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# Skewed X Inactivation in a Female MZ Twin Results in Duchenne Muscular Dystrophy

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## Summary

**One of female MZ twins presented with muscular dystrophy. Physical examination, creatine phosphokinase levels, and muscle biopsy were consistent with Duchenne muscular dystrophy (DMD). However, because of her sex she was diagnosed as having limb-girdle muscular dystrophy. With cDNA probes to the DMD gene, a gene deletion was detected in the twins and their mother. The de novo mutation which arose in the mother was shown by novel junction fragments generated by *Hin*dIII, *Pst*I, or *Taq*I when probed with cDNA8. Additional evidence of a large gene deletion was given by novel *Sfi*I junction fragments detected by probes p20, J-Bir, and J-66 on pulsed-field gel electrophoresis (PFGE). Immunoblot analysis of muscle from the affected twin showed dystrophin of normal size but of reduced amount. Immunofluorescent visualization of dystrophin revealed foci of dystrophin-positive fibers adjacent to foci of dystrophin-negative fibers. These data indicate that the affected twin is a manifesting carrier of an abnormal DMD gene, her myopathy being a direct result of underexpression of dystrophin. Cytogenetic analysis revealed normal karyotypes, eliminating the possibility of a translocation affecting DMD gene function. Both linkage analysis and DNA fingerprint analysis revealed that each twin has two different X chromosomes, eliminating the possibility of uniparental disomy as a mechanism for DMD expression. On the basis of methylation differences of the paternal and maternal X chromosomes in these MZ twins, we propose uneven lyonization (X chromosome inactivation) as the underlying mechanism for disease expression in the affected female.**

## Introduction

Duchenne muscular dystrophy (DMD)/Becker muscular dystrophy (BMD) is an X-linked recessive disease which usually affects males and is characterized by the inability to produce normal dystrophin, a component of the membrane cytoskeleton of myofibers (Bonilla et al. 1988*a*, 1988*b*; Hoffman et al. 1988; Knudson et al. 1988; Sugita et al. 1988; Watkins et al. 1988; Hoffman and Kunkel 1989). Although the vast majority of young boys presenting with a Duchenne-like pattern of weakness are afflicted with DMD, there are other rare autosomal recessive dystrophies—the limb-girdle and Duchenne-like dystrophies—which mimic the clinical presentation and progression of DMD (Zatz et al. 1988). While DMD is expected to affect males exclusively, such autosomal recessive conditions are expected to affect males and females equally. For this reason, girls presenting with a DMD-like dystrophy are diagnosed as having limb-girdle dystrophy rather than DMD.

While the large majority of sex-based differential diagnoses are correct, there are expected exceptional cases. Females heterozygous for DMD have been found with "true" DMD. Many of these "manifesting carriers" have been found to have gross chromosomal rearrangements involving translocations between the X chromosome and an autosome (Boyd et al. 1987), one breakpoint invariably involving the DMD gene. While these manifesting carriers with translocations all have normal dystrophin genes on their intact X chromosomes, their

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normal X chromosomes appear to be preferentially inactivated. Such a mechanism has been supported by the finding of markedly decreased levels of dystrophin in translocation-bearing manifesting carriers (Hoffman et al. 1988; Arahata et al. 1989*a*, 1989*b*). Even in the absence of gross chromosomal rearrangements, statistical probability dictates that a very small number of females heterozygous for DMD will randomly inactivate a majority of their normal dystrophin genes and thereby manifest either typical DMD or a milder variant. Such karyotypically normal manifesting carriers are in fact observed (Nisen et al. 1986).

In addition to manifesting carriers resulting from translocations and random (unfortunate) normal X inactivation, a third group of manifesting carriers appears to be triggered by the MZ twinning process. A number of MZ female twin pairs heterozygous for DMD have been identified where one of the twins is clinically normal and the other has severe, typical DMD (Gomez et al. 1977; Burn et al. 1986; Chutkow et al. 1987; Pena et al. 1987). In the present paper we identify an additional set of such MZ twins discordant for DMD and thoroughly characterize them at both the dystrophin-gene and dystrophin-protein levels. Given the relatively large numbers of such MZ twins discordant for DMD, we suggest that the preferential inactivation of the normal X in one of the twins frequently accompanies the twinning process.

## Material and Methods

### *Dystrophin Analysis*

Dystrophin immunoblotting was performed according to a method described elsewhere (Hoffman et al. 1988, 1989). Dystrophin anti-dystrophin immune complexes were visualized using alkaline phosphatase–conjugated second antibodies (Sigma). Dystrophin immunofluorescence was done using affinity-purified anti-60-kD-dystrophin antibodies according to a method described elsewhere (Watkins et al. 1989).

