Deletions in the 5' region of dystrophin and resulting phenotypes

Francesco Muntoni, Paola Gobbi, Caroline Sewry, Tim Sherratt, Jo Taylor, Satpal K Sandhu, Stephen Abbs, Roland Roberts, Shirley V Hodgson, Martin Bobrow, Victor Dubowitz

Abstract

Deletions in the dystrophin gene give rise to both Duchenne and Becker muscular dystrophies. Good correlation is generally found between the severity of the phenotype and the effect of the deletion on the reading frame: deletions that disrupt the reading frame result in a severe phenotype, while in frame deletions are associated with a milder disease course. Rare exceptions to this rule, mainly owing to frameshift mutations in the 5' region of the gene (in particular deletions involving exons 3 to 7) which are associated with a milder than expected phenotype, have been reported previously. In order to characterise better the relationship between genotype and phenotype as a result of mutations arising in the 5' region of the gene, we have studied a large cohort of patients with small in frame and out of frame deletions in the first 13 exons of the dystrophin gene. Fifty-five patients with a deletion in this area were identified; approximately one third of them had a phenotype different from that theoretically expected. Patients were divided into two groups: (1) patients with a severe clinical phenotype despite the presence of a small, in frame deletion and (2) patients with a mild phenotype and an out of frame deletion. Noticeable examples observed in the first group were Duchenne boys with a deletion of exon 5, of exon 3, and of exons 3-13. In the second group we observed several patients with an intermediate or Becker phenotype and out of frame deletions involving not only the usual exons 3-7 but also 5-7 and 3-6. These data indicate that a high proportion of patients with a deletion in the 5' end of the gene have a phenotype that is not predictable on the basis of the effect of the deletion on the reading frame. The N-terminus of dystrophin has at least one actin binding domain that might be affected by the small, in frame deletions in this area. The effect of the in frame deletions of exon 3, 5, and 3-13 on this domain might account for the severe phenotype observed in these patients. Other mechanisms, such as unexpected effect of the deletion on splicing behaviour, might, however, also be implicated in determining the phenotype outcome.

Duchenne and Becker muscular dystrophies (DMD and BMD) both arise from mutations in the dystrophin gene.^{1,2} In the majority of patients affected by DMD and BMD, a deletion affecting one or more exons of the dystrophin gene is found after either Southern blotting or polymerase chain reaction (PCR) study of genomic DNA.³⁻⁵ In general the effect of the deletion on the reading frame was found to be in good agreement with the severity of the observed phenotype: deletions causing a frameshift were generally found to be associated with a severe DMD phenotype (irrespective of the size of the deletion), while deletions not affecting the reading frame were found to be associated with the milder BMD phenotype.⁶

Exceptions to this general rule have also been identified for both types of mutations. In particular, BMD or intermediate DMD/BMD phenotypes have been observed in some patients with frameshift deletions, most noticeably in boys with deletions of exons 3-7,⁷⁻¹⁵ but also involving exons 51, 49-50, 47-52, and 45.¹⁶ The reasons for the relatively mild phenotype in this group of patients may be different, and various hypotheses have been put forward. The first possibility is that alternative splicing could restore the reading frame, creating a new in frame deletion that results in the production of a partially functional protein⁸; a second hypothesis is the presence of a second promoter downstream from the deletion, possibly localised within the large intron between exon 7 and exon 8⁷; a further mechanism postulated is the occurrence of a new in frame translational start site downstream from the deletion.^{7,11}

More interestingly, rare patients with in frame deletions but a DMD phenotype have been described. Almost all these patients have relatively large deletions in the 5' region extending into the mid rod domain, for example, deletions removing exons 3-31 and 3-25,¹⁷ 4-41 and 4-18.¹⁶ Most likely, the explanation for the severe phenotype observed in these patients is that the deletion has removed a large portion of the molecule essential for dystrophin function. However, the relative large size of these deletions makes it difficult to identify more precisely which are the important regions of this molecule.

The aim of our study has been to identify cases, from our large cohort of patients with DMD and BMD, who had a phenotype in disagreement with the frameshift hypothesis. We have concentrated our analysis on patients with deletions in the 5' region of the gene,

Department of Paediatrics & Neonatal Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK
F Muntoni
P Gobbi
C Sewry
T Sherratt
V Dubowitz

Neuromuscular Unit, Division of Medical and Molecular Genetics, Guy's Hospital, London, UK
J Taylor
S S Sandhu
S Abbs
R Roberts
S V Hodgson
M Bobrow

Correspondence to Dr Muntoni.

