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Triplet Repeat Expansion at the *FRAXE* Locus and X-linked Mild Mental Handicap

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Summary

We have recently shown that the expression of the *FRAXE* fragile site in Xq28 is associated with the expansion of a GCC trinucleotide repeat. In the families studied, *FRAXE* expression is also associated with mild mental handicap. Here we present data on families that previously had been diagnosed as having the fragile X syndrome but that later were found to be negative for trinucleotide repeat expansion at the *FRAXA* locus. In these families we demonstrate the presence of a GCC trinucleotide repeat expansion at the *FRAXE* locus. Studies of the *FRAXE* locus of normal individuals show that they have 6–25 copies of the repeat, whereas affected individuals have >200 copies. As in the fragile X syndrome, the amplified CpG residues are methylated in affected males.

Introduction

The most common form of X-linked mental retardation, the fragile X syndrome, has been shown to be due to the expansion of a CGG trinucleotide repeat at the 5′ end of a gene known as "FMR-1" (Oberlé et al. 1991; Verkerk et al. 1991; Yu et al. 1991). The CGG amplification in patient DNA results in the methylation of the CpG residues and in transcriptional silencing of the gene (Hansen et al. 1992; Sutcliffe et al. 1992). Gene function may also be lost by point mutation or deletion events, but these are rare and are not associated with chromosome fragility (Gedeon et al. 1992; Wöhrle et al. 1992; De Boulle et al. 1993). Mentally retarded individuals with fragile sites at Xq27/28 but without amplification at the *FRAXA* locus have been shown to express fragility at sites more distal in Xq28, designated "*FRAXE*" (Sutherland and Baker 1992; Flynn et al. 1993) and "*FRAXF*" (Hirst et al. 1993). Cloning of the *FRAXE* site has shown that the fragility is also associated with the amplification of a CpG-rich triplet repeat (GCC) and that the methylation of CpG residues is associated with the observed phenotype (Knight et al. 1993).

The triplet repeat at *FRAXA* generally expands when passed through the female line, and reductions in copy number are rare. It remains to be seen whether this is also the case for *FRAXE* carrier females, where 3/10 transmitting females studied to date have passed on reduced copy numbers. By contrast, 5/5 males transmitting the *FRAXE* mutation have transmitted a triplet repeat reduced in GCC copy number. This apparent contraction in male meiosis may be due to the fact that only small expansions are present in the sperm, a situation that previously has been described for the CGG expansions at the *FRAXA* locus (Reyniers et al. 1993).

We were interested in determining whether *FRAXE* expansion or contraction events could explain the apparent dissociation between fragile-site expression and phenotype in a previously reported family (Voelckel et al. 1989). In this family, the proband was a Down syndrome child. Cytogenetic analyses revealed the expression of a fragile site at Xq27/q28 in the mother, but not in the proband. Further investigation revealed that many other members of the family express the fragile site. It is interesting that all of the females expressing fragility are phenotypically normal. One male, a lorry driver by occupation, expresses the fragile site and was originally ascertained as being intellectually and physically normal. Another male, who does not express the fragile site, is mildly retarded. No members show expansions at the *FRAXA* locus.

We also reinvestigated individuals of a second family who show expression of a fragile site at Xq28 but who are negative for expansion at the *FRAXA* locus (Nakahori et al. 1991; Dennis et al. 1992). Clinically affected individuals in this family are mildly retarded compared with the more severe phenotype usually associated with fragile X syndrome (Dennis et al. 1992).

Material and Methods

Samples of peripheral blood lymphocytes and lymphoblastoid cell lines were collected for both cytogenetic and DNA investigations. The DNA samples were digested with *Hin*dIII, for detection of GCC amplifications, and with *Hin*dIII+*Bss*HII, *Hin*dIII+*Sac*II, and *Hin*dIII+*Not*I, for

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methylation analyses as described elsewhere (Knight et al. 1993). The digested samples were run on 20-cm × 25-cm 0.8% agarose gels and were allowed to migrate at 65 V for 20 h or until the 5-kb marker reached the center of the gel (to give maximum resolution of any expanded allele). Electrophoresed samples were transferred onto Hybond-N membrane (Amersham) and hybridized with OxE20 as described elsewhere (Knight et al. 1993). PCR analysis across the *FRAXE* GCC repeat of normal X chromosomes was performed using the flanking oligonucleotides 598 and 603 as described elsewhere (Knight et al. 1993).