### *Southern Blot Analysis*

DNA extracted from lymphocytes was digested with the appropriate restriction enzyme, electrophoresed on a 0.8% agarose gel in Tris-borate-EDTA buffer, and alkali-transferred to Hybond-N (Amersham). The blot was hybridized (Church and Gilbert 1984) overnight to probes labeled with $^{32}$P-dCTP (3,000 Ci/mmol; ICN) to a specific activity of $10^9$ cpm/μg by using the random primer labeling method of Feinberg and Vogelstein (1983). Following washing at 65°C in 40 mM sodium phosphate pH 7.2, 1% SDS, the blot was exposed to X-ray film overnight.

### *Pulsed-Field Gel Electrophoresis (PFGE)*

Lymphocytes were isolated from whole blood by using Ficoll-Paque (Pharmacia), mixed with an equal volume of 1% low-melting-point agarose in PBS, and injected into a plug mold. The plugs were incubated in a solution of 0.5 M EDTA pH 8, 1% sarkosyl, 2 mg proteinase K (Boerhinger Mannheim)/ml for 48 h at 55°C and then were treated with 0.04 mg phenylmethylsulfonylfluoride/ml for 1 h. DNA was digested with *Sfi*I at 50°C and electrophoresed on a 1% agarose gel in 0.01 M Tris, 0.5 mM EDTA pH 8 by using transverse alternating-field electrophoresis with 60-s pulses at 170 mA for 18 h.

### *Probes*

Probes J66 and p20 were obtained from Dr. G. van Ommen (van Ommen et al. 1987; Wapenaar et al. 1988); JBir, pERT 87, and cDNA 8 probe (44-1) were obtained from Dr. L. Kunkel (Kunkel et al. 1985; Monaco et al. 1985; Koenig et al. 1987). The following variable-number-of tandem-repeat (VNTR) probes obtained from Lifecodes Corporation (New York) were used in the identity test: pAC 256 (D17S79), pAC 255 (D2S44), pAC 299 (D14S13), and pAC 225 (DXYS14). The M27-beta probe was a gift from Drs. I. Craig and Y. Boyd (Fraser et al. 1989).

### *Cytogenetic Analysis*

Standard techniques were used to prepare trypsin-Giemsa-banded karyotypes from peripheral blood lymphocytes. Cytogenetic resolution at about a 500-band level was obtained.

## Results

### *Case Presentation*

An extensive pedigree analysis of these 19-year-old female twins revealed no family history of any form of muscular dystrophy. The twins, discordant for muscular dystrophy, were the product of an uneventful pregnancy and normal delivery. Both were the same birth weight, but the affected twin was noted to be floppy. She experienced delayed motor development with progressive general muscle weakness. She walked at 18 mo (her twin walked at 13 mo), had difficulty riding a tricycle, and was noted to fall easily. At age 7 years she presented with mild contractures of the hips and ankles. Neurological abnormalities were confined to the

motor system, with proximal atrophy of the sternal head of the pectoralis major, prominent scapular winging, proximal weakness of both upper and lower extremities, positive Gowers sign, and poor stair-climbing ability. At age 13 years she was confined to a wheelchair. Her creatine phosphokinase values were 42–92 times the upper limit of normal. Muscle-biopsy histopathology revealed an active myopathy described as similar to that of DMD. However, because of her sex, she was diagnosed as having limb-girdle muscular dystrophy.

Her clinical presentation was consistent with a diagnosis of DMD; such rare cases of manifesting carriers of DMD are not unknown. Possible mechanisms which could explain her manifesting carrier state include (1) a balanced translocation, (2) uniparental disomy, or (3) uneven lyonization. Each possibility was tested experimentally.

### *Cytogenetic Analysis*

Karyotype examination revealed that both twins had two cytogenetically normal X chromosomes, as shown in figure 1; therefore, neither a translocation nor a 45,X (Turner syndrome) could explain her disease expression.

### *Identity Analysis*

DNA fingerprint analysis was done (1) to examine uniparental disomy and (2) to confirm monozygosity. One multilocus and four single-locus highly polymorphic VNTR probes were used to determine probability of identity. As shown in figures 2 and 3, pAC 299, 255, 256, and 225 detect the same RFLP in each twin (lanes 1 and 2). DNA samples were mixed in lane 3, and the resulting banding pattern confirms identity. On the basis of identical RFLP patterns with five probes and parental heterozygosity (data not shown), the probability of identity was calculated to be 99.6%. Further analysis was done using the XY VNTR probe to determine whether each twin inherited a maternal and a paternal X chromosome. As shown in figure 3, each twin inherited two RFLPs from the maternal X (3.22 and 2.4 kb) and two RFLPs from the paternal X (3.7 and 2.6 kb). Therefore, uniparental disomy was eliminated as a mechanism for expression of DMD in the proband.