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Table 1 Summary of clinical, genetic and biochemical data of Hammersmith Hospital patients

Patient	Last seen	Diagnosis	Clinical status	Exons deleted	Frame	Dystrophin
HH 1	13y	Int	Off feet at 13.5y	3	in frame	WB: ↓↓ amount
HH 2*	7y	Int/BMD	FS 29/40	5	in frame	WB: ↓ amount
HH 3*	10y	Int/BMD	Gowers in 9"	5	in frame	ND
HH 4†	20y	DMD	Off feet at 7y (femur fracture)	5	in frame	ND
HH 5 ‡	22y	Int	Off feet at 13.5y	5	in frame	ND
HH 6	11y	DMD	Off feet at 9y	3-13	in frame	ND
HH 7	22y	BMD	Ambulant	3-6	frameshift	WB: ↓ mwt ↓ amount
HH 8	12y	DMD	Off feet at 11y 3mth	3-6	frameshift	WB: absent
HH 9	7y‡	BMD	Myoglobinuria and cramps	3-7	frameshift	IC: ↓↓ intensity
HH 10	17y	BMD	Ambulant	3-7	frameshift	WB: ↓ mwt ↓ amount
HH 11	30y	BMD	Off feet at 26y	3-7	frameshift	WB: ↓ mwt ↓ amount
HH 12	28y	BMD	Ambulant	3-7	frameshift	IC: ↓ intensity WB: ↓ mwt ↓ amount
HH 13	17y	Int	Off feet at 13y 10mth	3-7	frameshift	ND
HH 14	28y	Int	Off feet at 13y 3mth	3-7	frameshift	WB: ↓ mwt ↓ amount
HH 15	18y	Int	Off feet at 15y	3-7	frameshift	WB: ↓ mwt ↓ amount
HH 16	4y	Int/BMD	FS 30/40	3-7	frameshift	IC: ↓ intensity
HH 17	24y	BMD	Ambulant	3-7	frameshift	WB: ↓ amount ↓ mwt
HH 18	9y	Int	Off feet at 14.5y	5-7	frameshift	WB: absent

*First cousins. †First cousins. ‡Died at the age of 7 after anaesthetic reaction. ND=not done. FS=functional score. WB=Western blot. IC=immunocytochemistry. Int=intermediate DMD/BMD phenotype. mwt=molecular weight.

because other studies had previously identified several patients with mutations in this area who had an unusual disease course. We have only analysed patients with single exon deletions, or with small multiple exon deletions, so that as much information as possible on specific domains of dystrophin could be gathered.

Material and methods

PATIENTS

This study was performed by comparative analysis of the clinical, molecular, and pathological data on 370 patients, with DMD and BMD, in whom a deletion was found, who attended the Neuromuscular Clinic at the Hammersmith Hospital.

The age at which patients lost ambulation was the main clinical parameter used to differentiate DMD from intermediate and BMD phenotypes. Patients who lost independent ambulation before the age of 13 were classified as DMD, those who were still ambulant at the age of 16 were classified as BMD, and those losing ambulation between the ages of 13 and 16 years were classified as intermediate. Those patients who were still too young to be assigned to one of these three categories were classified using a combination of clinical and biochemical parameters. In particular, all children were evaluated using a protocol for the quantitative assessment of muscle function developed by the Physiotherapy Department of the Hammersmith Hospital; the assessment was based on measurement of Total Muscle Strength, Myometry and evaluation of Motor Ability Score.¹⁸

In addition, the quantity and quality of dystrophin expression in the skeletal muscle biopsy was evaluated in all patients in whom this information was available. Muscle biopsies were obtained, by needle, from the quadriceps.¹⁹

DNA ANALYSIS

Screening for dystrophin gene deletion was performed on genomic DNA using multiplex PCR and cDNA probe hybridisation to Southern blots, as previously described by Hodgson *et al.*¹⁴

GEL ELECTROPHORESIS AND WESTERN BLOTTING

Muscle biopsies were frozen in liquid nitrogen, crushed, and treated with solubilisation buffer containing protease inhibitors, as described by Clerk *et al.*²⁰ A sample of solubilised muscle containing 60 µg of total protein was electrophoresed on 4%–20% linear gradient polyacrylamide gels as previously described.²¹ Separated proteins were electroblotted onto nitrocellulose membrane; blots were incubated with dystrophin antisera, diluted in PBS/0.05% Tween 20, and bound antibody was visualised as already described.²¹

