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Brother/Sister Pairs Affected with Early-Onset, Progressive Muscular Dystrophy: Molecular Studies Reveal Etiologic Heterogeneity

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Summary

An autosomal recessive (AR) form of muscular dystrophy that clinically resembles Duchenne/Becker types exists, but its frequency is unknown. We have studied three unrelated affected brother/sister pairs and their families for deletions and polymorphisms with the entire dystrophin cDNA and other DNA probes from the Xp21 region to test for involvement of the DMD locus. In family 1 a large intragenic deletion was found in the affected male. The affected sister was heterozygous for this deletion, but the mother was not, implying germinal mosaicism. In family 2, no deletion was detected in the affected male. RFLP analysis revealed that the affected male and an unaffected sister shared a complete Xp21 haplotype while the affected sister had inherited a recombinant Xp21 region resulting from a crossover between pERT 87-15 and J-Bir. Only the 5′ region of the dystrophin gene was shared with the affected boy. X-inactivation studies using a polymorphism in the 5′-flanking region of the HPRT gene, in conjunction with methylation-sensitive enzymes, revealed random X inactivation in the affected girl's leukocytes. In a muscle biopsy from the affected male, the dystrophin protein was present in normal amount and size. Family 3 was informative for four RFLPs detected with dystrophin cDNA probes which span the entire gene. The affected male was found to share the complete dystrophin RFLP haplotype with his unaffected brother, while his affected sister had inherited the other maternal haplotype. It is concluded that the clinical presentation of early-onset, progressive muscular dystrophy in a male and in his karyotypically normal sister can be caused by mutations at different loci. While in family 1 a deletion in the dystrophin gene is responsible, this gene does not appear to be involved in families 2 and 3.

Introduction

Since the cloning and sequencing of the dystrophin gene and the elucidation of its product, molecular studies at the DNA and protein levels have demonstrated that a defect in the dystrophin gene on Xp21 is responsible for the majority of cases of Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD), as well as for many of the "outlier" patients that are not easily classified (Cross et al. 1987; den Dunnen et al. 1987; Hoffman et al. 1987, 1988; Koenig et al. 1987; Darras et al. 1988*a*; Miranda et al. 1988). Alternative causes have to be considered for those exceptional cases in whom cDNA probes fail to detect a deletion and in whom the dystrophin protein appears to be present in normal size and abundance by immunoblotting.

Convincing evidence for the existence of autosomal recessive (AR) mutations that produce a DMD/BMD-like phenotype has recently been reported in consanguineous families and inbred populations from central and northern Africa and at low frequency in the United Kingdom (Hamida et al. 1983; Salih et al. 1983; Gardner-Medwin and Johnston 1984; Somer et al. 1985). In an AR form of the disorder, males and females in the same sibship should be affected to the same

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degree. Although affected females were among the earliest reported cases of DMD, X-linked recessive inheritance was supported by the familial patterns and the preponderance of affected males (reviewed by Emery 1987). The fact that maleness is one of the diagnostic criteria for DMD is particularly relevant for sporadic cases. In 1970, Penn et al. (1970) critically reviewed the literature on 104 females reported to have a DMD-like disorder. They excluded all but 19 on clinical grounds. For those remaining, other possible diagnoses, such as spinal muscular atrophy, polymyositis, or X-chromosome abnormality could not be excluded, owing to lack of adequate laboratory tests, serum enzyme levels, and karyotypes.

Possible causes for girls to have an early-onset, progressive muscular dystrophy include abnormalities of the X chromosome. Very few female patients with 45,X, 46,XY, or Turner mosaic karyotypes have been reported (Ferrier et al. 1965; Jalbert et al. 1966; Wulfsberg and Skoglund 1986). But in recent years more than 20 affected females have become known who have apparently balanced X/autosome translocations with breakpoints in Xp21 and preferential inactivation of the normal X (reviewed in Boyd et al. 1987). In these girls, the translocation breakpoint presumably involves the dystrophin gene. In addition, females heterozygous for a mutation in the dystrophin gene could express the disorder either if X inactivation were nonrandom secondary to a translocation with a breakpoint elsewhere on the X or for a nonchromosomal reason, e.g., a cell-lethal mutation in another X-linked gene.

