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Fragile X Syndrome and the $(CGG)_n$ Mutation: Two Families with Discordant MZ Twins

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Summary

The fragile X phenotype has been found, in the majority of cases, to be due to the expansion of a CGG repeat in the 5′ UTR region of the *FMR-1* gene, accompanied by methylation of the adjacent CpG island and inactivation of the *FMR-1* gene. Although several important aspects of the genetics of fragile X have been resolved, it remains to be elucidated at which stage in development the transition from the premutation to the full mutation occurs. We present two families in which discordance between two sets of MZ twins illustrates two important genetic points. In one family, two affected MZ brothers differed in the number of CGG repeats, demonstrating in vivo mitotic instability of this CGG repeat and suggesting that the transition to the full mutation occurred postzygotically. In the second family, two MZ sisters had the same number of repeats, but only one was mentally retarded. When the methylation status of the *FMR-1* CpG island was studied, we found that the majority of normal chromosomes had been inactivated in the affected twin, thus leading to the expression of the fragile X phenotype.

Introduction

The fragile X syndrome is the commonest form of familial mental retardation (Sherman 1991), and it is associated with facial dysmorphology, macroorchidism, and the expression of a folate-sensitive fragile site at Xq27.3 (Sutherland 1985). The fragile X locus and the associated mental retardation gene, *FMR-1*, was isolated and characterized in 1991 (Dietrich et al. 1991; Heitz et al. 1991; Kremer et al. 1991; Oberlé et al. 1991; Verkerk et al. 1991; Yu et al. 1991). The fragile X phenotype was found, in the majority of cases, to be due to the expansion of a CGG repeat in the 5′ UTR of the *FMR-1* gene, accompanied by methylation of the adjacent CpG island and inactivation of the *FMR-1* gene (Fu et al. 1991).

The *FMR-1* CGG repeat is highly polymorphic, where the normal range of repeats is 6–54 copies; premutated alleles are 52–200 repeats and are not associated with fragile X phenotype; alleles with more than 200 repeats are methylated and regarded as fully mutated, being 100% penetrant in males and approximately 50% penetrant in females. Normal transmitting males (NTMs) will pass on the premutated allele to all their daughters, where the repeat will expand further and can lead to the expression of the syndrome in the grandsons (Fu et al. 1991). This phenomenon is known as the Sherman paradox (Sherman et al. 1984, 1985).

An important aspect of the genetics of the fragile X mutation remains to be elucidated: at which stage in development does the transition from the premutation to the full mutation occur—during oogenesis or postzygotically? Here we present two cases of MZ twins which highlight some important features of the mechanism of transmission, instability, and expression of the fragile X CGG mutation.

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Material and Methods

Two Spanish families were referred for genetic counseling: SFX6, having affected MZ twin boys, and SFX24, having one son and one female cousin affected. The female cousin was an MZ twin whose twin sister was phenotypically normal.

DNA was extracted from peripheral blood leukocytes by using the "salting out" procedure (Miller et al. 1988). For Southern analysis with probes StB12.3 (Oberlé et al. 1991) and F33 (Heitz et al. 1991), *Eco*RI and *Eco*RI/*Eag*I double digests of DNA were run, blotted, and hybridized using standard techniques (Sambrook et al. 1989). Densitometric analysis was performed with a Preference HR Sebia. Calculations of the densitometric differences between the active and inactive normal *FMR-1* gene were performed according to the following formula: active 2.8 kb/(active 2.8 kb + inactive 5.2 kb). The linked markers used, which flank the fragile X site, were 55E (Patterson et al. 1988), 4D8 (Boggs et al. 1984), pRN1 (Hupkes et al. 1989), VK21A/C (Suthers et al. 1989), and p1A1.1 (Bell et al. 1989).

PCR of the CGG repeat was carried out essentially as described by Fu et al. (1991), but with the alternative primer, P2, as the reverse primer (5′-TTGTAGAAAG-CGCCATTGGAGCCC-3′) and with 25 two-step cycles of denaturation at 95°C for 1.5 min and annealing and elongation at 65°C for 2 min. Whole-blood cultures were exposed to either a continuous deficient system or 5-fluorodeoxiuridine (FUdr), according to previous protocols (Sutherland 1979).