### *DNA Analysis*

The DNA from the affected girl, as well as that from other family members, was analyzed with cDNA probes to the entire DMD gene to detect deletions or duplications of the dystrophin gene. As shown in figure 4, a junction fragment of approximately 5 kb was detected by using *Hin*dIII and probe cDNA8 (44-1) in both twins

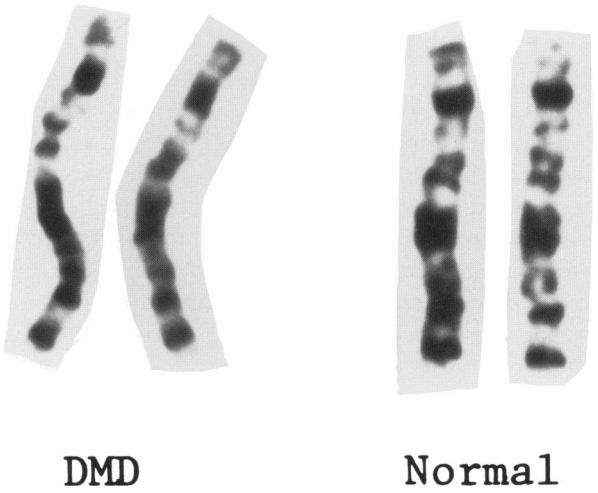


**Figure 1** GTG-banded X chromosomes prepared from peripheral blood lymphocytes of the twins.

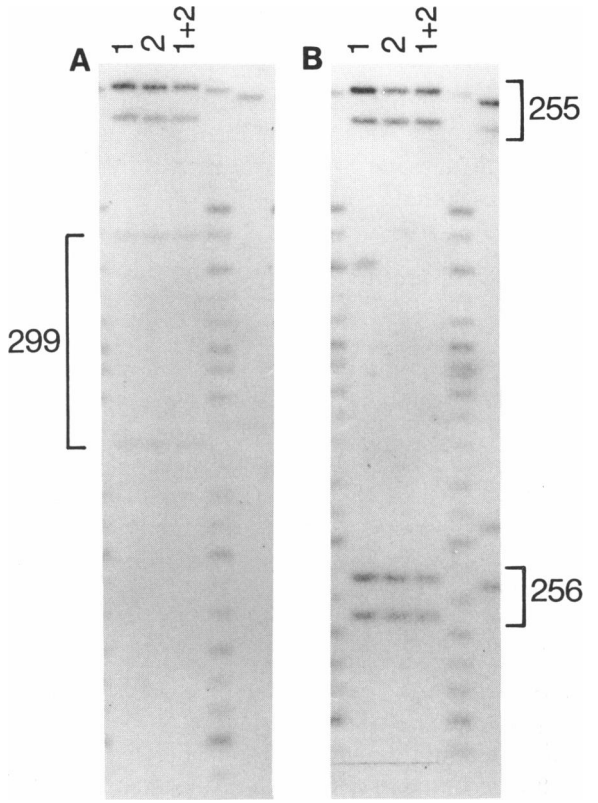


**Figure 2** DNA fingerprint test for identity. DNA was digested with *Pst*I, electrophoresed, Southern blotted, and probed with (*A*) pAC255 and 299 and (*B*) pAC255 and 256. Lane 1, Unaffected twin. Lane 2, Affected twin. Lane (1+2), Mixture from both twins. Lane 4, Markers. Lane 5, Standard control.

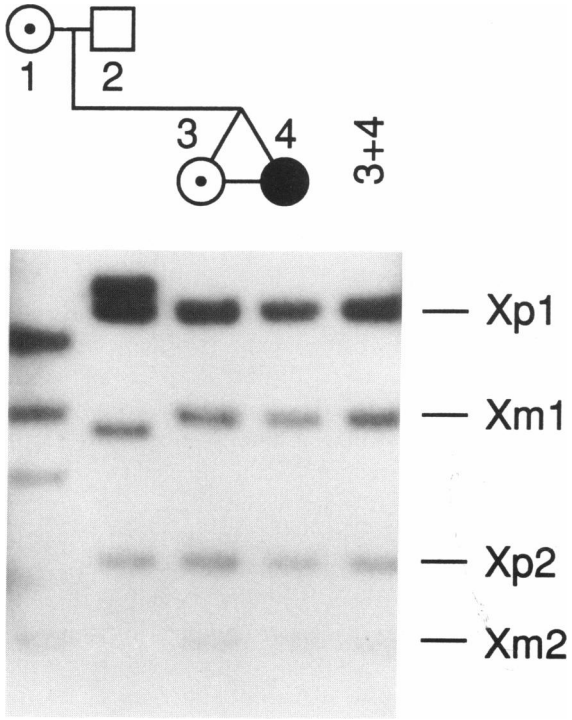


**Figure 3** DNA fingerprint test. *Pst*I-digested DNA was probed with pAC225. Lane 1, Mother. Lane 2, Father. Lane 3, Unaffected twin. Lane 4, Affected twin. Lane 5, Mixture from both twins. Maternal and paternal X chromosomes are denoted by Xm and Xp, respectively.