By RFLP analysis with flanking and intragenic probes and by deletion screening with the entire cDNA, we have studied three families with affected male/female sib pairs, for involvement of the dystrophin gene. In all three families the affected girls had normal female karyotypes, there were no other affected family members, and each sib pair had early-onset, progressive muscular dystrophy with diagnosis before 10 years of age. Possible nonrandomness in X inactivation in the affected females was evaluated by studying the methylation patterns of polymorphic restriction fragments derived from the 5′ control regions of X-linked housekeeping genes (Fearon et al. 1987).

Case Histories

Family 1

Patient B-1.—This patient was the 3.2-kg product of a normal pregnancy and delivery. Although meningitis at 8 mo of age was effectively treated, the patient's psychomotor development was delayed. He walked at 3 years with a waddling gait pattern. At 2 years of age he was noticed to have pseudohypertrophy of the calves, diminished deep tendon reflexes, and proximal muscle weakness. Serum CK levels were 21,000 IU/L initially and 5,700 IU/L recently. Muscle histology was consistent with muscular dystrophy. Proximal muscle weakness has been progressive. He became nonambulatory at 12 years of age. EKGs have been normal. At 8 years, psychometric testing using the WISC scale revealed a verbal IQ of 49, performance IQ of 63, and full-scale IQ of 51.

Patient B-2.—Patient B-2, patient B-1's half-sister, was born at term weighing 3.7 kg after an uncomplicated pregnancy and delivery. She began to walk at 14 months. At 5 years of age she was found to have pseudohypertrophy of the calves and toe walking. Serum CK levels have been 2,800 and 6,480 IU/L. At 5½ years she had hyporeflexia, mild proximal muscle weakness, tight heel cords, and developmental delay. Muscle biopsy at 5 years of age was histologically compatible with muscular dystrophy. EMG and karyotype were normal. Stanford-Binet Intelligence Scale, form L-M, performed at 7$^{4}/_{12}$ years revealed a mental age of 3.1 years and an IQ of 38, and at 9$^{5}/_{12}$ years it revealed a mental age of 4.3 years and an IQ of 46.

The children have a common mother (B-12) and different fathers, as confirmed by HLA testing. Their mother has no physical or neurological abnormalities. Tests on three occasions have shown her serum CK levels to be normal.

Family 2

Patient T-2.—This patient was the 4.1-kg product of a normal full-term pregnancy. She sat at 7 mo and walked at 13 mo. She was slow to acquire speech. She repeated kindergarten and is now an average student in seventh grade. She was never able to run or jump but did walk upstairs by alternating feet. She later lost this ability. She walked with a waddling gait and severe lordosis. At 10 years of age she was noted to have mild to moderate loss of muscle bulk, particularly of the proximal girdle muscles; symmetrical weakness proximal and distal; a positive Gower sign; absent or diminished DTRs; and marked scapular winging. She had no myotonia and an otherwise normal neurological examination. She started to use a wheelchair at 11 years of age. Presently, at age 13 she can no longer lift her arms but still is able to take a few steps with support. Her pseudohypertrophy of the calves seems to have varied in size over the years.

A muscle biopsy at age 9 years of age revealed a severe myopathy with degeneration and regeneration, and generalized endomysial fibrosis. Fibers of both types, I and II, were atrophic or hypertrophic. Oil-Red-Q and PAS stains were normal. EMG showed severe myopathy with spontaneous activity. Serum CK level was greater than 10,000 IU/L at 9 years of age and 2,300 IU/L at 12 years of age (normal range 17–119). Her karyotype was 46,XX with G-banding. High-resolution chromosome analysis of a lymphoblastoid cell line established in our laboratory revealed no structural abnormalities.

Patient T-1.—Patient T-1, patient T-2's younger brother, started to walk at 13 mo and did not fall as much as T-2 did. He was able to alternate feet going upstairs and later lost this ability. He always had large calves and appeared muscular. Weakness was first noticed in his shoulders. At 8 years of age he had symmetrical weakness in proximal and distal muscles and mild bilateral scapular winging. Initially, it seemed that the progression of his disease was slower than that in his sister T-2, but then it progressed more rapidly and presently, at 11 years of age, he falls as much as T-2 did at that age. Serum CK levels were 1,640 IU/L at the time of diagnosis and 1,707 IU/L recently (normal range 32–157). Muscle biopsy at 8 years of age showed the same changes as seen in T-2: variation in fiber size, mild degree of endomysial fibrosis, multiple degenerating fibers undergoing phagocytosis, and many regenerating fibers, all symptoms consistent with DMD. EMG was indicative of myopathy. T-1 had no speech problems, and his IQ was 119.

