly smaller size (17→48) was reported by Davies *et al*²⁰ in a very mild Becker case. According to these observations a large part of the rod in the dystrophin molecule seems to be dispensable, but the two ends of the rod seem to be necessary for maintaining partial activity of the deleted molecule.

As far as the BMD cases are concerned the only out of frame deletion encompasses exons 3→7. Similar deletions were reported by Malhotra *et al*²¹ and by Koenig *et al*.³ The clinically benign course in these cases was attributed to alternative splicing, as recently confirmed by sequencing.²²

One relatively large deletion (exons 45→55) was found in the present investigation in a man, aged 31, who suffered only from myalgia after heavy muscular exercise and who has mild hypertrophy of the calves. The same deletion (45→55) was found in an unrelated BMD patient, aged 64, who walked with great difficulty, and in another BMD patient, now 25 years old and still walking, who came to

Figure 3 BMD families showing variability in the clinical phenotype. The severity of the disease is indicated as follows: A, asymptomatic (raised CK level or calf hypertrophy or both); M, mild (same symptoms as above plus clinically detectable weakness and fatigue after physical exercise); Mo, moderate (same symptoms as above plus waddling gait, difficulties in climbing stairs, and clear muscular weakness); S, severe (same symptoms as above plus muscular wasting, loss of most muscular strength, and great difficulty in walking); W, wheelchair bound. Numbers indicate the age at which the patients lost the ability to walk.

Figure 4 Screening for gross mutations of the muscle promoter region by PCR amplification followed by agarose gel electrophoresis of the resulting 568 bp. Samples 4 to 38 are from 35 BMD non-deleted patients. Samples 1, 2, and 3 are from three BMD patients showing reduced amount of normal sized dystrophin. Positive controls (DNA from healthy males) were run in lanes C and negative controls in lanes N. The last lanes on the right correspond to the DNA molecular weight marker (λ DNA, digested with HindIII).

medical attention at the age of 18 because of a very high CK level.

In the BMD families with recurrence of the disease, large variability in the clinical features of the related patients is observed in different generations (fig 3). It is a general observation that the clinical course is very similar among brothers; therefore the occurrence of cases with different grades of severity in different generations raises the suspicion that additional factors may play a role in the expression of the clinical phenotype. Although the absence or alteration of dystrophin has been identified as the primary cause of DMD and BMD, the subsequent steps of the molecular pathogenesis are still unknown. It is not difficult to accept that genetic variability may exist in molecules involved in key processes like recognition and destruction of damaged muscle fibers, muscular regeneration, or simply in the cellular response to different hormones.

The collaboration of Dr E Hoffman, Department of Molecular Genetics and Biochemistry, School of Medicine, University of Pittsburgh

in providing western blot confirmation of dystrophin assessment by immunohistochemistry and the active participation of Drs E Pegoraro and M Fanin in clinical and laboratory assessment of cases are gratefully acknowledged. The work was done with financial support of the Telethon Research Fund, UILDM (Italian Muscular Dystrophy Association), and the Italian Ministry of University and Scientific Research (MURST).

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