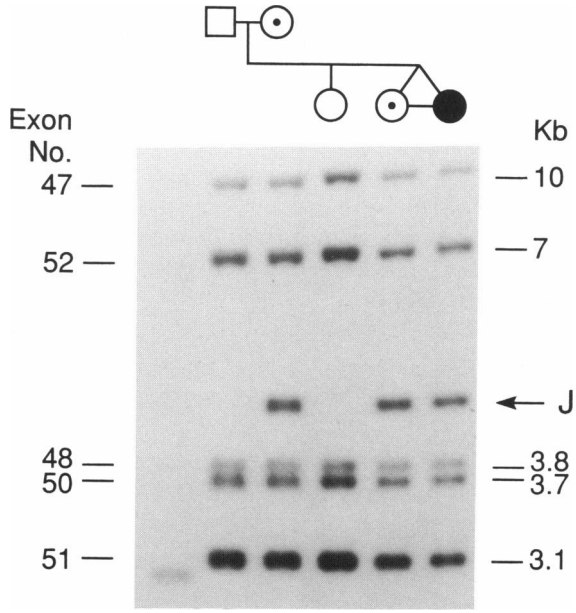


**Figure 4** cDNA deletion analysis. DNAs from the father, mother, sister, unaffected twin, and affected twin were digested with *Hin*dIII, Southern blotted, and probed with cDNA8. J = junction fragment.

and their mother. *Pst*I and *Taq*I also produced junction fragments, eliminating the possibility of a polymorphism. Such a junction fragment could have resulted from either a deletion or a duplication; however, in this case the differences in banding intensity suggested a deletion.

A Southern blot containing *Hin*dIII fragments of DNA from the twins, a female control, and a male control was hybridized to probe cDNA8, and radioactivity in each *Hin*dIII fragment was quantitated using a Betascope 603 Scanner (data not shown). The amount of DNA (5 μg) quantitated was equalized as determined both spectrophotometrically and by ethidium bromide staining intensity. The results suggest that the *Hin*dIII fragments, representing exons 47–52, contain 1.7–2.0-fold more radioactivity in the female control than in the twins. Therefore, exons 47–52 appear deleted in the twins and their mother.

PFGE was used to confirm this deletion and estimate its size. With this method large gene deletions in heterozygous females are represented by additional bands of altered size (den Dunnen et al. 1987; Chen et al. 1988). As shown in figure 5, high-molecular-weight DNA was digested with *Sfi*I, pulsed on a transverse alternating-field electrophoretic system, and probed with p20 (Wapenaar et al. 1988). The normal pattern is exemplified by the father and consists of a doublet of 460 and 590 kb. Both the mother and the twins display the normal pattern as well as a doublet of larger size (860–990 kb), which confirms and extends the cDNA analysis indicating a deletion in the DMD gene. Additional long-range mapping of this rearrangement reveals that JBir, a genomic probe located 292 kb 5′ of p20, also detects the same banding pattern, while pERT 87-8, located 730 kb 5′ of p20, detects only the normal banding pattern of 280 kb in all family members tested. J66 reveals the normal bands at approximately 530 and 330 kb as well as the same 860–990-kb doublet. These data, taken together, confirm the presence of a deletion and allow a size estimate for the deletion of approximately 300 kb, which includes the presence of a *Sfi*I site located between p20 and J66 as described by van Ommen et al. (1987).

Linkage analysis was done to predict carrier risks for other family members (data not shown). A total of 13 polymorphisms were examined in this family. Five of the polymorphisms examined, including a flanking probe at each end of the DMD gene, proved to be in-

