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Molecular and Phenotypic Analysis of Patients with Deletions within the Deletion-rich Region of the Duchenne Muscular Dystrophy (*DMD*) Gene

E. F. Gillard,*,‡ J. S. Chamberlain,§ E. G. Murphy,† C. L. Duff,* B. Smith,* A. H. M. Burghes,*,‡,[1] M. W. Thompson,*,‡ J. Sutherland,* I. Oss,* S. E. Bodrug,*,‡ H. J. Klamut,* P. N. Ray,* and R. G. Worton,*,‡

*Genetics Department and Research Institute, and †Division of Neurology, Department of Pediatrics, Hospital for Sick Children, and ‡Departments of Medical Genetics and Medical Biophysics, University of Toronto, Toronto; and §Institute for Molecular Genetics, Baylor College of Medicine, Houston

Summary

Eighty unrelated individuals with Duchenne muscular dystrophy (DMD) or Becker muscular dystrophy (BMD) were found to have deletions in the major deletion-rich region of the *DMD* locus. This region includes the last five exons detected by cDNA5b–7, all exons detected by cDNA8, and the first two exons detected by cDNA9. These 80 individuals account for approximately 75% of 109 deletions of the gene, detected among 181 patients analyzed with the entire dystrophin cDNA. Endpoints for many of these deletions were further characterized using two genomic probes, p20 (DXS269; Wapenaar et al.) and GMGX11 (DXS239; present paper). Clinical findings are presented for all 80 patients allowing a correlation of phenotypic severity with the genotype. Thirty-eight independent patients were old enough to be classified as DMD, BMD, or intermediate phenotype and had deletions of exons with sequenced intron/exon boundaries. Of these, eight BMD patients and one intermediate patient had gene deletions predicted to leave the reading frame intact, while 21 DMD patients, 7 intermediate patients, and 1 BMD patient had gene deletions predicted to disrupt the reading frame. Thus, with two exceptions, frameshift deletions of the gene resulted in more severe phenotype than did in-frame deletions. This is in agreement with recent findings by Baumbach et al. and Koenig et al. but is in contrast to findings, by Malhotra et al., at the 5′ end of the gene.

Introduction

Deletions of the *DMD* gene in 6%–10% of individuals with Duchenne muscular dystrophy (DMD) or Becker muscular dystrophy (BMD) were first detected with the DNA probes pERT87 (DXS164; Kunkel et al. 1985) and XJ (DXS206; Ray et al. 1985) (Monaco et al. 1985; Kunkel et al. 1986; Hart et al. 1986; Thomas et al. 1986). The use of genomic probes JBir (DXS270) and J66-H1 (DXS268; Monaco et al. 1987) increased the number of detectable gene deletions to 17% by Southern analysis and to more than 50% by field-inversion gel electrophoresis (den Dunnen et al. 1987). The latter results indicated that a deletion hot spot existed in the 950 kb between probes JBir and J66H1. The position of the hot spot was further defined by genomic clone p20 (DXS269) within this region, which revealed deletions of the *DMD* gene (not detected with pERT87 or XJ) in 16% of DMD/BMD individuals (Wapenaar et al. 1988).

Koenig et al. (1987) found the overall deletion frequency for the *DMD* gene to be 50%, by using a series of ~1-kb dystrophin cDNA subclones on *Hin*dIII digests of DNA samples from 104 patients. Deletions of the gene were found to occur most frequently in two regions

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Address for correspondence and reprints: Ronald G. Worton, Ph.D., Department of Genetics, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8.

1. Present address: Department of Neurology, Ohio State University, 1654 Upham Drive, Columbus, OH 43210.

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of the 14-kb cDNA. The first of these (cDNA1b) corresponded to the pERT87/XJ region and accounted for 28% of deletions, while the second (cDNA8) mapped between JBir and J66H1 and accounted for 51% of deletions. Similar findings were reported by Darras et al. (1988), who found an overall deletion frequency of 66% (21/33 patients) for the *DMD* gene. Of these 21 patients, 57% had deletions of exons detected by cDNA8 and 29% had deletions of exons detected by cDNA1–3. Deletions of the *DMD* gene were also detected in *Pst*I digests of DNA samples from 59 of 107 BMD/DMD patients by using two cDNA clones, Cf23a and Cf56a (Forrest et al. 1987*a*, 1987*b*, 1988). These two clones correspond approximately to cDNA7 and cDNA8 of Koenig et al. (1987). In conjunction with two 5′ cDNA clones, gene deletions were detected in 70% of DMD/BMD individuals (Forrest et al. 1987*a*). Thus, partial deletions of the dystrophin gene account for 50%–70% of mutations at the *DMD* locus.

Accompanying the heterogeneity in the size and location of deletions at the molecular level is a considerable variation in the severity and progression of the disorder among affected individuals. An understanding of the relationship between a given deletion of the *DMD* gene and the resulting phenotype would be of considerable value for clinical prognosis and would provide further insights into the role of dystrophin in the disease. To date, studies have revealed no apparent correlation between the size of *DMD* deletions and the severity and progression of the disorder (Hart et al. 1987; Darras et al. 1988; Lindlof et al. 1988).

On the basis of a study of three BMD and three DMD patients with deletions in the DXS164 locus, it was proposed that the milder BMD phenotype resulted from *DMD* gene deletions that maintained the translational reading frame, while the more severe DMD phenotype resulted from *DMD* gene deletions that shifted the translational reading frame (Monaco et al. 1988). In our own laboratory, 29 patients with deletions confined to the first 10 exons of the gene were studied. Six patients with mild (BMD) and eight with intermediate phenotypes were found to have a gene deletion that is predicted to shift the reading frame (Malhotra et al. 1988), indicating that serious exceptions to the frameshift hypothesis exist.

Recently intron sizes and intron/exon boundaries have been described for eight exons of the major deletion-rich region (Chamberlain et al. 1988; Baumbach et al. 1989), thus enabling us to examine the reading-frame hypothesis for patients who have deletions of the *DMD* gene within this region. In the present report we determine the relationship between deletions within the major deletion-prone region (hot spot) of the *DMD* locus and their associated phenotypes, and we examine the feasibility of predicting the severity of the disease from DNA studies of individuals with gene deletions in this region.