Results

Molecular Alterations at the FRAXE Locus

Molecular alterations at the *FRAXE* locus can be visualized after digestion of DNA samples with *Hin*dIII and hybridization of the Southern blots with the probe OxE20 (Knight et al. 1993). OxE20 lies ~600 bp distal to the GCC repeat and within the same 5.2-kb *Hin*dIII fragment (fig. 1). A PCR survey of 86 unrelated normal X chromosomes of Caucasian background shows that the range of GCC copy number is 6–25 copies, with the most common allele being 15 (fig. 1).

Two families have been analyzed for molecular alterations at the *FRAXE* locus. Both families previously had been described as having the fragile X syndrome but later were found to be negative for trinucleotide repeat expansion at the *FRAXA* locus (Voelckel et al. 1989; Dennis et al. 1992). Table 1 shows a summary of the phenotypes originally reported for the male *FRAXE* carriers in these families (Voelckel et al. 1989; Dennis et al. 1992).

Family 1.—*Hin*dIII genomic blots from selected individuals of the family originally described by Voelckel et al. (1989) were hybridized with probe OxE20. The results of the analysis are presented in figure 2. Individual 2 (lane 2) is a normal female and shows the expected 5.2-kb *Hin*dIII fragment, which, because of double dosage, is of increased intensity. In contrast, all females expressing a fragile site in Xq27/28 show two fragments, one of normal size (5.2 kb) and one of increased molecular weight. These females are phenotypically normal, presumably because they have one normal X chromosome.

Individual 10 was originally reported as being physically and intellectually normal and yet expresses the fragile site in 43% of his cells. DNA analysis reveals that he has an expansion of Δ = ~2.3 kb. It is of interest that he has passed on a much smaller fragment (Δ = 500 bp) to his phenotypically normal, cytogenetically negative carrier daughter. His half-brother, individual 8, is mentally retarded and originally presented with an encephalopathy and no signs suggestive of a dysmorphic syndrome (Voelckel et al. 1989). His karyotype is normal, with no fragile site, even after use of specific inclusion media. This individual also has an amplified fragment (Δ = 550 bp), but this