Results

Family SFX6

Two mentally retarded twin boys had dysmorphic features indicative of fragile X syndrome, but there was no family history of the disease. The parents were unrelated and 27 years old at the time of birth of the twins. At 6 years of age fragile X was suspected, and the twins were found to express the fragile site in 2% of metaphases, whereas the mother was negative. In order to provide a future prenatal diagnosis, the family was studied using flanking linked markers (55E, 4D8, pRN1, VK21A and VK21C, and p1A1), but they were uninformative.

When the probe StB12.3 became available, the family was restudied (fig. 1). The mother was found to be a carrier with a premutation: an increment of 200 bp on the normal 2.8-kb and 5.2-kb bands, having a total of

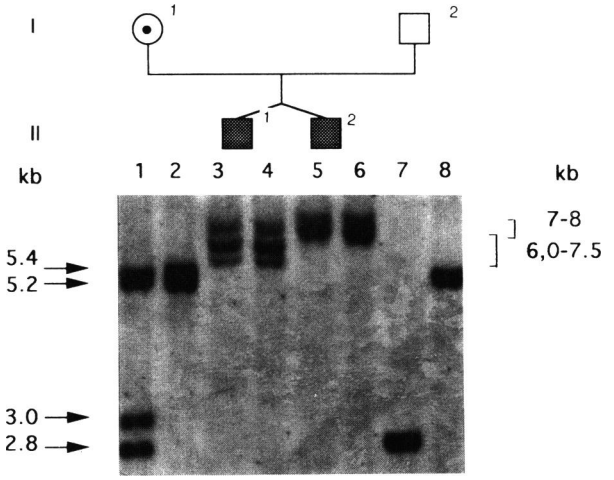

Figure 1 Segregation of the fragile X mutation in family SFX6. Peripheral blood leukocyte DNA was hybridized with probe StB12.3. Lanes 1, 3, 5, and 7, *Eco*RI and *Eag*I double digest. Lanes 2, 4, 6, and 8, *Eco*RI only. Normal fragments are 2.8 kb, unmethylated, and 5.2 kb, methylated, for the double digests. For the twins there is a clear difference in the size range of the smear for the fragments containing the repeats (6–7.5 kb and 7–8 kb). Blackened squares denote affected males, and the circle with a dot denotes the carrier female. Lanes 1 and 2, Mother. Lanes 3 and 4, First twin. Lanes 5 and 6, Second twin. Lanes 7 and 8, Father.

68 repeats (by PCR analysis; data not shown). The affected twins had an expansion to the full mutation. A difference between the two boys, in the size range of repeats in peripheral blood leukocytes, was found; one had a smear at 6–7.5 kb, representing approximately 1,000–1,600 repeats, and the other had a smear at 7–8 kb, representing approximately 1,400–1,800 repeats. One possible explanation could be that the restriction digests were incomplete, but, when they were hybridized using the control probe F33, this was found not to be the case (data not shown). As the mother of the twins had five unaffected brothers, we studied the grandmother and found that she was not a carrier, which allowed us to conclude that the dead grandfather must have been an NTM (data not shown).

Family SFX24

Family SFX24 presented with an affected male and a family history of mental retardation in an affected female cousin (fig. 2). The affected boy (III-3) was found cytogenetically to have 10% expression of the fragile site, whereas the affected cousin (III-5) was negative. Family SFX24 was not studied with the linked markers. With probe StB12.3 the progression of the mutation through three generations could be followed. The