Material and Methods

Clinical Evaluation

In the present study patients were classified as DMD if they were wheelchair bound before age 12 years and as BMD if they were still ambulant at age 16. Patients were classified as intermediate if they became wheelchair bound between the ages of 12 and 16 years. Those patients too young to allow a definitive diagnosis were grouped separately. Phenotypic severity was also assessed on a numerical scale from 1 to 5 (most severe to least severe) by one of us (E.G.M.), on the basis of *all* the available clinical data for each patient, as summarized in the Appendix. Patients younger than 12 years were also scored for severity by comparison with members of each class when they were of a similar age. This assessment was performed blind (i.e., without referring to deletion data). To a first approximation, these categories represent the following phenotypes: 1 = severe DMD; 2 = mild DMD to severe intermediate; 3 = intermediate; 4 = severe BMD; 5 = mild BMD. Family history was considered positive (denoted by a plus sign [+] in the Appendix) if there were affected individuals in more than one generation or in more than one branch of the family. The majority of patients were diagnosed at The Hospital for Sick Children, Toronto, although 13 of the 80 patients in the study were referred from other genetic centers in Canada. Patients are indicated throughout the text by the family number (in parenthesis), followed by the patient number.

DNA Analysis

DNA was digested with restriction enzymes (Boehringer Mannheim) under the manufacturer's recommended conditions. Samples were subjected to electrophoresis in 0.6%–0.7% agarose gels and were transferred to Hybond-N™ (Amersham) membrane. Probes were labeled by random hexanucleotide–primed synthesis (Feinberg and Vogelstein 1983, 1984). Prehybridization (3 × SSC, 0.05 M $NaPO_4$, 1% SDS, 0.5% instant milk powder, 50% formamide, and 0.3 mg sonicated denatured herring sperm DNA/ml) and hybridization (3 × SSC, 0.05 M $NaPO_4$, 1% SDS, 0.5% instant milk powder, 50% formamide, and 10%

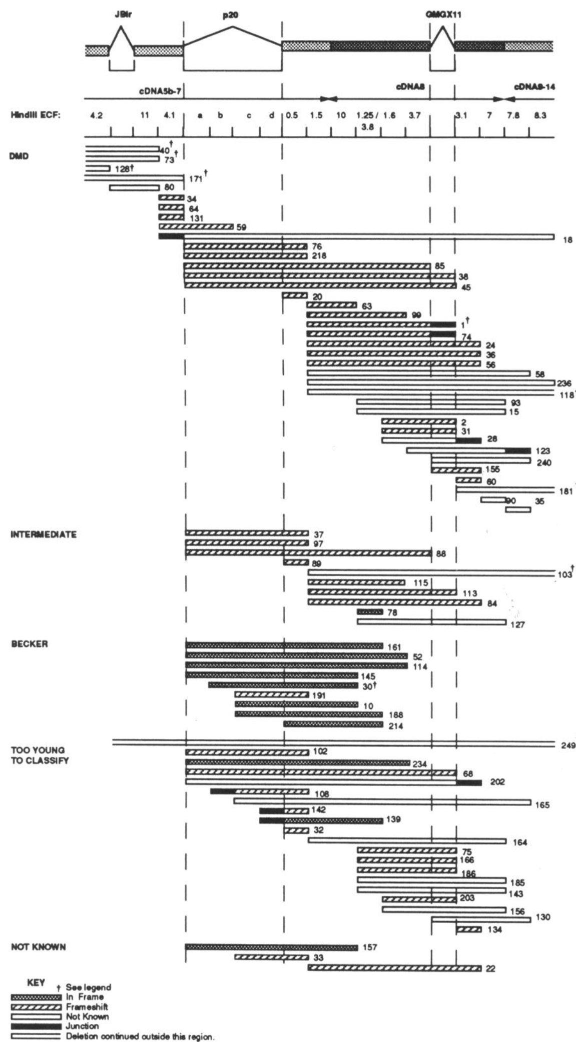

Figure 1 Deletions of the deletion-prone region. Letters a–d represent genomic fragments detected by probe p20 on *Hin*dIII, *Eco*RI, and *Pst*I digests as follows: a = 4.4/12-kb *Hin*dIII; b = 8.3-kb *Hin*dII, 3.2-kb *Eco*RI, and 8.0-kb *Pst*I; c = 2.5/2.9-kb *Hin*dIII and 2.5-kb *Pst*I; d = 3.8-kb *Hin*dIII and 6.7/6.9-kb *Eco*RI. The predicted reading-frame status of each deletion is indicated as frameshift (▨), in frame (▩), or unknown (▭). Junction fragments are indicated at ends marked in black (▬), and deletions extending outside the region are open ended (▭). Additional data for families where numbers are followed by a superscript dagger (†) are as follows: 1—Two of the three affected males are classed as DMD while the third is classed as intermediate; 30—The 3.2-kb *Eco*RI fragment is not deleted. The 8.0-kb *Pst*I fragment is deleted, and a *Pst*I junction fragment is detected; 40 and 128—also deleted for all exons 5′ to the region indicated, with the exception of the first two exons; 73—also deleted for all exons 5′ to the region indicated, with the exception of the first 10 exons. A junction fragment is detected by the 10th exon; 171—also deleted for the rest of cDNA5b-7 and for five *Hin*dIII ECFs of cDNA4-5a (18 kb, 12 kb, 4.7 kb, 5.2 kb, and 20 kb); 118 and 181—also deleted for five *Hin*dIII ECFs of cDNA9-14 (2.3 kb, 1.0 kb, 8.8 kb, 6.0 kb, and 3.5 kb); 103—also deleted for the 2.3-kb *Hin*dIII ECF of cDNA 9-14; 249—also deleted for the rest of cDNA5b-7, for three *Hin*dIII ECFs of cDNA4-5a (18 kb, 12 kb, and 4.7 kb) and for six *Hin*dIII ECFs of cDNA9-14 (2.3 kb, 1.0 kb, 8.8 kb, 6.0 kb, 3.5 kb, and 6.6 kb).

dextran sulfate) were performed at 42°C for 2 h and at 42°C overnight, respectively. Filters were washed successively, for 20 min each at 55–65°C, in 2 × SSC/0.1% SDS, 0.5 × SSC/0.1% SDS, 0.2 × SSC/0.1% SDS, and 0.1 × SSC/0.1% SDS as appropriate. Films were exposed with intensification screens for 1–7 d at −70°C. Prior to reprobing, filters were stripped in 0.1 × SSC at 80°C for 10 min.