ANTIBODIES

Antiserum P6 was a polyclonal whole rabbit antiserum raised to a fusion protein.²¹ Monoclonal antibodies DYS1 and DYS2 were obtained from Novocastra Laboratories, Newcastle upon Tyne.^{22,23}

IMMUNOCYTOCHEMISTRY

Muscle biopsies were mounted transversely and frozen in isopentane precooled in liquid nitrogen. Unfixed frozen section (5 µm) were incubated with the various dystrophin antisera for 30 minutes. Bound antibodies were detected as described by Sewry *et al.*²⁴

Results

We selected, from among our large cohort of patients with DMD and BMD, 55 patients who have been found to have deletions involving one or more exons in the 5' region of the

A schematic map of the first 14 exons at the 5' end of the DMD gene. The shape of each exon has been drawn so that the effect of the deletion on the reading frame can be predicted. Rectangular shaped exons can be removed without affecting the reading frame, while exons with indentation can cause a frameshift if removed, except for when the exon they join carries a suitable indentation. The different phenotypes we found in our patients with deletions in this region are also indicated.

dystrophin gene. Fifteen of them had a large deletion encompassing more than 20 exons in the 5' region. A second group of five patients had relatively small deletions, but their effect on the reading frame could not be established (mainly owing to the presence of a junction fragment). A third group (18 patients) had deletions removing only a few exons; in these patients the effect of the deletion on the frame was in good agreement with the observed phenotype. For example, a patient with in frame deletion of exon 13 had very mild BMD, seven patients with a frameshift deletion of exons 8–9 showed a DMD phenotype, and so did patients with frameshift deletions of exons 8–20, 1–6, 2–13, and 10–11.

Seventeen patients with deletions involving either one single exon or small multiple exon deletions in this region were found to have a phenotype different from the one expected on the basis of the frameshift hypothesis. We identified two major categories of exceptions: (1) patients with small in frame deletions but a phenotype more severe than BMD; and (2) patients with deletions causing frameshifting and a phenotype milder than the one expected. The clinical and genetic data in these two categories of patients are summarised in table 1. A schematic representation of the first 14 dystrophin exons is indicated in the figure; the effect of the various deletions in this area on the reading frame can be inferred from the figure.

PATIENTS WITH IN FRAME DELETIONS BUT A RELATIVELY SEVERE PHENOTYPE

In this category we found both single exon and multiple exon in frame deletions (table 1). Among the former, one patient had a deletion of exon 3 and an intermediate phenotype. He became wheelchair bound at the age of 13.5 years; Western blot analysis showed trace amounts of a dystrophin detected with an antibody directed against a region distal to the deletion, indicating that an in frame message was indeed produced.

Four patients had a deletion removing exon 5 and a severe or relatively severe phenotype. In particular, three patients were classified as having an intermediate phenotype, while one

boy was classified as having DMD; he became wheelchair bound at the age of 7 after a femur fracture and he was never able to walk again. Western blot analysis performed on one patient in this group showed reduced dystrophin with an antibody recognising an epitope distal to the region deleted.

Regarding the multiple exon deletions, we have identified one patient with an in frame deletion involving exons 3–13 and a Duchenne phenotype. The muscle of this boy, who became wheelchair bound at the age of 9, could also be visualised with antibodies raised against a portion of the protein distal to the deletion, confirming an in frame deletion.

PATIENTS WITH OUT OF FRAME DELETIONS BUT A RELATIVELY MILD PHENOTYPE

All persons in this group had deletions involving multiple exons (table 1). Nine patients had a deletion removing exons 3–7 and a phenotype ranging from BMD to intermediate (five were BMD and four intermediate). Of the patients with a BMD phenotype, three were still ambulant at the age of 16, 23, and 28, respectively, when last assessed. One patient lost the ability to walk at the age of 26, while another died at the age of 7 following a reaction to general anaesthesia. At that point he had no apparent weakness and only minimal functional disability, mainly characterised by occasional episodes of cramp and myoglobinuria after exercise. The remaining three patients in this group had an intermediate phenotype, and none had DMD. Western blot analysis was performed in seven of them and showed a marked reduction in dystrophin abundance in all. There was apparently no correlation between the differences in size and amount of dystrophin (as judged by Western blot) and the severity of the phenotype (that is, both BMD and intermediate patients produced similar amounts of the protein). Immunocytochemical analysis of the muscle, performed in four of these patients, showed low dystrophin levels produced in all fibres.