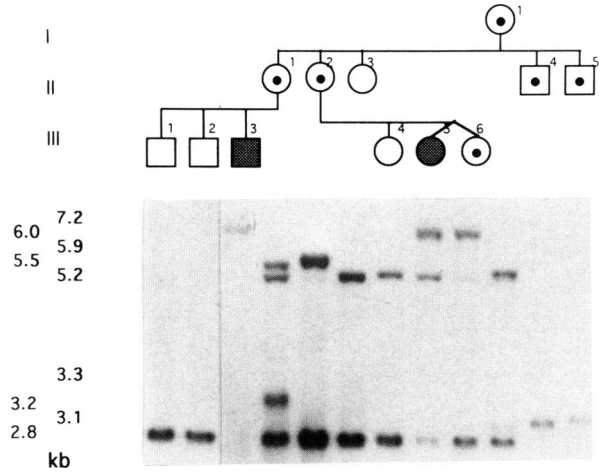

Figure 2 Segregation of the fragile X mutation in family SFX24. *Eco*RI and *Eag*I double digested DNAs were hybridized with probe StB12.3. Normal fragments are 2.8 kb, unmethylated, and 5.2 kb, methylated, for the double digest. The initial premutation, a 220-bp band in lane 10 (grandmother), can be traced through the family by following its increments (lanes 11 and 12) until it finally reaches full mutation (lanes 3, 5, 8, and 9). The blackened circle and blackened square denote affected individuals, and the circles with a dot and the squares with a dot denote carrier females and NTMs, respectively.

grandmother (I-1) was found to have a premutation with an increase of 220 bp, compared with the normal bands, possessing a total of 74 repeats (as visualized by PCR; data not shown). Of her five children, two were NTMs with slightly larger unmethylated increments, of 260 bp and 300 bp, when compared with the normal 2.8-kb band (II-4 and II-5). Among her three daughters we found the three possible states of *at-risk* females, one having inherited the normal X chromosome (II-3), one being a premutated carrier (II-1), and one being a phenotypically normal carrier, with a full mutation (CGG > 200 repeats) (II-2). The premutated carrier, who had a 500-bp increase over normal band (approximately 170 repeats in total), had one affected son (III-3) and two normal sons (III-1 and III-2). The carrier with the full mutation had one normal daughter (III-4) and MZ twin daughters (III-5 and III-6), both of which carried the full mutation but only one of which (i.e., III-5) was mentally retarded. By Southern blotting, the only difference between the twins was that the unaffected twin had a higher proportion of the unmethylated normal fragment (.86) than did her affected sister (.28) (fig. 3). This also holds true for the phenotypically normal mother (.98) (data not shown). Analysis of both families, using six microsatellite markers located on different chromosomes, for which allelic frequencies are known for the Spanish population (Fuentes et al. 1993), gave a 99.9% probability of both sets of twins being MZ.

Discussion

The two fragile X families presented here highlight several features of the genetics of the fragile X syndrome and also pose some questions on the timing of the transition to the full mutation of the CGG repeat. There is a clear difference in the size range of the repeat for the affected MZ twins of family SFX6, most likely resulting from mitotic instability, although it must be taken into account that (*a*) the expansion in the leukocytes may not be representative of other tissues and (*b*) the time of study (the twins were 10 years old) may not reflect the status of the repeats at some critical period

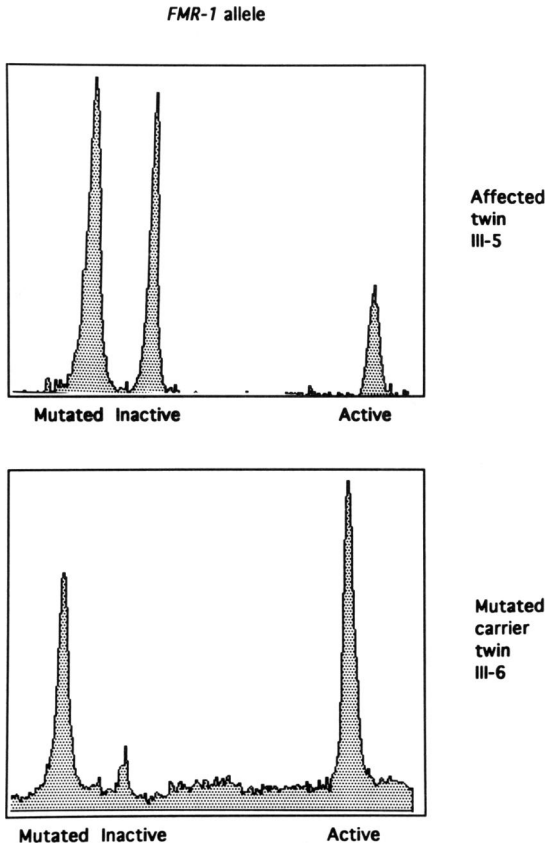

Figure 3 Family SFX24: densitometric analysis of MZ twins carrying the full fragile X mutation, showing the difference in the methylation status of the normal *FMR-1* gene. III-5 is affected, and III-6 is a carrier.