Sources of DNA Probes

The cosmid clones from the GMGX11 region were isolated from an XXXXY size-selected (30–60-kb) *Sau*3A partial digest library in LoristB (Cross and Little 1986; Malhotra et al. 1988), by screening with subclone cDNA8 of the *DMD* cDNA. Subclones cDNA5b-7, cDNA8, and cDNA9 (Koenig et al. 1987) were received from American Type Culture Collection. Probe p20 has been described elsewhere (Wapenaar et al. 1988). GMGX11 was isolated as a 1.2-kb insert from an *Eco*RI flow-sorted X-chromosome library in lambda NM1149 during a screen for "single-copy recombinant phage" (Gillard et al. 1987), was subcloned into pUC19, and was localized to Xp21-Xp22.3 by hybridization to somatic cell hybrid DNA samples. It was subsequently shown, by deletion mapping (data not shown), pulse field analysis (Burmeister et al. 1988), and analysis of translocation breakpoints in females with X;autosome translocations (Boyd et al. 1988), to lie with the *DMD* locus.

Results

Deletion Analysis

Eighty patients are described in the present study and represent a selected subgroup of the 109 patients with deletions of the *DMD* gene who were detected in a systematic cDNA analysis of 181 BMD/DMD individuals. Ten duplications of the *DMD* gene were also detected in this analysis, two of which were duplicated for part of the selected region (Hu et al. 1989; X. Hu and R. G. Worton, personal communication). The selected group includes patients deleted for any or all of the last five exons detected by cDNA5b-7, all exons detected by cDNA8, or the first two exons detected by cDNA9. These partial deletions of the *DMD* gene are shown schematically in figure 1. Patients are grouped according to phenotype and are identified by family

number. The effect of each deletion on the translational reading frame, where known, is indicated by appropriate shading of the deleted region. Further clinical details can be found in the Appendix.

To determine the extent of the deletions diagrammed in figure 1, southern blots, containing *Hin*dIII and *Eco*RI digests of DNA from all 80 patients, were analyzed following hybridization to each cDNA subclone. Southern blots containing *Pst*I digests of DNA from a subset of 50 patients were also analyzed. The order of exon-containing fragments (ECFs) was deduced for each enzyme by aligning and ordering the full set of overlapping deletions. Exons have been defined by ECF size rather than by exon number, since the number of exons that precede this region is not yet rigorously defined. *Hin*dIII ECFs are indicated in figure 1. The 1.25-kb and 3.8-kb *Hin*dIII ECFs represent a single exon with a *Hin*dIII site in cDNA8. *Eco*RI and *Pst*I ECFs corresponding to each *Hin*dIII ECF with defined intron/exon boundaries are indicated in figure 2.

The use of more than one enzyme lessens the likelihood of missing comigrating or small ECFs (Darras et al. 1988) and also allows a distinction to be made between junction fragments and RFLPs. This distinction is important, since a junction fragment suggests the possibility of a partial exon deletion. Individuals with junction fragments visualized by cDNA probes were therefore excluded from the frameshift analysis, although those individuals with junction fragments detected only by genomic probes p20 or GMGX11 were included. Putative junction fragments are shaded black in figure 1.

The results of hybridization with genomic probes p20 and GMGX11 are also included in figure 1. In this study, 29/80 deletion patients have deletions of p20, and 37/80 have deletions of GMGX11. The large number of deletions with endpoints within p20 made it possible to order genomic fragments a–d detected by this probe, again by ordering overlapping deletions. Hybridization with p20 revealed *Hin*dIII and *Eco*RI RFLPs, in addition to those previously described with *Msp*I and *Eco*RV. The sizes and relative order of these fragments are described in the legend to figure 1.

The clone cDNA5b–7 detects comigrating ECFs of 1.5 kb and 12 kb on *Hin*dIII and *Eco*RI digests, respectively. To eliminate any ambiguity this may cause, a 0.5-kb *Hin*cII subclone of cDNA5b–7, which detected *Hin*dIII ECFs of 0.5 kb, 1.5 kb, and 4.1 kb and *Eco*RI ECFs of 4.2 kb, 12 kb, and 25 kb was therefore used to visualize the most 3′ ECFs of cDNA5b–7. This 0.5-kb subclone revealed a novel *Eco*RI fragment of approxi-

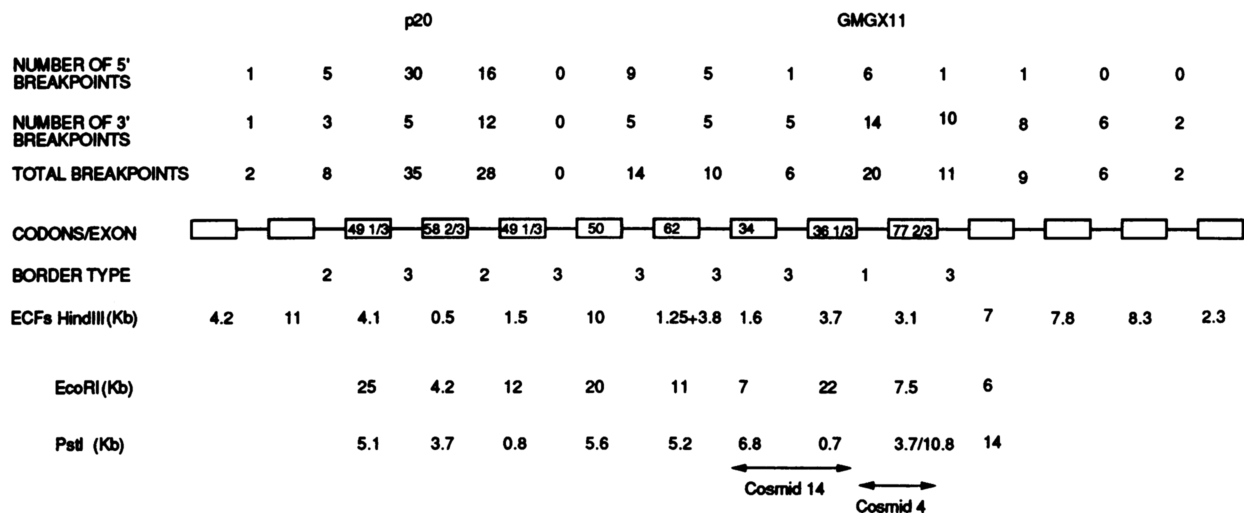

Figure 2 Localization of deletion breakpoints. The position of 5′ and 3′ deletion breakpoints is indicated with respect to *Hin*dIII, *Eco*RI, and *Pst*I ECFs. The open boxes represent ECFs. The numbers within the boxes represent the number of codons in that exon, where known (Baumbach et al. 1989). The lines between boxes represent intronic sequences. Exon boundaries were defined by comparison of intron/exon boundaries (Baumbach et al. 1989) with the protein sequence (Koenig et al. 1988). The exon border number indicates the relative position of each intron/exon border with respect to the translational reading frame. A border of "3" means that the intron/exon border occurs between intact codons in the mRNA (i.e., after codon position 3). A border of "1" or "2" means that the border occurs after the first or second nucleotide of the codon, respectively (nomenclature from Koenig et al. 1989). The translational reading frame is maintained if a deletion juxtaposes the 3′ exon border of the exon preceding the deletion to a 5′ exon border of the same border type. A frameshift occurs if a deletion juxtaposes differing 3′ and 5′ border types. ECF sizes are indicated in kilobases for *Hin*dIII, *Eco*RI, and *Pst*I restriction digests. The *Pst*I RFLP is detected by cDNA8 (Darras and Francke 1988).

mately 6.6 kb replacing the 12-kb *Eco*RI band in DNA from four patients (families 20, 32, 89, and 218) with deletions of the 0.5-kb *Hin*dIII ECF. The finding of a similar novel fragment in all four patients raises the possibility of a common junction fragment for three of the four (the deletion in family 218 extends further into the p20 intron). If this is the case, a common mechanism of deletion is an interesting possibility for these patients.