In addition to this relatively large group of patients, we identified three persons with frameshift deletions internal to the relatively common 3–7 deletion: two of them had a 3–6 deletion (patients HH 8 and HH 9), while one was lacking exons 5–7 (patient HH 7). Of these patients, one had a typical BMD phenotype, being still ambulant at the age of 21, and a clear, although faint band on the Western blot corresponding to a lower molecular weight dystrophin. The other showed a DMD disease course, since he lost ambulation by the age of 11 years, and showed complete absence of dystrophin on immunocytochemistry.

The boy with the 5–7 deletion had an intermediate phenotype (lost ambulation at 14.5 years) and his muscle biopsy showed a complete lack of dystrophin.

Discussion

The majority of patients with DMD and BMD have deletions of one or more dystrophin exons. Although there is good agreement between the

Table 2 Correlation between phenotype and deletions in the 5' region of the dystrophin gene

Exons deleted	Frame	Expected phenotype	Observed phenotype	References
2-7	+	Mild	Severe	9
3	+	Mild	Mild (1/6)	3
			Intermediate (4/6)	7 9 12
			Severe (1/6)*	HH patient 25
3-9	+	Mild	Mild (1/2)	13
			Intermediate (1/2)	15
3-13	+	Mild	Severe (2/2)	HH patient 14
5	+	Mild	Intermediate (3/5)	HH patients
			Severe (1/5)	
5-13	+	Mild	Intermediate (1/1)	3
6-13	+	Mild	Intermediate (1/2)	9
			Severe (1/2)	8
10-13	+	Mild	Severe (1/1)	12
13	+	Mild	Mild (2/2)	HH patient 3
1	-	Severe	Severe (3/6)	7 26
			Mild (3/6)	9 15 Boyce 1991
3-6	-	Severe	Severe (2/3)	HH patient 7
			Mild (1/3)	HH patient
3-7	-	Severe	Mild (23/47)	7-16
			Intermediate (20/47)	
			Severe (4/47)	
5-7	-	Severe	Intermediate (2/3)	HH patient 7
			Severe (1/3)	26
7	-	Severe	Severe (2/2)	7 14
7-13	-	Severe	Severe (1/1)	8
8-9	-	Severe	Intermediate (2/11)	HH patient, 7
			Severe (9/11)	HH patients 7 14
12	-	Severe	Severe (1/1)	8

HH=Hammersmith Hospital. The asterisk indicates a patient with a missense point mutation. The symbol + or - in the frame column indicates the effect of the deletion on the reading frame. The exons in bold indicate cases described in the present article.

effect of the deletion on the reading frame and the observed phenotype, several exceptions have been previously reported. These rare cases are not only important because they highlight the pitfalls in giving a prognosis based exclusively on the genetic study results, but also because the mechanism responsible for the discrepancy found in these patients might help to indicate the function of specific dystrophin domains. In particular, in frame deletions removing single or few exons, but associated with more severe than expected phenotypes, might indicate that a specific portion of dystrophin is crucial for its function.

In this study we found that approximately one third of patients with mutations in the 5' region of the gene do not conform to the frameshift rule. We have divided our patients into two major categories: those with a small in frame deletion and a severe phenotype and those with out of frame deletions associated with relatively mild phenotypes.

In the first group of patients we have found deletions removing exons 3, 5, and 3-13. Only one patient had a deletion removing exon 3 alone; he had an intermediate phenotype. Mutations involving exon 3, which encodes for 31 amino acids, have only been reported in five other patients (table 2). Their phenotype ranged from DMD to BMD.^{7 9 12 25} The biochemical studies performed in these cases, as in our own, indicate the presence of dystrophin detected with antibodies directed towards regions 3' of the deletion, further indicating that the frame was maintained. Four of these patients had a deletion of exon 3^{7 9 12} and one had a missense mutation.²⁵

Regarding the four patients with a deletion removing exon 5, they also had a relatively severe phenotype despite having a small, in frame deletion (exon 5 contains 31 amino acids). This deletion, as far as we are aware,

has not been reported by other groups. Three of the patients with exon 5 deletions were classified as intermediate, while one had a DMD phenotype; dystrophin analysis performed in one of these patients confirmed the production of significant amounts of dystrophin in the region distal to the deletion.