The mother (T-12) of T-1 and T-2 is Hispanic descent, and their father (T-11) is of Irish-English descent. An older sister (T-3) is clinically normal. CK levels in unaffected family members were in the normal range: T-12, 23 IU/L (normal 17–128); T-11, 114 IU/L (normal 32–267); T-3, 79 and 84 IU/L (normal 17–128).

Family 3

Patient R-1.—This patient was delivered at term with normal Apgar scores. He sat at 9 mo and began walking at 10 mo. He has always been a toe walker and had a waddling gait. When examined at 4½ years of age, he was intellectually bright and verbally advanced. He was able to ride a two-wheeled bicycle but had difficulty running. He was found to have mild weakness of the deltoids, weakness of dorsiflexion of both feet, inability to walk on his heels, and some weakness in his hip flexors. His waddling gait and accentuated lordosis were consistent with proximal muscle weakness. At 5½ years of age he had mild pseudohypertrophy of the calves and difficulty hopping on one foot. He was able to climb stairs without using the railing. Gower sign was positive. Serum CK was 9,300 IU/L (normal <82). Aldolase was 123 U/L (normal 1–6). LDH, alkaline phosphatase, and SGOT were also elevated.

Electromyography revealed myopathic changes. A muscle biopsy from the quadriceps revealed considerable variation in fiber size and small numbers of atrophic and regenerating fibers. No fibrosis or cytoplasmic inclusions were present. Twelve percent of the fibers had internal nuclei. Both type I and type II fibers were equally involved in the dystrophic process.

Subsequently, his clinical course has been slowly progressive. Presently, at 8 years of age, he has increased proximal muscle weakness and a waddling gait with exaggerated lumbar lordosis.

Patient R-2.—Patient R-2, the sister of patient R-1, was found to be toe walking at 5 years of age. Presently, at 6 years of age, she has no clinical evidence of muscle weakness but does have pseudohypertrophy and a serum CK level of 14,000 IU/L. Her karyotype was normal 46,XX with routine banding.

A 4-year-old brother (R-3) is said to be normal and to have normal CK levels. There is no consanguinity. The father (R-11) is of Eastern European Jewish ancestry, and the mother (R-12) is Scottish. Tests on three occasions have shown the mother's serum CK levels to be normal.

Materials and Methods

Blood samples were obtained from both affected children and from the mother in family 1; from both affected children, their normal sister, and both parents in family 2; and from both affected children, their normal brother, and both parents in family 3. DNA was extracted from white blood cells according to a method described elsewhere (Baas et al. 1984). In addition, lymphoblastoid cell lines were established, by transformation with Epstein-Barr virus, on the affected children in families 2 and 3.

Initially, a search for RFLPs was carried out in order to determine whether the affected siblings had inherited the same Xp21 chromosomal region from their mother. Random genomic DNA probes flanking the DMD locus as well as intragenic genomic probes were used. A total of 15 RFLPs were analyzed using probes and methods described elsewhere (Darras et al. 1987, and 1988*a*, 1988*b*; van Ommen et al. 1987).

When the dystrophin cDNA (Koenig et al. 1987) be-

came available, seven contiguous segments covering the entire gene were used as probes to screen the affected males for deletions and their mothers for polymorphisms. Digestion with *Bgl*II, *Hin*dIII, *Taq*I, and *Pst*I and Southern blotting, labeling, and hybridization were carried out as described elsewhere (Darras and Francke 1988).

Randomness of X inactivation was studied by determining the methylation patterns of polymorphic restriction fragments hybridizing with 5′-region probes of X-linked housekeeping genes, according to a method described by Vogelstein et al. (1985). First, the families were studied for RFLPs detected with 5′-region probes from the human PGK and HPRT genes. Probes pSPPT 19.1 (PGK) and pPB1.7 (HPRT) were made available by J. Singer-Sam (Keith et al. 1986) and T. Friedmann (Jolly et al. 1982). None of the females were heterozygous for a PGK RFLP. Only family 2 was informative for the HPRT polymorphism.