Analysis of Deletion Breakpoints

The location of deletion breakpoints (fig. 1) with respect to ECFs is summarized schematically in figure 2. Although the region is clearly deletion prone, there is no single intron that stands out as the major contribution to 5′ or 3′ breakpoints. The three introns that contribute most breakpoints are the "p20 intron," the intron adjacent to it on the 3′ side, and the "GMGX11" intron. For two of these introns, genomic clones p20 and GMGX11 allow further delineation of breakpoints within the intron. Ten of 29 deletions detected by p20 have one breakpoint within p20, and 9 of these 10 deletions extend toward the 3′ end of the gene. This is comparable to an earlier study in which 15/49 BMD/DMD patients with p20 deletions had one breakpoint within p20 itself (Wapenaar et al. 1988). Clone p20 is a deleted derivative of a cosmid clone, and therefore these 10 breakpoints must lie within about 40 kb on the chromosome (Wapenaar et al. 1988). Wapenaar et al. (1988) placed p20 15 kb 5′ to the 0.5-kb *Hin*dIII ECF, and in our study there are four additional breakpoints within this 15 kb. All four of these deletions extend toward the 3′ end of the gene.

Ten of the 14 3′ breakpoints in the GMGX11 intron lie on the 3′ side of GMGX11, and two others are detected as junction fragments by GMGX11 (fig. 1). A cosmid clone (cosmid 4; fig. 2) has been isolated that contains GMGX11 and the 3′-adjacent 3.1-kb *Hin*dIII ECF at opposite ends. Three of the six 5′ breakpoints in the GMGX11 intron also lie within this cosmid, indicating that, in the absence of a cloning artifact, at least 13 breakpoints lie within the 40 kb covered by this clone. The 1.6-kb and 3.7-kb *Hin*dIII ECFs have also been localized within a single cosmid (cosmid 14; fig. 2), suggesting that, in the absence of a cloning artifact, the intron between them, containing six breakpoints, is no more than 40 kb in size.

Correlation of Genotype Analysis with Phenotype

Eight of the nine BMD patients in our study have deletions of genomic clone p20, and the deletions in all nine patients cluster toward the 5′ end of the deletion hot spot. This is in agreement with earlier findings with cDNA clones Cf23a and Cf56a, which showed that the more 5′ clone Cf23a preferentially detected deletions associated with a BMD phenotype while the more 3′ clone Cf56a preferentially detected deletions associated with a DMD phenotype (Forrest et al. 1988). It is notable that all 13 in-frame deletions in this study commence in the "p20 intron" (fig. 1), with the exception of the most seriously affected patient, (78)703. Half of *all* BMD patients in an earlier study (Read et al. 1988) had deletions with a breakpoint in this intron.

Thirty-eight independent patients are classified as DMD, BMD, or intermediate and have deletions confined to the eight exons with defined intron/exon boundaries (Baumbach et al. 1989). Eight BMD and one intermediate patient have in-frame deletions of the *DMD* gene, while 21 DMD, 7 intermediate, and 1 BMD patient have frameshift deletions of the *DMD* gene (fig. 1). These data, with two exceptions, are in accordance with the frameshift hypothesis of Monaco et al. (1988). The most notable exception is BMD patient (191)1406 with an apparent frameshift deletion of the *DMD* gene, who underwent surgery and bracing at age 14 years and was wheelchair bound by age 18 years. He represents the most severe end of the BMD spectrum (severity 4). The exon deleted in BMD patient (191)1406 was also detected in a BMD patient (one of three exceptions to the frameshift hypothesis) in the study of Baumbach et al. (1989).

Only one apparent in-frame gene deletion is associated with a more severe (intermediate) phenotype. It is interesting that this deletion represents the smallest in-frame deletion of the *DMD* gene in this group: 62 codons in patient (78)703 (severity 2). The deletion of the gene in 78(703) is unique among the 80 patients in our group. The association of his severe phenotype (severity 2) with the apparent in-frame deletion of a single exon suggests that the exon within the 1.25/3.8-kb *Hin*dIII ECF might encode a critical part of the protein. This is unlikely, however, given that this deletion is included within three other in-frame deletions of the *DMD* gene that result in a milder BMD phenotype.

The severity of phenotype was assessed on a numerical scale from 1 to 5, independently of the deletion data, by one of us (E.G.M.), as described in Material and Methods. All patients (including those younger than age 12 years but not yet wheelchair bound) were graded in this way, with the exception of the youngest individual, (249)1760, who is not yet 3 years of age. The correlation between phenotypic severity and the pre-

dicted effect on the translational reading frame of the exon(s) deleted could be examined in 51 independent families by this classification (fig. 3). Patients with junction fragments or with deletions that extend outside the eight exons with defined intron/exon boundaries were excluded. Among these 51 families there are no exceptions (other than the two patients already described above) to the hypothesis that a severe phenotype (severity 1–3) is associated with deletions of the *DMD* gene that apparently disrupt the translational reading frame while a mild phenotype (severity 4 or 5) is associated with deletions of the *DMD* gene that apparently maintain the translational reading frame.

The most severely affected group of patients (severity 1) are those deleted for the 4.1-kb *Hin*dIII ECF. The most disparate group, that deleted for the 0.5-kb *Hin*dIII ECF, includes 11 patients with severities ranging from 1 to 4. This group includes BMD patient (191)1406 with a frameshift deletion of the *DMD* gene. It is noteworthy that a wide spectrum of phenotypes is also associated with the deletion of exons 3–7 (Malhotra et al. 1988), which includes patients who fail to conform to the frameshift hypothesis.