One patient had an in frame deletion removing exons 3-13 and showed a typical DMD disease course. The phenotype of this patient was more severe than the one observed in patients with deletion of exon 3 or 5; another patient with the same deletion also showed a similar severe phenotype.¹⁴

We have reviewed published cases with in frame deletions in this 5' region: the results are summarised in table 2. Analysis shows that in frame deletions in this region can result in great phenotypic variability, and not always in BMD, confirming what is reported in this study. In particular, deletions of exons 2-7, 3, 3-9, 5-13, 6-13, and 10-13 have been frequently associated with disease courses more severe than expected (table 2).

Regarding patients with single exon deletion, from the data regarding our own cases and those reported by others, one is tempted to conclude that both exon 3 and exon 5 are very important for dystrophin function. Since this region of the gene contains at least one putative actin binding site,^{27 28} one possibility is that the overall conformation of this actin binding domain would be affected by deletions or mutations in this region, as already suggested.²⁵ The fact that our patients with a deletion of exon 3 and of exon 5 produced significant levels of the protein might further reinforce this view. However, both the fact that patients with an apparently identical deletion involving exon 3 can have a variable phenotype and the finding of BMD patients whose deletions include exons 3 and 5 (such as the deletion of exons 3-9, table 2), makes this possibility less likely. Other factors, such as unexpected effects of the deletion on splicing behaviour, might also be implicated. In this respect it should be noted, however, that the study of peripheral blood lymphocyte mRNA showed that one patient with a deletion of exon 5 (patient HH 3) does not have alternative splicing around the area of the deletion (that is, mRNA shows that exons 4 and 6 are joined together²⁹). Another possible explanation for the difference in severity between patients with deletion of exon 3 and 5 and patients with larger deletions encompassing these two exons might be the effect of the former deletions on the tertiary structure of dystrophin, affecting both actin binding domains.^{27 28} Further characterisation of muscle mRNA in these patients is required before a final conclusion of the function of these exons can be drawn.

Two patients with a deletion of exon 13 have been found to have a mild BMD phenotype (table 2); it can be concluded that this exon does not contribute significantly to dystrophin function.

Regarding multiple exon deletions, all patients with in frame deletions encompassing exons 10 and 11 invariably had a DMD pheno-

type, while a more variable disease course was associated with deletions occurring in the first nine exons of the DMD gene. It might therefore be that sequences contained in exons 10 and 11 are also essential for dystrophin function. Patients with larger in frame deletions entirely removing the region of the major actin binding domain (such as exons 3–13) invariably have a severe phenotype.

With regard to patients with out of frame deletions and milder than expected phenotypes, we found nine patients with deletions of exons 3–7. As already extensively described, we also found that the phenotype in this group of patients is rather variable, ranging from intermediate to BMD patients. We found no DMD in this category of patients, although there have been rare reports of such an association (table 2).

Deletions internal to the typical exon 3–7 region have rarely been reported.⁷¹⁶²⁶ Although the conclusion of these studies was that the phenotype resulting from this type of internal deletion were not responsible for unusual phenotypes,¹⁶²⁶ we have found that both deletions removing exons 5–7 and 3–6 (all out of frame) were associated with a wide spectrum of disease severity, ranging from a very mild BMD phenotype (one patient with a 3–6 deletion) to an intermediate or DMD phenotype (one patient with exon 5–7 and another with exon 3–6 deletion). The reason for this is not known, but some of the mechanisms that have been proposed to account for the relatively mild phenotype of patients with 3–7 deletions might also hold true in this category of patients. In this respect, it should be noticed that in one study performed in patients with a deletion of exon 3–7, minor alternative splicing phenomena in this 5' region of the cDNA were found (in particular splicing of exons 2–10, and of exons 1–8, which restore the reading frame but also minimal splicing out of exons 1–10, still being out of frame⁸). Other studies, however, have not confirmed these results.⁷¹¹¹⁶ Why patients with out of frame deletions removing exons 3–7, 3–6, and 5–7 might have a phenotype milder than patients with single exon in frame deletions in this area has already been discussed,²⁵ but is still unclear.

On the basis of our data, we can conclude that deletions in the 5' region of the dystrophin gene are frequently associated with unpredictable phenotypes; this information should be considered when counselling cases with such mutations. The data regarding severe patients with in frame deletions of single exons in this region might also suggest an important function of these sequences for protein stability or function.

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