For the study of X-inactivation patterns, total leukocyte DNA from the affected females and from controls was digested with *Bam*HI and *Pvu*II. The samples were then split, and one-half was further digested with *Hpa*II. The *Msp*I/*Hpa*II site within the *Bam*HI-*Pvu*II fragments derived from the 5′ region of the HPRT gene will not be cleaved if the internal C is methylated—i.e., derived from the inactive X chromosome—as has been demonstrated by Fearon et al. (1987). If only one of the two allelic fragments is sensitive to *Hpa*II digestion, it is thought to be derived from a preferentially active X chromosome. If, after *Hpa*II digestion to completion, both fragments are equally reduced in intensity and both sets of smaller fragments are present, one can conclude that X inactivation is random.

Results

Family 1

RFLP studies with 15 probes that recognize 18 different polymorphisms in Xp21 were mostly uninformative, in part because the affected girl's father was not available. The family was only informative for the *Msp*I RFLP at the *OTC* locus, where both children had received different alleles from their mother. *OTC* is known to be at least 15 cM proximal to the 5′ end of the dystrophin gene.

Deletion screening and RFLP analysis with the cDNA probes was highly informative (fig. 1). A large deletion found in patient B-1 extends from region 5b-7 (1.5- and 10.0-kb *Hin*dIII and 3.3- and 3.5-kb *Bgl*II fragments deleted) to probe 9 with the 8.3-, 7.8-, 2.3-, and 1.0-kb *Hin*dIII (fig. 1, *right*) and 16.0- and 8.9-kb *Bgl*II fragments missing. All fragments that hybridize with probe 8 are deleted as well (fig. 1, *center*). This deletion has been included in a previously published series (coded O) (Darras et al. 1988*a*). Relative intensities of fragments hybridizing with probes 5b-7 and 9 provided evidence that the affected sister B-2 is heterozygous for the same deletion but that their mother B-12 is not a carrier in her leukocytes (fig. 1, *right*). RFLP data derived with probe 8 further confirm these results. B-12 is heterozygous for the *Taq*I/8 RFLP (not shown). For the *Pst*I/8 RFLP she is homozygous (3.4 kb/3.4 kb) (fig. 1, *left*), but her affected daughter B-2 has only the 10.8-kb, presumably paternal, allele, indicating that her maternally derived X chromosome carries the deletion.

We conclude that in family 1 the DMD phenotype is caused by a large dystrophin-gene deletion present in two half-siblings whose mother is a germ-line mosaic for this deletion. X-inactivation studies were not informative in the affected girl. However, the myopathy in her is more slowly progressive than that in her brother, who was wheel-chair bound by 12 years of age; this finding is consistent with her being a manifesting carrier with predominant inactivation of the normal allele. Both sibs have the same degree of moderate developmental delay that is at the severe end of the spectrum seen in DMD and that may be unrelated to their dystrophin-gene deletion.

Families 2 and 3

No deletion was found in the affected males with genomic and cDNA probes. In the absence of direct evidence for involvement of the dystrophin gene, Xp21 RFLPs were studied to determine whether the affected sibs had inherited the same dystrophin gene–containing chromosome region from their mother.

Family 2.—The mother (T-12) was informative for six RFLPs, five of them intragenic and spanning most of the gene and one of them in the proximal flanking region. The *Taq*I/8 RFLP is shown in figure 2, and the Xp21 haplotypes are shown in figure 3. The affected boy T-1 and his unaffected sister T-3 share the same maternal haplotype. The affected girl T-2 has received a recombinant X chromosome with the proximal region of the dystrophin gene identical to her brother's but with the central and distal portions differing from his.

X-inactivation patterns were studied in this family. T-2 was heterozygous for the *Bam*HI/*Pvu*I RFLP detected with the 5′ HPRT probe. Further digestion with