There is no strong correlation between IQ and deletion type in our patient group (Appendix and figs. 1, 3). Patient (58) 661, with an IQ of 30–50, had a unique deletion with an as yet undetermined effect on the translational reading frame. His deletion is completely encompassed by two other deletions of the *DMD* gene, one of these in a patient with a measured IQ value of 77. Two overlapping deletions with measured IQ values of 86 and 91 include all the exons deleted in 58(661) and extend beyond his deletion toward opposite ends of the *DMD* gene. This rules out a gene with neurological function contained within an intron deleted in the retarded boy. The mean IQ for the 19 patients with measured IQ values and frameshift deletions of the *DMD* gene is 88.2, which does not differ significantly from the mean of 86.5 for the group as a whole. This argues against a neurological function for that portion of the *DMD* gene 3′ to the deletion hot spot, unless the hotspot region of the gene is removed by differential splicing in neurological tissue.

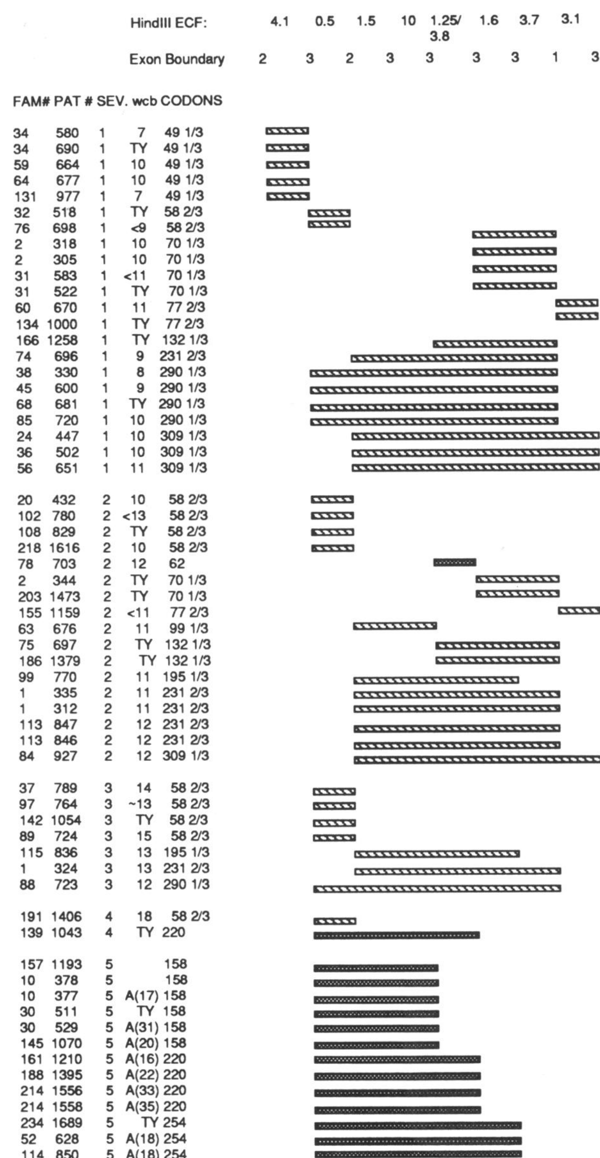

Figure 3 Correlation of phenotype with number of codons deleted. Patients are grouped according to the severity of their phenotype on a scale from 1 to 5 as described in Material and Methods. The *Hin*dIII ECFs deleted in eachpatient are indicated by hatched (▨; frameshift) or shaded (▩; in frame) bars. Exon boundaries were defined by comparison of intron/exon boundaries (Baumbach et al. 1989) with the protein sequence (Koenig et al. 1988). The number of codons deleted and the age at which each patient became wheelchair bound (wcb) are indicated. Further clinical details are given in the appendix.

Discussion

Clustering of Deletion Breakpoints

It has been noted that, in proportion to its size in the genome, deletions of the *DMD* gene occur at a frequency comparable to those of other X-linked genes, such as *FVIIIc, HPRT,* or *OTC.* (Koenig et al. 1987; Darras et al. 1988). On the basis of the assumption that deletion breakpoints are uniformly distributed, it can be argued that the greatest number of breakpoints should occur in the largest introns (with exon sizes being negligible in relation to the gene as a whole). In

our group of 181 patients, 123 deletion breakpoints fall in the estimated 650 kb between JBir and GMGX11 (Burmeister et al. 1988; Wapenaar et al. 1988), which corresponds to a minimum breakpoint density of 1.0 breakpoint/million bases (Mb)/patient. Since 60% of our 181 patients have partial deletions of the 2.4-Mb *DMD* gene (0.5 breakpoints/Mb/patient), the breakpoint density of the deletion-rich region is double that for the gene as a whole.

At least 13 breakpoints lie in the 40 kb (cosmid 4) between GMGX11 and the adjacent 3.1-kb *Hin*dIII ECF, six breakpoints lie in the 40 kb or less (cosmid 14) between the 1.6- and 3.7-kb *Hin*dIII ECFs, 10 breakpoints lie in the 40 kb of p20 itself, and 4 breakpoints lie in the 15 kb between p20 and the 0.5-kb *Hin*dIII ECF. The breakpoint densities of these four intervals of the deletion-rich region are 1.8, 0.8, 1.4, and 1.5 breakpoints/Mb/patient, respectively, while the weighted mean (1.4 breakpoints/Mb/patient) is two- to threefold higher than the estimate for the gene as a whole.

For comparison, six deletion breakpoints in intron 7 were detected among the same 181 patients. Intron 7 lies within the "minor deletion hot spot" at the 5′ end of the *DMD* gene and is 110 kb in size (Burghes et al. 1987). The breakpoint density for this intron is 0.3 breakpoints/Mb/patient, a little below the average for the gene as a whole. Relatively few intron sizes are known, but the mean intron size (35 kb; Koenig et al. 1987) and the estimated breakpoint density for the gene as a whole suggest that 3.2 breakpoints should occur per "average" intron in our patient group. There are very few deletion breakpoints in cDNA10-14, which suggests that the breakpoint density for this region may be even lower than that observed for intron 7.

Clustering of breakpoints could be due to either sequence-specific (homologous) or structure-specific (nonhomologous) recombination hot spots. Nonhomologous recombination mechanisms are attractive for the relative ease with which they can account for the diversity of deletions observed in this study. By comparison, one might predict that homologous recombination mechanisms would result in a relatively small number of endpoints. However, given the high frequency of deletions and the considerable heterogeneity of deletion endpoints, it seems probable that both types of mechanism contribute to the generation of deletions.