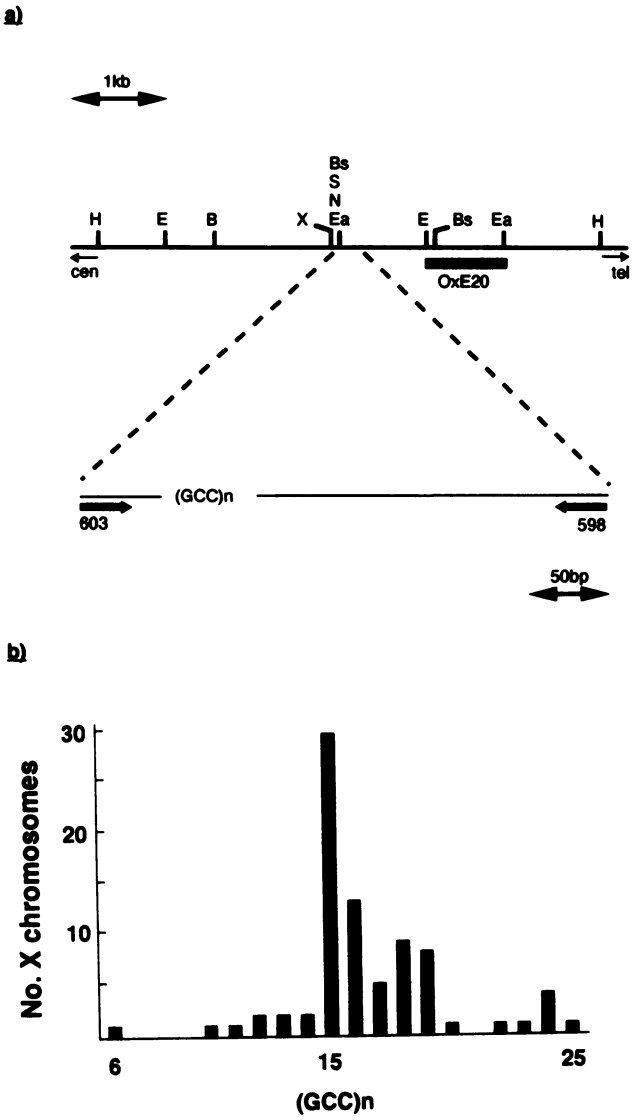

Figure 1 *a*, Map positions of probe OxE20 and PCR primers 598 and 603, relative to the *FRAXE* GCC repeat. H = *Hin*dIII; E = *Eco*RI; B = *Bam*HI; X = *Xho*I; Bs = *Bss*HII; S = *Sac*II; N = *Not*I; and Ea = *Eag*I. *b*, Distribution of GCC copy number in 86 unrelated normal X chromosomes of Caucasian origin obtained by PCR across the repeat, with use of primers 598 and 603.

is much smaller than that of individual 10 and corresponds to a GCC copy number of 200, the lower limit of the mutant range previously reported to be associated with mental retardation (Knight et al. 1993).

Individuals 1, 8, and 10 from this family were further investigated for the methylation status of the CpG island immediately adjacent to the *FRAXE* site. DNA samples were digested with *Hin*dIII and then with the methylation-sensitive enzymes *Bss*HII, *Sac*II, and *Not*I contained within the *FRAXE* CpG island. The resulting blots were hybridized with OxE20 (fig. 3). The expected smaller fragments are found in the normal male, indicating an unmeth-

Table 1

Reported Phenotypes of *FRAXE* Males from Family 1 and Family 2

FRAXE Male	Reported Phenotype
Family 1 (Voelckel et al. 1989):	
Individual 8	Encephalopathy; no dysmorphic features
Individual 10	Appeared normal although no formal testing; lorry driver by trade
Family 2 (Dennis et al. 1992):	
Individual 3	8½ years; severe behavioral problems; attended residential school for children with maladjustment and behavioral problems
	14 years; nondysmorphic; on the Vineland adaptive behavior scales, scored at age level 5–6 years in communication, 7–8 years in daily living skills, 4–5 years in socialization; in psychological testing, variable scores on different items of the WISC test, some being normal and others being in the range of severe learning difficulty
Individual 10	According to his mother, spoke his first words at age 1 year but did not walk until age 2 years; from age 2–5 years, spent long periods rocking and making repetitive noises; attended residential school because of behavioral difficulties
	28 years; would not answer "personal questions"; claimed to read books and have a social life; regular job as a machine operator
Individual 11	Attended special school for learning difficulties; at age 26 years, scored at adult levels in all domains of Vineland scale; difficulty with writing a long or complicated letter; worked as a photographic darkroom processor
Individual 12	Described by sister as somewhat "backward" in childhood but did not attend a special school; on the Vineland adaptive behavior scale, according to his sister's report, had adult scores in daily living skills but had significantly lower scores in communication and socialization; worked as a factory cleaner

ylated CpG island. For normal females, smaller fragments are also seen, and the signal from the 5.2 kb is reduced by ~50%, presumably because of methylation of the inactive X chromosome (data not shown). In figure 3, individual 1, a phenotypically normal, cytogenetically positive carrier female, shows normal unmethylated fragments in addition to methylated fragments in all of the double digests. This is consistent with her normal phenotype, with the normal X chromosome being active and the abnormal X chromosome remaining inactive. The analysis of the affected individual 8 shows that his amplified fragment is also methylated across the CpG island; again, this is consistent with the observed mental handicap. In contrast, the data for individual 10 are less easy to explain. This individual is reported to have a normal phenotype, and yet his amplified fragment is refractory to digestion, indicating the methylated status of his *FRAXE* CpG island.