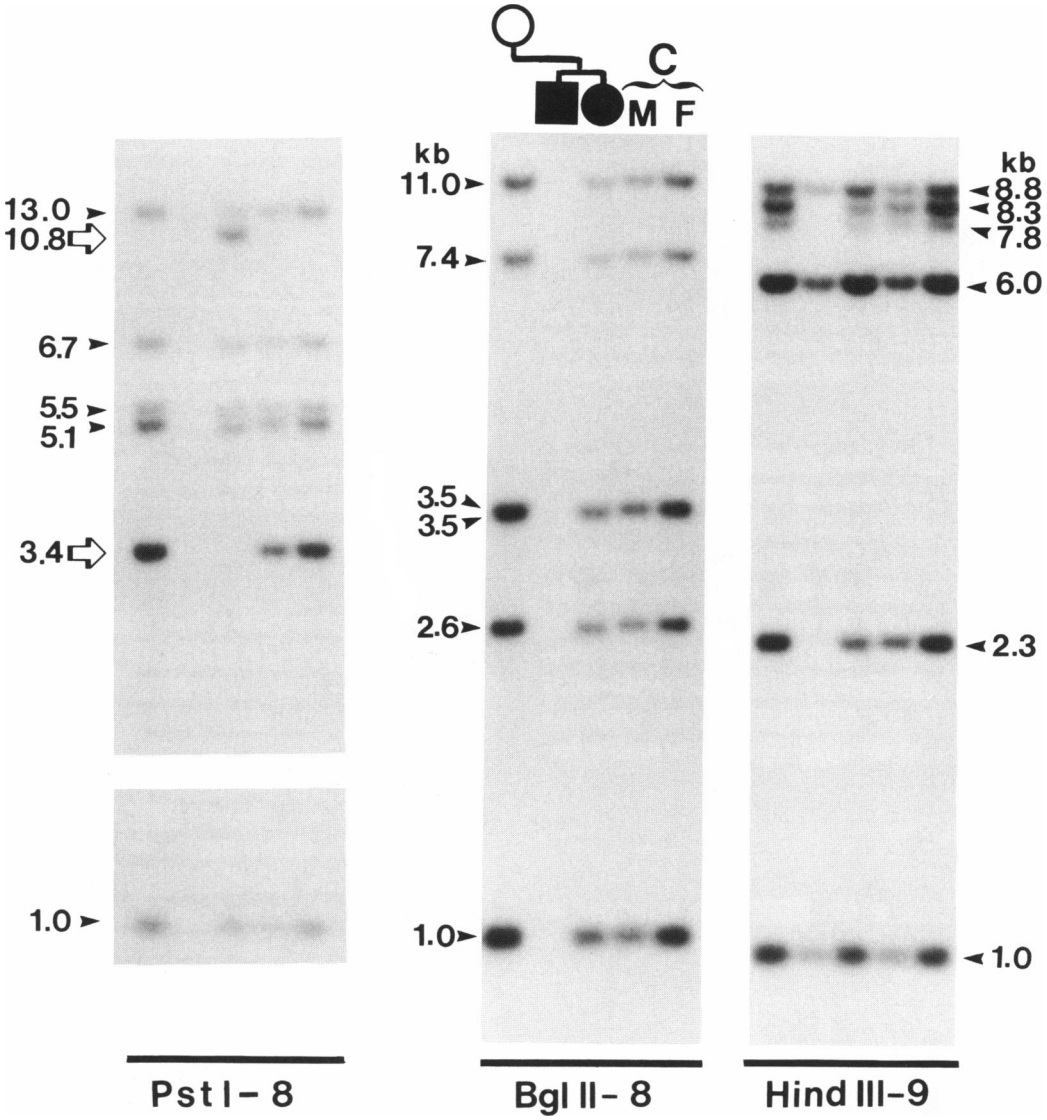

Figure 1 Deletion in family 1. DNA samples of family 1 and of male (M) and female (F) controls were digested with *Pst*I, *Bgl*II, and *Hin*dIII and were loaded on gels in the order depicted on top of the center panel. Southern filters were hybridized with cDNA probes 8 or 9. The affected male is missing all fragments seen with probe 8 (*left* and *center*) and some fragments (*Hin*dIII 8.3, 7.8, and 2.3 kb) with probe 9 (*right*). Compared with the controls, the affected female has single-copy intensity of the *Hin*dIII/9 fragments missing in her brother and two-copy intensity of those not missing. Furthermore, she has not inherited a 3.4-kb *Pst*I/8 allele from her mother but only has the 10.8-kb, presumably paternal, allele (*left*, open arrows). The mother's DNA (first lanes) shows two-copy intensity for all fragments. Therefore, she is not a deletion carrier. However, since both affected children have inherited the same dystrophin deletion from their mother, she must be a germ-line mosaic.