Table 1

Characteristics of Reported Cases of Fragile X Twins

Family	Reference	Age (years)	Sex	Mosaicism	Difference in Methylation	Difference in Repeat No.	Mental Retardation
1	Devys et al. 1992	9	Male	+	−	+	+/+
2	Devys et al. 1992	30	Female	−	+	−	+/+
3	Malmgren et al. 1992	?	Female	−	−	+	+/−
4	Present report	10	Male	−	−	+	+/+
5	Present report	15	Female	−	+	−	+/−

NOTE.—+ = present; and − = absent.

of expression during development. This observation contrasts with two previous reports on three pairs of MZ twins (Devys et al. 1992; Malmgren et al. 1992), which concluded that MZ twins have identical band patterns (table 1). However, the report by Devys et al. (1992) stated that the twin boys whom they studied were *almost* identical, indicating that some difference in the CGG repeat pattern was detected.

The fact that the MZ affected brothers of family SFX6 differ in the smallest size of the repeat (1,000 copies, compared with 1,400) suggests that the mitotic instability is present very early during embryogenesis, such that, at the time of separation the cell mass would consist of a population of cells with different repeat sizes and each twin would then receive variations in the number of repeats. The exact moment at which the CGG expansion to the full mutation occurs, whether during maternal meiosis or during early embryogenesis, has not been clearly defined. The data presented here for the affected MZ brothers do not allow us to make any definitive conclusion. However, when the case presented here is considered together with (1) the existence of mosaic individuals possessing both premutated and mutated alleles, (2) the findings that mothers with the full mutation have been found to have mosaic sons (Rousseau et al. 1991) and that daughters of fragile X patients have only premutations (Willems et al. 1992), and (3) the Reyniers et al. (1993) data showing that fragile X syndrome males with the full mutation in their leukocytes have only the premutation in their sperm, it seems more likely that the transition to full mutation is a postzygotic event.

The postzygotic transition to full mutation would be accompanied by some type of parental influence on the X chromosome, such that only maternally derived chromosomes undergo transition to the full mutation. In two other disorders, which have recently been found to involve trinucleotide expansion, parental influence has been suggested. In myotonic dystrophy (DM), regression from the full mutation is only seen in paternally derived chromosomes (Brook 1993), and the most severe form of the disease only presents when it is passed on by the mother (congenital DM). For Huntington chorea, paternal chromosomes are apparently more unstable (Huntington's Disease Collaborative Research Group 1993).

The role that methylation plays in the expression of the fragile X syndrome can be illustrated by the twin sisters of family SFX24, where, although both sisters are MZ and possess a full mutation, only one is affected. In the phenotypically unaffected twin the majority of normal *FMR-1* alleles occur on the active, unmethylated X chromosome (presumably by random X inactivation). For the retarded twin the picture is the reverse, with the majority of the unmethlylated chromosomes having the mutated *FMR-1* gene (again by random X inactivation), apparently leaving too few functional copies of the gene, leading to mental retardation (fig. 3). A similar situation has been demonstrated for MZ twin sisters, discordant for Duchenne muscular dystrophy, by using in situ hybridization (Zneimer et al. 1993).

The discordance in methylation status seen between the sisters could be explained by a skewed X inactivation in the zygote, especially since at the time of separation the cell mass is small, such that the affected twin may have inherited a significantly higher proportion of cells in which the normal X chromosome had been inactivated. Another possible explanation could be that the separation itself is due to the setting up of two distinct foci of development, due to the presence of two different cell populations, each carrying the opposite X chromosome active (Bocklage 1981).

Since the cloning of the fragile X site and the *FMR-1* gene, vast improvements have been made in genetic counseling and prenatal diagnosis for the fragile X syn-

drome. The prognosis for a female fetus carrying a full mutation is still an area that needs resolving, as we are, as yet, unable to predict whether she would be affected. For chorionic villus sampling (CVS) this is further complicated by the fact that CVS material is undermethylated compared with fetal tissue. Further investigation on the nature of the CGG repeat, the methylation of the CpG island, and the time at which transition occurs would provide great insight into the molecular mechanism of this mutation.

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