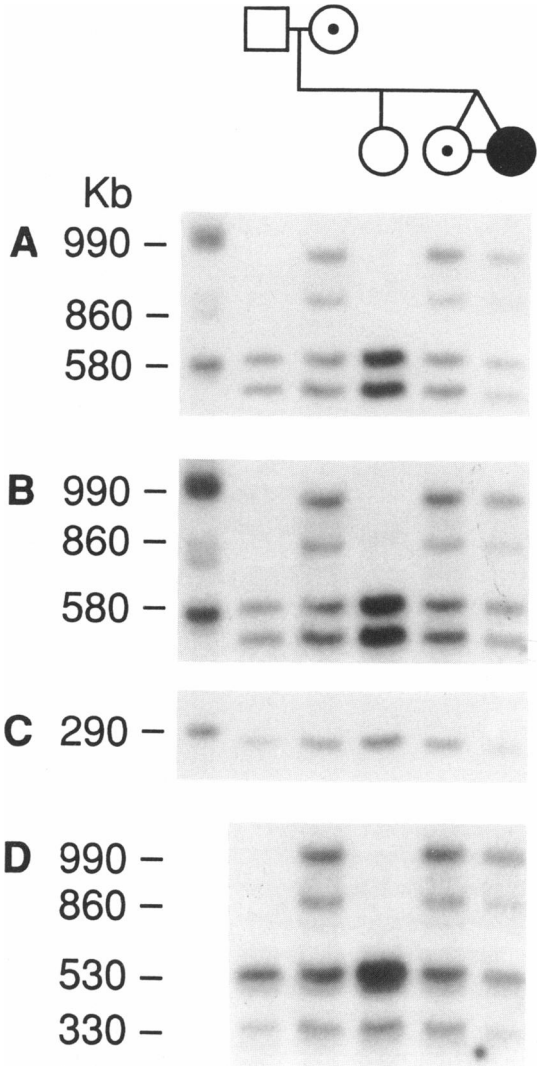


**Figure 5** Pulsed-field gel electrophoresis. Agarose-embedded high-molecular-weight DNA was digested with *Sfi*I and subjected to transverse alternating-field electrophoresis. The Southern blot was probed with p20 (*A*) and then stripped and reprobed with J-Bir (*B*), pERT 87-8 (*C*), and J-66 (*D*) separately.

formative and, therefore, allowed the affected chromosome to be distinguished. The twins inherited the maternal X chromosome containing the new mutation, while the older sister inherited the grandpaternal X chromosome and was, therefore, not a carrier. Although the grandmother did not have the deletion in her somatic cells, she carries an approximate 10% risk of germinal mosaicism (Hall 1988).

### *Dystrophin Analysis*

A muscle biopsy of the affected twin was analyzed for dystrophin content and distribution by using both immunoblotting of total muscle protein and immunofluorescence of cryosections. As shown in figure 6, dystrophin is visualized by immunoblotting of normal muscle samples at the expected molecular weight of 427 kD (Koenig et al. 1988) (lanes 1 and 3). The muscle biopsy of the affected twin (lane 2) shows a greatly reduced level of dystrophin relative to that of the adjacent normal controls. The low quantity of dystrophin detected by immunoblotting could be due either to a small percentage of fibers expressing normal quantities of dystrophin or to all fibers expressing very low levels of dystrophin. To distinguish between these possibilities, indirect immunofluorescence for dystrophin was done on cryosections of the affected twin's muscle biopsy. As shown in figures 7*A* and 7*B*, islands of completely dystrophin-positive fibers were seen adjacent to regions of completely dystrophin-negative fibers. Dystrophin-negative fibers generally present as islands of small regenerating/degenerating fibers, surrounded by extensive fibrous tissue typical of a progressive myopathy. Dystrophin-positive islands of fibers appeared much more normal, in both size and size distribution (figs.

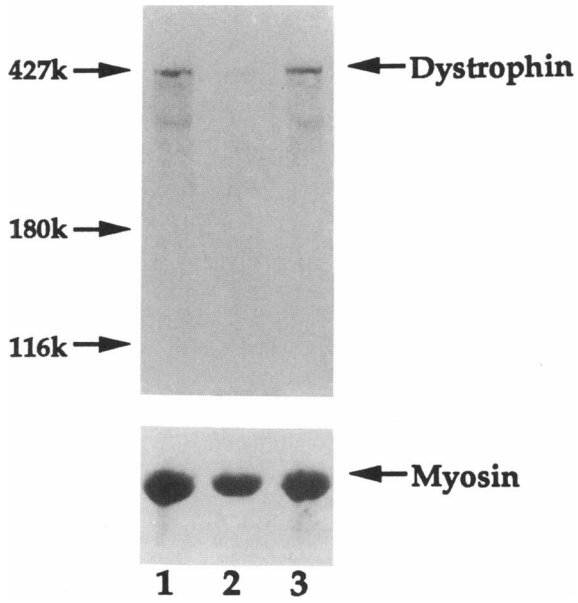


**Figure 6** Immunoblot visualization of dystrophin in affected twin. Shown is an immunoblot analysis of total muscle protein from a muscle biopsy of the affected twin (lane 2) and from normal human control muscle (lanes 1 and 3). The muscle protein content of each lane is controlled for by the posttransfer Coomasie blue staining of the myosin heavy chain in the same gel used for the immunoblot. Molecular-weight markers are prestained proteins (116 kD and 180 kD; Sigma) and dystrophin (427 kD; Koenig et al. 1988).