*Hpa*II revealed a digestion pattern consistent with random X inactivation (fig. 4). The findings that T-2 is at least as severely affected as her brother T-1 but shows random X inactivation in blood leukocytes and that T-1 and his unaffected sister T-3 share the same Xp21 haplotype suggest that in this family the disorder is autosomal. Consistent with this conclusion is the demonstration, as determined by immunoblotting by E. P. Hoffman and L. M. Kunkel according to published procedures (Hoffman et al. 1988), of dystrophin protein of normal size and abundance in a muscle specimen from T-1.

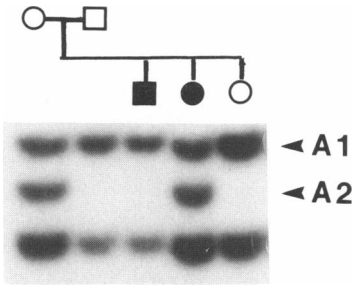

Figure 2 Family 2. The mother, who is heterozygous for the *Taq*I/8 RFLP, transmitted the A1 (6.5-kb) allele to her affected son and normal daughter and the A2 (5.6-kb) allele to her affected daughter (dark symbol).

Family 3.—This family was uninformative for flanking RFLP markers, but four of the intragenic RFLPs detectable with the cDNA probes (Darras and Francke 1988) were informative. They span most of the cDNA (fig. 5). Haplotype analysis (fig. 6) reveals that the affected brother (R-1) and the unaffected (R-3) brother have inherited the same dystrophin gene from their mother and that the affected sister has received the other allele. These results are considered strong evidence against involvement of the dystrophin gene in this disorder.

Discussion

Molecular analyses of the dystrophin gene and of

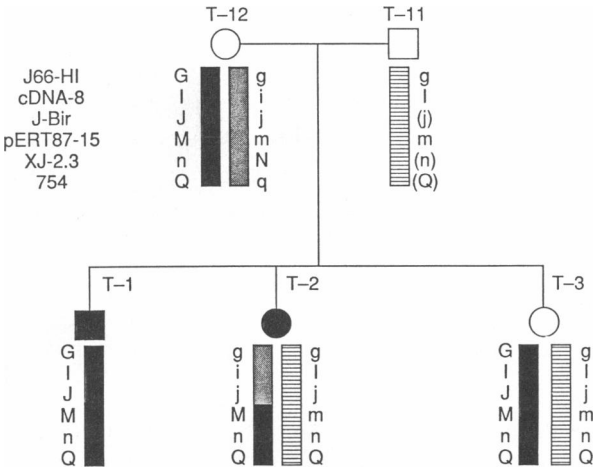

Figure 3 Family 2. T-1 and T-2 are the affected siblings. Haplotypes have been deduced for dystrophin gene RFLPs (J66-HI to XJ-2.3) and flanking marker DXS84 (754). For T-11 the alleles in parentheses are inferred. T-1 and T-3 share maternal haplotypes, and T-2 has received a recombinant Xp21 chromosome region. The meiotic exchange point is within the dystrophin gene. These results were confirmed on repeat blood samples.

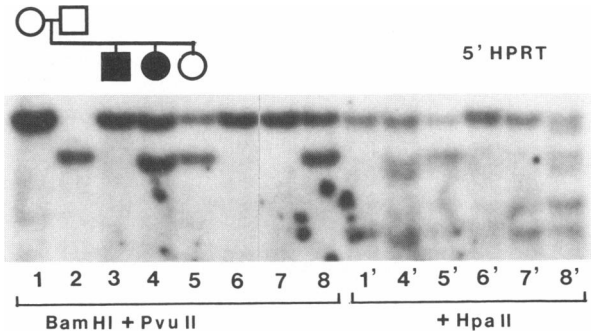

Figure 4 Family 2. The affected girl (lane 4) is heterozygous for the *Bam*HI+*Pvu*II RFLP seen with the 5′ probe from the HPRT gene. After further digestion with *Hpa*II (lane 4′), both fragments are reduced in intensity, and smaller fragments are present (not all are shown) in a pattern identical to that of the female control (lane 8′), indicating random X inactivation in her leukocyte DNA.

RFLPs associated with it have revealed genetic heterogeneity in three families with brother/sister pairs affected by early-onset, progressive muscular dystrophy. While the mutation clearly involves the dystrophin gene in family 1, we have found strong evidence against involvement of this gene in families 2 and 3.