Deletions might occur as a consequence of nonhomologous recombination between widely separated sequences that are physically close in the nucleus as a result of anchorage to the nuclear matrix. One model, involving loss of a complete chromatin loop, predicts deletions of approximately equal size with different endpoints (Vanin et al. 1983). Anand et al. (1988) proposed that deletions might occur by deletion of newly replicated DNA loops, the size of the deletion being dependent on the length of DNA replicated. One endpoint of such a deletion is defined by the region immediately adjacent to the nuclear-matrix attachment site, while the second is potentially defined by a DNA-polymerase pause site. The attachment site itself is not deleted, so deletions appear to fan out from the attachment site.

The 5′ and 3′ deletions in our study are not apparently staggered as would be predicted by the model of Vanin et al. (1983) but appear rather to fan out from specific regions. This is notable for three introns—namely, the two introns flanking the 0.5-kb *Hin*dIII ECF and the GMGX11 intron (fig. 2)—with some directional bias to the deletions commencing in the p20 and GMGX11 introns. These data would tend to support the model of Anand et al. (1988). The apparent directional bias in our study could perhaps be explained by an asymmetric distribution of DNA-polymerase pause sites.

The Frameshift Hypothesis

Our data, with two exceptions, support the hypothesis that, for eight exons within the deletion-rich region, a more severe phenotype results from a frameshift deletion of the *DMD* gene than from one that maintains the translational reading frame. The most notable exception in our series is BMD patient (191)1406, who has an apparent frameshift deletion of one exon of the *DMD* gene. The same exon was deleted in a BMD patient who represents one of three exceptions to the frameshift hypothesis in the study of Baumbach et al. (1989). This suggests that there may be unique features of deletions of this exon that account for its occasional failure to conform to the frameshift hypothesis.

The low incidence of BMD patients with frameshift deletions observed for the eight exons in our study (1 patient in 38) is in contrast to that observed for the first 10 exons of the gene (6 of 29 patients, Malhotra et al. 1988). Each of the six BMD patients in their study had a deletion of exons 3–7, a deletion expected to create a frameshift if exon 2 is spliced to exon 8 in the mRNA. Three mechanisms could account for these unexpected findings (Malhotra et al. 1988): (1) splicing of exon 2 to exon 9 or of exon 1 to exon 8, resulting in an mRNA with an in-frame deletion, (2) reinitiation of protein synthesis at an in-frame putative translational start site in exon 8, and (3) transcription from a postu-

lated promoter in intron 7 that might initiate protein synthesis from the same putative translational start site.

Altered splicing is the most likely explanation to account for the two patients in our study who apparently fail to conform to the frameshift hypothesis. Point mutations that adversely affect mRNA splicing, by either the generation of novel splice sites or utilization of cryptic splice sites, have been described for the β-globin gene. Three point mutations within intron 2 that generate new donor sites for mRNA splicing result in transcripts that also utilize a cryptic acceptor site located 5′ to each of the three mutations. Consequently, intron sequence is included in the processed mRNA, despite the presence of normal donor and acceptor sites flanking intron 2 (reviews by Kazazian and Antonarakis 1988; Orkin 1987).

The reading frame of the deletion in BMD patient (191)1406 would be restored if alteration of a normal splice-site consensus resulted in failure to include either exon that flanks his deletion. This deletion is unlikely to disrupt the normal donor site of the preceding exon, however, as its 5′ breakpoint lies within p20. The reading frame could also be restored by inclusion of intron sequences in the message, an inclusion resulting from activation of a cryptic splice site or from generation of a novel splice site within flanking introns.

The severe phenotype in patient (78)803, who has an apparent in-frame deletion of the gene, may also be explained by altered splicing. The translational reading frame in this patient might be disrupted by inclusion of intron sequences within the mRNA, either by activation of a cryptic splice site or by the generation of a novel splice site in flanking introns. Both exons flanking the deletion in (78)703 code for an integral number of codons, so that alterations to flanking consensus sites to include either of these exons in the mRNA would not disrupt the translational reading frame.

Deletion Analysis for Diagnosis of DMD

Deletion studies such as this reveal a number of practical tips for the routine diagnostic-service laboratories. All deletions in this study could have been detected by hybridization of cDNA probes to *Hin*dIII, *Eco*RI, or *Pst*I digests, although none of these restriction enzymes is ideally suited to such an analysis. The large (20 kb or more) ECFs detected by *Eco*RI are difficult to resolve, while the small (less than 1 kb) ECFs detected by *Pst*I and *Hin*dIII are difficult to visualize. Both the 25-kb *Eco*RI ECF and the 1.5-kb *Hin*dIII ECF hybridize weakly to cDNA5b-7. *Bgl*II digests are not a suitable alternative for screening deletions in this region, because of the failure of cDNA8 to resolve two *Bgl*II ECFs (Darras and Francke 1988).

Further constraints are involved when the entire cDNA is to be used for deletion analysis, making the use of multiple blots (with different enzymes and varying concentrations of agarose and conditions for electrophoresis) almost obligatory. Thus it seems likely that the multiplex polymerase chain-reaction (PCR) recently outlined by Chamberlain et al. (1988) will prove the method of choice for preliminary screening for deletions. Sixty-six of the 80 deletions described here could have been detected by multiplex PCR using the primer sets described by Chamberlain et al. (1988), which detect exons 8, 17, and 19 and *Hin*dIII ECFs of 4.1 kb, 0.5 kb, and 1.25/3.8 kb. This number can be increased to 75 of the 80 deletions (94%) by multiplex PCR using more recent primer sets (which additionally detect exon 4, exon 12, and the 3.1-kb *Hin*dIII ECF).

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Appendix

Table AI

Clinical Information on Affected Individuals from Each of the 80 Families with Deletions within the Deletion-prone Region