Family 2.—Initial studies of this family revealed that affected individuals were negative for triplet repeat expansion at the *FRAXA* locus, despite the apparent segregation of fragile-site expression with mild mental handicap (Nakahori et al. 1991). The clinical features of individuals in this family have been reported by Dennis et al. (1992). The extensive pedigree and the analysis with probe OxE20 are presented in figure 4. The proband in this family is a mentally retarded male who expresses a fragile site in 10% of his cells. He was referred at the age of 8½ years, with behavioral problems, mild learning difficulties, and obesity. DNA analysis shows that he has a very faint smear of fragments at the *FRAXE* locus, with a predominant fragment size of ~6.5 kb (Δ = 1.3 kb). His mother is also cytogenetically positive, and she has a larger expansion, Δ = 2.2 kb. Similarly, her sister, niece, and mother are fragile-site positive with expansions of the *FRAXE* site. The remaining two affected males analyzed (individuals 10 and 11) show a GCC triplet repeat expansion consistent with the observed phenotype.

Discussion

Families segregating for *FRAXE* have been ascertained through screening programs for the fragile X syndrome. The studies presented here, together with those previously reported by Knight et al. (1993), suggest that the expression of *FRAXE* and the methylation of this site may be responsible for the mental handicap observed in these families. However, in family 1 presented here there is a phenotypically normal male who expresses the *FRAXE* site, shows amplification of the GCC repeat, and is methylated across the *FRAXE* CpG island. This individual is currently undergoing more detailed clinical evaluation. If the expansion of GCC repeats and methylation of the *FRAXE* locus do indeed result in mental impairment, then the phenotype of this individual may be extremely mild and reflect the clinical variability of this disease. Thus, the situation may be similar to fragile X syndrome, where the degree of men-