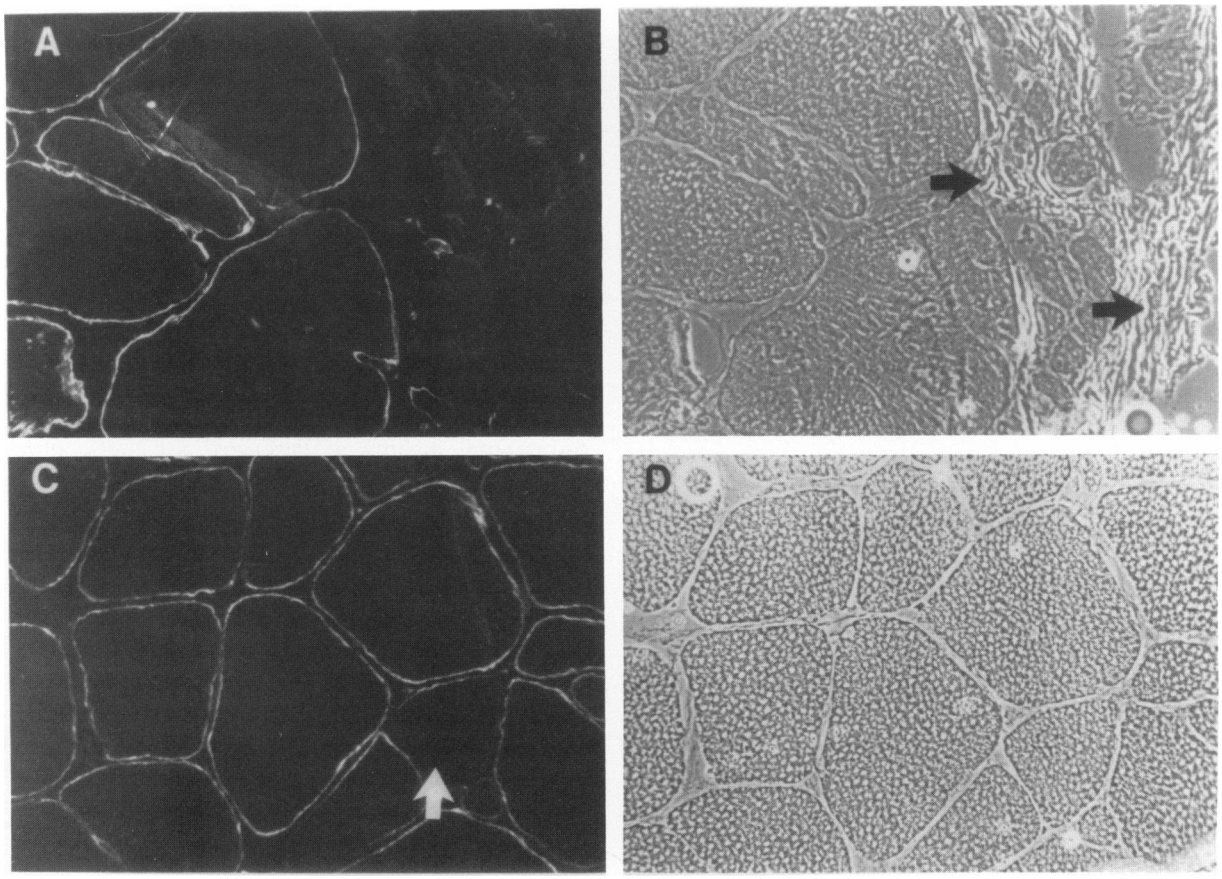


**Figure 7** Indirect immunofluorescence of dystrophin labeling in cross sections of muscle from the affected twin. *A* and *B*, Islands of positive and negative fibers, seen in close apposition to each other. The small dystrophin-negative fibers and the extensive fibrous tissue are clearly seen in the accompanying phase micrograph (*B*, arrows) (× 255) *C* and *D*, Fibers contained within the dystrophin-positive islands, often appearing normal in size and dystrophin complement. Occasional fibers showing patchy dystrophin labeling may be seen (arrow) (× 255)

7*C* and 7*D*), though occasional dystrophin-negative fibers were seen in dystrophin-positive islands. These data, taken together with the molecular genetic data, clearly document that the affected twin is a manifesting carrier of an abnormal DMD gene. Furthermore, her myopathy is the direct result of underexpression of dystrophin.