Family, Patient (date of birth)	Phenotype	Severity	Codons Deleted[a]	Family History[b]	Age of Patient (years): Diagnosis	Last Seen	TAL[c]	WCB[d]	Luque Instrumentation[e]	Muscle Biopsy[f]	Electromyography	ECG	IQ
1, 324 (6/4/69)	Intermediate	3	231 2/3	+	6		11	13	...[g]	Dystrophic	Myopathic	Normal	
1, 335 (6/5/72)	DMD	2	231 2/3	+	2	15	10	11	13	Dystrophic	Myopathic	Normal	106
1, 312 (4/9/63)	DMD	2	231 2/3	+	8	22		11	14	Dystrophic	Myopathic	Normal	93
2, 318 (11/1/73)	DMD	1	70 1/3	+	6.5	15	9	10	13	...	...	...	
2, 305 (15/12/72)	DMD	1	70 1/3	+	...[h]	15		10	13	...	...	...	
2, 344 (24/5/79)	Too young	2	70 1/3	+	1	9	Refused	Ambulatory		...	...	...	
10, 378 (12/8/67)	BMD	5	158	−2						Dystrophic	Myopathic	Normal	
10, 377 (11/2/70)	BMD	5	158	−2	...[i]	17	...	Ambulatory		...	...	...	
15, 310 (30/9/65)	DMD	1	ND	+	2	18	9	11	14	...	...	Normal	
18, 415 (4/8/77)	DMD	1	ND	+	4.5	11	8	11		Dystrophic	Myopathic	Normal	77
20, 432 (28/2/66)	DMD	2	58 2/3	+	3	19	9	10	15	Mild	Myopathic	Normal	93
22, 515 (27/6/78)			309 1/3	+									
24, 447 (2/4/68)	DMD	1	309 1/3	−	7[j]	18	8	10	14				
28, 478 (25/1/69)	DMD	1	ND	+	2	16	Refused	10	...[k]	Dystrophic	Myopathic	Normal	
30, 529 (11/3/58)	BMD	5	158	+	6	31		Ambulatory[l]		+	+		87
30, 511 (18/7/77)	Too young[m]	5	158	+	4	10				Dystrophic	Myopathic	Abnormal	102
31, 583 (28/8/68)	DMD	1	70 1/3	+	...[n]	17	9		11			Abnormal	
31, 522 (26/4/75)	Too young	1	70 1/3	+	6.5	6.5[o]				Severe	Myopathic	Normal	80
32, 518 (2/12/76)	Too young	1	58 2/3	−	7.5	7.5				+	...	Abnormal	77
33, 507 (23/4/67)			58 2/3	−2	9								
34, 580 (13/7/77)	DMD	1	49 1/3	+	1	11	...	7	Not yet[p]	...	...		
34, 690 (29/10/85)	Too young	1	49 1/3	+	...[q]	3				...	...		
35, 380 (1/6/72)	DMD	1	ND	+	6	14	...	8.5	11	Dystrophic	Myopathic	...	
36, 502 (29/9/71)	DMD	1	309 1/3	+	...[r]	16		10	12	−	−	−	
37, 789 (7/10/73)	Intermediate	3	58 2/3	−	...[s]	15	11	14	Soon	+	+	−	
38, 330 (3/3/71)	DMD	1	290 1/3	−	6	15	8	8	Missed	Dystrophic	Myopathic	−	
40, 562 (4/4/68)	DMD	1	ND	−	3.5	18	8	10	13	Dystrophic	Myopathic	Normal	87
45, 600 (29/8/74)	DMD	1	290 1/3	−	3	14	9	9	P(14)	Dystrophic	Myopathic	Normal	
52, 628 (22/11/69)	BMD	5	254	−	...[t]	18	−	Ambulatory	−	−	−		
56, 651 (29/8/73)	DMD	1	309 1/3	−	6	15	10	11	12	Dystrophic	Myopathic		72
58, 661 (1/3/77)	DMD	1	ND	−	3	11	−	8	−	Severe	Severe		30–50
59, 664 (9/9/73)	DMD	1	49 1/3	−	5	15	8	10	12	Severe		Abnormal	
60, 670 (25/10/63)	DMD	1	77 2/3	−	5	14	9	11	14	Dystrophic	Myopathic	Normal	91
63, 676 (12/2/69)	DMD	2	99 1/3	−	3	18	11	11	15	+	+		...[u]
64, 677 (3/1/67)	DMD	1	49 1/3	−	3	19	9	10	14	+	+		80

(continued)

Appendix (continued)

Table AI

FAMILY, PATIENT (date of birth)	PHENOTYPE	SEVERITY	CODONS DELETED[a]	FAMILY HISTORY[b]	AGE OF PATIENT (years): Diagnosis	Last Seen	TAL[c]	WCB[d]	Luque Instrumentation[e]	MUSCLE BIOPSY[f]	ELECTROMYOGRAPHY	ECG	IQ
68, 681 (6/5/83)	Too young	1	290 1/3	–	3	5				+	+	Abnormal	85
73, 685 (28/9/77)	DMD	1	ND	–	4	11	–	11	P(11)	Moderate	Mild		95
74, 696 (14/3/78).....	DMD	1	231 2/3	–	4	10	. . .[v]	9?	Not yet	+	+	Normal	93
75, 697 (30/9/77)	Too young	2	132 1/3	–	6	11	10	. . .[w]		Severe	Severe	Normal	70–80
76, 698 (1/5/73)......	DMD	1	58 2/3	–	4	14	6		9	+	+		109
78, 703 (1/6/73)......	Intermediate	2	62	–	5	15	9	12	P(16)	Severe	Myopathic	Normal	
80, 705 (12/3/77).....	Dystrophic	2	ND	–		11	–	9	. . .[x]	Moderate	Myopathic	Abnormal	92
84, 927 (14/6/72).....	Intermediate	2	309 1/3	–	7[y]	16	10	12	12	Dystrophic	Myopathic	Normal	
85, 720 (8/10/75).....	DMD	1	290 1/3	–	7	13		10		Severe	Myopathic		81
88, 723 (16/8/68).....	Intermediate	3	290 1/3	–	6	18	8	>10.5[z]	12	+	+		80
89, 724 (16/8/72).....	Intermediate	3	58 2/3	–	5.5	16	12.5	. . .[aa]	Not yet	+	+		
90, 725 (13/10/69)	DMD	1	ND	–	6	18	–	7	13	–	–	Normal	
93, 757 (25/10/70)....	DMD	2	ND	–	5	17	9	10	12	+	+	Normal	80
97, 764 (18/9/67).....	Intermediate	3	58 2/3	–	5	19	9	~13	15		–		
99, 770 (19/9/76).....	DMD	2	195 1/3	–	3	12	10	11	Not yet	+	+		
102, 780 (14/6/78)....	Too young	2	58 2/3	–	6.5	9				+	–		
103, 782 (10/3/71)	Intermediate	2	ND	–		16	10		13	+	+	Normal	86
108, 829 (27/9/76) ...	Too young	2	58 2/3	–	8.5	8.5				+	+		
113, 847 (25/10/76) ...	Intermediate	2	231 2/3	–2	. . .[bb]	12	Refused	~12	Soon	–	–		
113, 846 (18/4/73)	Intermediate	2	231 2/3	–2	. . .[cc]	15	–	By 12	15	+	+		
114, 850 (23/8/68)....	BMD	5	254	–	. . .[dd]	18	–	–	–	Moderate	+	Normal	93
115, 836 (14/1/71).....	Intermediate	3	195 1/3	–	6	17	–	13	Not yet	+	+	Normal	
118, 889 (2/12/63)	DMD	1	ND	–2	3[ee]	21	9.5	11.5	Too weak				
123, 922 (5/7/73)	DMD	1	ND	–	5	15	7	8	12	+	+		
127, 963 (21/10/67) ...	Intermediate	3–4	ND	–	4	19	13		16	+	+	Normal	. . .[ff]
128, 964 (18/5/70)....	DMD	3	ND	–	3	17	9	11	–	+	+		102
130, 969 (19/4/83)....	Too young	1	ND	–	3.5	5.5	–	–	–	Severe	+	Abnormal	90
131, 977 (30/8/74)....	DMD	1	49 1/3	–	6	14	–	7	12	+	+		
134, 1000 (15/10/82)	Too young	1	77 2/3	–	1.5	6	–	Ambulatory[gg]	–	+	+		. . .[hh]
139, 1043 (11/5/81)	Too young[ii]	4	220	+	5.5	7		Ambulatory		+	+		
142, 1054 (12/5/79)	Too young	3	58 2/3	–	7	7	–	–	–	Dystrophic, fibrosis	Mild	Normal	82
143, 1061 (12/9/79)	Too young	2	ND	–	5	9				+	+		
145, 1070 (24/6/65)	BMD	5	158	–	17	22		Ambulatory		+[jj]	–		
155, 1159 (1/12/64)	DMD	2	77 2/3	+				By 11					
156, 1172 (31/1/81)	Too young	2	ND	–	6	9	–	–	–	+	+	Normal	70
157, 1193 (24/5/65)	. . .[kk]	5	158	–									