In family 1, the affected children had different unrelated fathers, which makes AR inheritance of their disorder unlikely. The affected girl was found to be heterozygous for the same dystrophin-gene deletion present in her brother. Manifesting heterozygotes had previously been reported in families segregating an X-linked recessive form of DMD (Gomez et al. 1977; Meola et al. 1981; Olson and Fenichel 1982; Wood et al. 1988). In family 1, however, the mother was clearly not a heterozygote, as demonstrated by RFLP analysis and the dosage of restriction fragments in her leukocyte DNA. We conclude that she represents another case of germline mosaicism—owing to mitotic origin of the mutation in early embryonic life—in addition to those reported previously (Bakker et al. 1987; Darras and Francke 1987; Lanman et al. 1987; Monaco et al. 1987; Wood and McGillivray 1988).

In families 2 and 3, the disorder is probably caused by an AR mutation. The family histories are noncontributory. There was no evidence for consanguinity, and in both families the parents are from different ethnic backgrounds. Thus, AR mutations must exist in different population groups and may not be so rare. Consistent with this idea are reports of AR progressive muscular dystrophy in various inbred populations and different ethnic groups (Shokeir and Kobrinsky 1976;

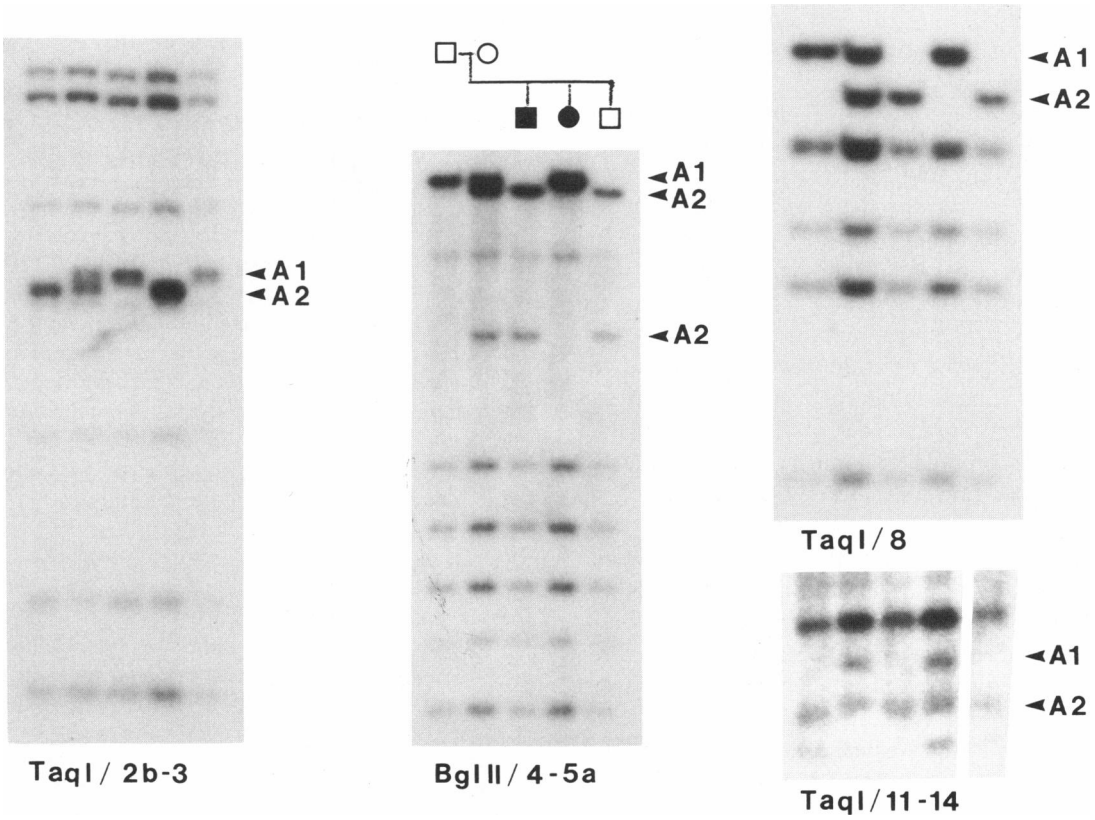

Figure 5 Informative intragenic RFLPs in family 3, after digestion with *TaqI* and *BglII* and hybridization with cDNA probes as indicated. Loading of samples is shown by pedigree. In all four panels, both males have inherited the same alleles, and their affected sister has received the other maternal alleles.