### *Methylation Analysis*

DNA from peripheral blood lymphocytes of each twin and from their parents was analyzed to examine methylation differences of their respective X chromosomes. With the restriction enzyme *Pst*I and probe M27-beta, which detects a DNA sequence that is hypermethylated on the active X and hypomethylated on the inactive X chromosome (Y. Boyd, personal communication), the twins are heterozygous (fig. 8, lanes 3 and 4). Both inherited the upper band from their father (lane 2) and the lower band from their mother (lane 1). With a methylation-sensitive restriction enzyme *Hpa*II recognizing the sequence C CGG but not the methylated sequence $C^{me}CGG$, the twins (lanes 7 and 8) show differences in their methylation patterns. The father's X (lanes 2 and 6) shows no change in banding pattern, appears hypermethylated at this site, and thus represents an active X. The mother's X chromosomes are indistinguishable with *Pst*I alone (lane 1); however, a new band of lower mobility (arrow) results from *Hpa*II digestion (lane 5). On *Hpa*II digestion, the affected twin (lane 7) shows loss of DNA from the upper paternal band; however, the lower band remains unchanged. In contrast, the unaffected twin (lane 8) shows no change in intensity of the upper paternal band; however, the lower maternal band is cleaved with *Hpa*II, resulting in a band of reduced mobility identical to that in the mother (lane 5). Thus, in the affected twin the maternal X chromo-

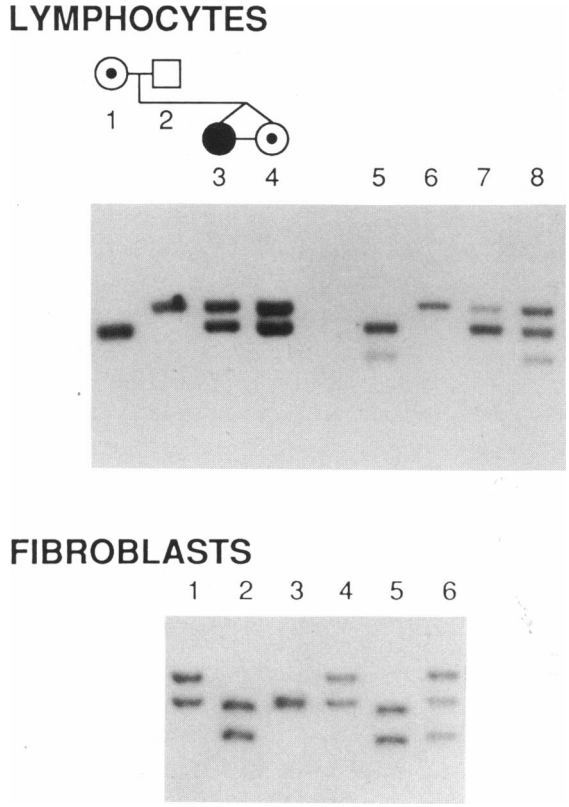


**Figure 8** Methylation analysis. *Top,* DNA isolated from peripheral blood lymphocytes of the mother (lanes 1 and 5), the father (lanes 2 and 6), the affected twin (lanes 3 and 7), and the unaffected twin (lanes 4 and 8), digested with *PstI* alone (lanes 1–4) or in combination with *HpaII* (lanes 5–8) and probed with $^{32}$P-M27-beta. *Bottom,* DNA isolated from skin fibroblasts derived from the affected twin (lanes 1–3) and the unaffected twin (lanes 4–6), digested with *PstI* alone (lanes 1 and 4) or in combination with either *MspI* (lanes 2 and 5) or *HpaII* (lanes 3 and 6) and probed with $^{32}$P-M27-beta.

some bearing the DMD gene deletion is predominantly hypermethylated at this site and therefore active. In contrast in the situation in the unaffected twin, the maternal X chromosome is predominantly undermethylated at this site and therefore inactive in a larger proportion of cells, while the paternal X carrying the normal DMD gene is predominantly hypermethylated at this site and therefore active in the majority of her cells.

Because different tissues may show different patterns of X inactivation, skin fibroblasts, which are mesodermal derivatives as are myoblasts, were examined to reflect X inactivation in muscle cells where dystrophin is expressed. Figure 8*B* shows DNA prepared from skin fibroblasts derived from each twin, restricted with *PstI* alone (lanes 1 and 4) or in combination with either *MspI* (lanes 2 and 5) or *HpaII* (lanes 3 and 6). These results clearly indicate differences in X chromosome methylation profiles in skin fibroblasts of these MZ twins (lanes 3 and 6), differences similar to those seen in lymphocytes.

## Discussion

Here we report female MZ twins discordant for DMD by both molecular genetic and dystrophin analyses and explore the possible mechanisms which produce manifesting carriers. Cytogenetic analysis eliminated both a translocation involving the X chromosome and Turner syndrome. DNA fingerprint analysis eliminated uniparental disomy as described for cystic fibrosis (Spence et al. 1988). The remaining possibility is uneven X inactivation. The Lyon (1962) hypothesis predicts that in females one X chromosome is inactivated at random in each cell, leaving only one X chromosome active. We postulate that the affected twin has an inactive normal X chromosome in the majority of her muscle precursor cells, while her identical twin has a higher proportion of the normal X chromosome active.