161, 1210 (3/4/72)	BMD	5	220	–	13	16		Ambulatory[ll]					
164, 1243 (13/11/83)	Too young	1	ND	+	3.5	5				+	+		
165, 1249 (5/6/78)....	Too young	4	ND	–	9[mm]	9.5		Ambulatory		Mild–moderate[nn]	+	Abnormal	91
166, 1258 (14/12/82)	Too young	1	132 1/3	–	4.5	5	–	–	–	Severe	Myopathic	Normal	81
171, 1292 (28/10/75)	DMD	2	ND	–	5	6		10		+	+		
181, 1394 (1/12/65)	DMD	2	ND	–2	6	. . .[oo]		10					
185, 1372 (29/6/80)	Too young	1	ND	–	7	8	Soon	Ambulatory[pp]		Moderate	+	Normal	
186, 1379 (2/5/83)....	Too young	2	132 1/3	–	4	5				Severe	+	Normal	109
188, 1395 (17/3/57)	BMD	5	220		14	22		Ambulatory[qq]		+	+	Normal	
191, 1406 (28/12/59)	BMD	4	58 2/3	–	. . .[rr]	29	14	18[ss]	18	–	–		
202, 1471 (23/11/81)	Too young	1	ND	–		6.5		Ambulatory[tt]		Severe	+	Normal	
203, 1473 (11/11/81)	Too young	2	70 1/3	–	6	7	–	–	–	+	+		
214, 1556 (19/10/54)	BMD	5	220	+	16	33		Ambulatory[uu]					
214, 1558 (15/3/52)	BMD	5	220	+	20	35		Ambulatory[uu]					
218, 1616 (2/12/73)	DMD	2	58 2/3	–	6	14.5		10	15	+	+		
234, 1689 (24/2/81)	Too young[vv]	5	254	–	7	7				Mild	Mild		69
236, 1695 (1/9/71)	DMD	1	ND	+	6	16	11	13	+	+			
240, 1721 (7/12/74)	DMD	1	ND	–2	4[ww]	14		8	–	+	. . .[xx]		
249, 1760 (24/9/86)	Too young	–	ND	+	1/12	2				. . .[yy]	. . .[yy]		

NOTE.—Information is included for all individuals irrespective of frameshift status.

[a] Frameshift status is indicted by the number of codons deleted. ND = not determined.

[b] Considered positive (+) if there were affected males in more than 1 generation or in more than one branch of the family; considered negative (–) if the affected male was an isolated case. Affected siblings in a nuclear family with no prior family history are identified as "–*N*," where *N* is the number of affected siblings.

[c] Achilles tendon lengthening and bracing.

[d] Wheelchair bound.

[e] P(age) denotes age proposed for the procedure.

[f] A plus sign (+) indicates that results are consistent with a progressive myopathy of the DMD/BMD type, whereas "Dystrophic" and "Myopathic" indicate a more definitive result.

[g] Not done, because of Von Willebrand disease.

[h] Diagnosed with cousin (2, 318).

[i] Diagnosed with elder brother.

[j] Ascertained at age 7.

[k] Not done, because of rapid deterioration.

[l] Trouble with stairs.

[m] Five living affected males aged 5–64 years.

[n] Not known: first seen in Toronto at age 12 years.

[o] Moved away; no followup.

[p] No spinal deformity.

[q] Diagnosed as infant.

[r] Ascertained at age 9; sudden onset of weakness at age 10.

[s] Not known: first seen in Toronto at age 11 years.

[t] Initial diagnosis elsewhere; first seen at age 11.

[u] Above-average intellect.

[v] Surgery not thought worthwhile.

[w] Almost WCB (at age 11).

[x] Developing scoliosis age 11.

[y] First seen at age 7.

[z] Walked until "at least 10.5."

[aa] "Walked to 12.5, gave up, then walked again until 15."

(continued)

Appendix (continued)

Table AI

[bb] First seen at age 8.
[cc] Followed up elsewhere.
[dd] Diagnosed at age 14, but problems were apparent prior to this.
[ee] Younger of two affected brothers.
[ff] Attending university.
[gg] Tightness of heel cords.
[hh] Special education.
[ii] No Gowers sign; slight difficulty climbing stairs; has affected cousin age 15 years; notes specify BMD.
[jj] Muscle pathology consistent with diagnosis of BMD.
[kk] Notes specify BMD.
[ll] Uncle WCB at approximately age 40 years.
[mm] Increasing problems for 2 years prior to diagnosis.
[nn] Biopsy consistent with BMD.
[oo] Living, never seen in Canada.
[pp] At 8.5 a little difficulty rising from floor.
[qq] Ambulant at age 22; difficulty climbing stairs.
[rr] First seen at age 17.
[ss] WCB from spinal fusion on; still in manual wheelchair at age 29.
[tt] Some tightness of heel cord.
[uu] A third brother WCB at age 32.
[vv] Notes specify BMD.
[ww] Diagnosed with elder brother.
[xx] For elder brother only.
[yy] For affected uncle only.

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