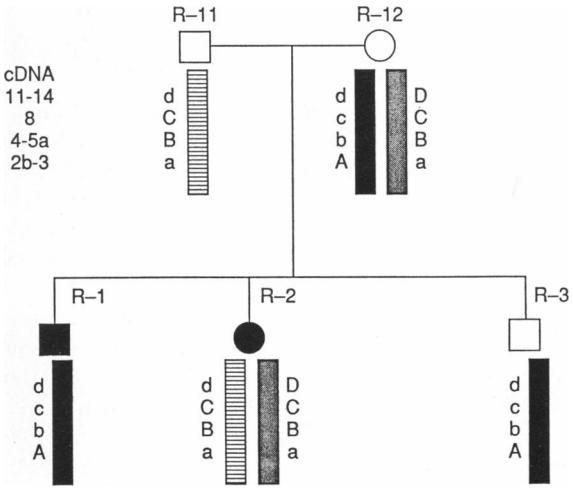

Figure 6 Family 3. The data shown in fig. 5 are represented here as haplotypes. The larger allelic fragments are indicated by capital letters, and the smaller alleles are in lowercase.

Hamida et al. 1983; Salih et al. 1983; Gardner-Medwin and Johnston 1984; Somer et al. 1985). Nevertheless, in a later publication, one family was reinterpreted as having limb-girdle muscular dystrophy (Jackson and Carey 1961; Jackson and Strehler 1968).

We are presenting the first molecular genetic evidence for the existence of an AR form of muscular dystrophy that may not be distinguishable from DMD or severe BMD on clinical grounds, simply because there is great variability in progression and clinical course in the X-linked muscular dystrophies (Brooke et al. 1983; Gardner-Medwin 1980; Hyser et al. 1987). However, some of the variability reported in these clinical studies could be due to the inclusion of AR cases. A retrospective review of the clinical histories of our patients suggests subtle differences compared with the most common presentation of DMD/BMD. For example, in families 2 and 3 toe walking was an early sign before onset of muscle weakness, consistent with the obser-

vation of Gardner-Medwin and Johnston (1984) in affected girls. Furthermore, weakness of the lower and upper extremities developed in parallel rather than sequentially, and winging of the scapula may be more apparent early in the course, as may be proximal muscle wasting as suggested by Stern (1972).

The difficulties that arise for genetic prediction and counseling are obvious. Had patient T-2 been male she undoubtedly would have been diagnosed as having DMD. With the brother also affected, future pregnancies would have been monitored for dystrophin-region markers shared by the two affected siblings, and the resulting counseling would have been erroneous. Likewise, the younger, unaffected brother in family 3, who shares the dystrophin RFLP haplotype with his affected brother, may have been considered affected had he been studied in utero.

To take the possibility of an AR form of the disorder into account when counseling families with sporadic cases, one needs to know the frequency of the AR versus the X-linked form. In a family previously considered a candidate for an AR dystrophy because affected and unaffected brothers shared the Xp21 haplotype (family D in Darras et al. 1987), we later found a deletion within the dystrophin gene by using cDNA probes. The deletion was not present in any female relatives, which both identified the single proband as a new mutation and proved the historical information of elevated serum CK levels in two of his sisters to be erroneous. When studied for deletions with cDNA probes, about two-thirds of all patients were found to have deletions (Darras et al. 1988*a*; Forrest et al. 1988). The proportion of AR muscular dystrophy patients among the one-third of cases in whom no deletion can be demonstrated cannot be estimated with any accuracy at the present time.

The next step of analysis has to be at the level of the dystrophin protein. Dystrophin deficiency is completely specific for DMD/BMD and has not been observed in any other muscle disorder (Hoffman et al. 1988). Thus, patients in whose muscle biopsies dystrophin of normal size and abundance can be demonstrated by immunoblotting, as was done in patient T-1, are candidates for a suspected AR mutation. Histological studies of muscle will be necessary to determine whether the dystrophin is in its normal location at the sarcolemma (Bonilla et al. 1988). Larger series of patients will have to be studied at the DNA and protein levels in order to determine how many males diagnosed with DMD do not have a mutation in the dystrophin gene. Those of either sex who are identified to have AR muscular dystrophy will provide useful material for unraveling the membrane biology of muscle and, potentially, the interactions of dystrophin with other structural muscle proteins.

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