Other female MZ twins discordant for X-linked diseases including DMD, hemophilia, glucose-6-phosphate dehydrogenase deficiency, and color blindness have been presumed to result from uneven lyonization (Lascari et al. 1969; Philip et al. 1969; Gomez et al. 1977; Phelan et al. 1980; Burn et al. 1986; Nisen et al. 1986; Chutkow et al. 1987; Pena et al. 1987; Ingerslev et al. 1989). The dystrophin data presented here strongly support uneven lyonization in muscle tissue as the mechanism responsible for the discordant phenotypes in these twins. Specifically, the regions of completely dystrophin-positive fibers adjacent to regions of completely dystrophin-negative fibers are probably the result of both myoblast migration and the inherent clonality of lineage normally seen in muscle development. Given that myofibers represent a syncytium of myonuclei and that the dystrophin protein shows only limited diffusion within myofibers (Watkins et al. 1989), a mosaic expression of dystrophin would be expected in most female carriers of DMD. Indeed, symptomatic carriers of DMD (Arahata et al. 1989*b*) and female mdx mice carriers (Watkins et al. 1989) show a mosaic pattern of dystrophin-positive and dystrophin-negative myofibers. As a result of both uneven lyonization in this manifesting carrier twin and the clonality of the development of muscle, primary myotubes in this patient probably were entirely dystrophin negative or dystrophin positive. Subsequent development of secondary and tertiary myotubes eventually resulted in islands of positive and negative fibers,

rather than in the different type of mosaic pattern seen in nonmanifesting, high–creatine phosphokinase carriers (Bonilla et al. 1988*b*; Arahata et al. 1989*b*).

It is important to speculate on the mechanism of the uneven lyonization observed in this patient. The patterns of gene methylation detected by probes specific for genes located on the X, including the transcribed genes for phosphoglycerate kinase (PGK) and hypoxanthine phosphoribosyltransferase (HPRT), have been used to distinguish active from inactive X chromosomes (Wolf et al. 1984; Yen et al. 1984; Cullen et al. 1986; Keith et al. 1986). Methylation of specific sites located in 5′ gene promoter regions generally hallmarks inactivation of these transcribed genes (Bird 1986; Grant and Chapman 1988). Since our twins proved homozygous for the probes which detect methylation sites in PGK and HPRT, M27-beta, a VNTR probe, was used to determine, on the basis of DNA methylation status, the ratio of active versus inactive maternal and paternal X chromosomes, with the active X hypermethylated at this site (Y. Boyd, personal communication). The methylation results suggest that the maternal X was preferentially inactivated in the unaffected twin, while the paternal X was preferentially inactivated in the affected twin. Differential uneven X inactivation most likely occurred early in the development of these MZ twins, prior to the differentiation of lymphocyte and fibroblast precursors from embryonic mesoderm, since both tissues show similar patterns of methylation. Moreover, that different parental X chromosomes were preferentially methylated supports and extends the dystrophin analysis, strongly suggesting that uneven X inactivation explains disease discordance in these twins.

Uneven X inactivation in female MZ twins may be directly related to the twinning process (James 1988; Jongbloet 1988). In the absence of documentation of placenta and fetal membranes, it is not possible to determine when twinning occurred in our twins. However, in most MZ twins the split is thought to occur at 3–8 d postfertilization (Smith et al. 1976). Random X inactivation in mammals is thought to occur early in embryonic development, by the 64-cell stage; however, neither the time nor the mechanism involved is completely understood. Nor is it clear that all tissues inactivate at the same time, in the same proportion, and/or in the same manner. Our twins certainly demonstrate disproportionate anomalous X chromosome inactivation. Burn et al. (1986) have hypothesized that random inactivation will occasionally produce clumps of cells with paternal X inactivation and maternal X inactivation which then "view" each other as "foreign" and separate into two distinct organisms. This process would not explain male MZ twinning. However, the growing number of case reports of female MZ twins discordant for an X-linked disorder suggest that the twinning process and lyonization are interrelated in a temporal or mechanistic manner such that one event can lead to the other. If twinning and lyonization are unrelated, then one would predict that female MZ twins concordant for DMD or any other X-linked disease would exist; we are unaware of such cases and would like to learn of them. Moreover, we know of no reported cases of DMD-carrier MZ twins in which both are nonmanifesting. It does appear that these cases of MZ twins with disproportionate X inactivation may help explain the mechanism of monozygous twinning.

## Acknowledgments

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