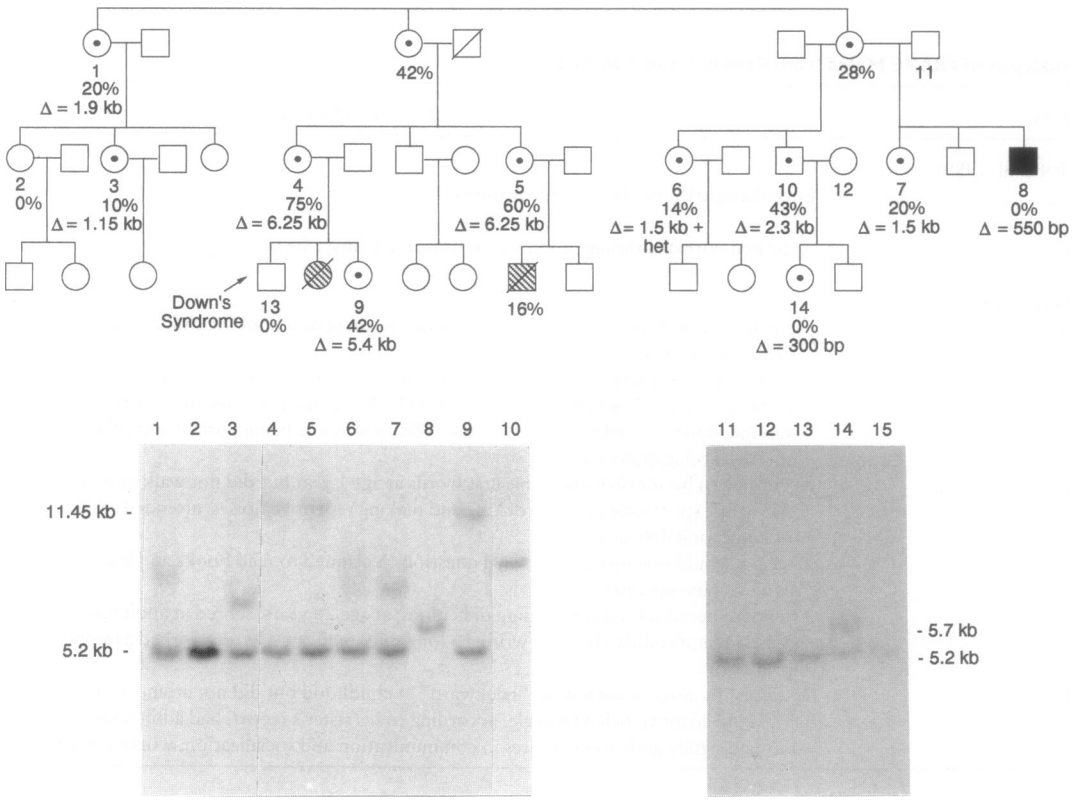

Figure 2 Analysis of *FRAXE* Family 1, with probe OxE20. DNA samples derived from peripheral blood lymphocytes were digested with *Hin*dIII. The numbers above the lanes correspond to the individual numbers on the pedigree. Lane 15, Normal male control. □ = Normal male; ○ = normal female; ⊡ = carrier male; ⊙ = obligate carrier female; ■ = affected male; ⊘ = deceased male; ▩ = male—pregnancy terminated; and ◍ = female—pregnancy terminated.

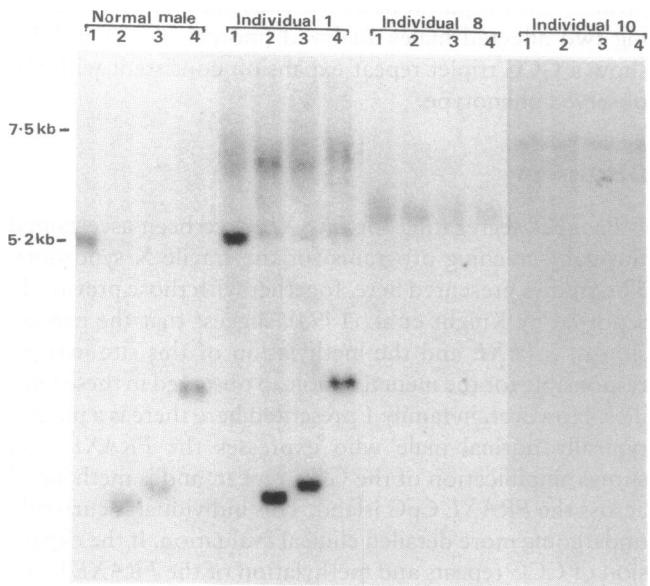

Figure 3 OxE20 methylation analysis across the *FRAXE* CpG island of a normal male and of individuals 1, 8, and 10 from family 1 (see fig. 2.). DNA samples were digested with *Hin*dIII (lane 1), *Hin*dIII+*Bss*HII (lane 2), *Hin*dIII+*Sac*II (lane 3), and *Hin*dIII+*Not*I (lane 4).

tal impairment varies from mild to very severe (Fryns et al. 1989). It is important that the parents of this individual be evaluated for their cognitive abilities, as high parental scores could also account for normal range values in their son. Alternatively, the normal phenotype observed in this male may be due to somatic mosaicism with normal expression of the *FRAXE* gene product in the critical tissues. Such mosaicism has been observed in rare individuals who express the *FRAXA* site and are phenotypically normal (D. Barton, personal communication). In contrast, the apparent association between *FRAXE* expression and mental handicap may be due to ascertainment bias, as suggested by Sutherland and Baker (1992).

In the few families studied thus far, the *FRAXE* individuals are more mildly affected than are fragile X syndrome (i.e., *FRAXA*) individuals. In light of the highly conserved nature of sequences in the *FRAXE* region (A. V. Flannery, unpublished data), it seems likely that the *FRAXE* phenotype results from the loss of expression of a gene. However, it is also possible that the *FRAXE* mutation causes reduced FMR-1 expression, resulting in a phenotype that is much milder than classic fragile X syndrome (i.e., *FRAXA*). The mechanism for this may be similar to that described by Hansen et al. (1993), who suggest that a large

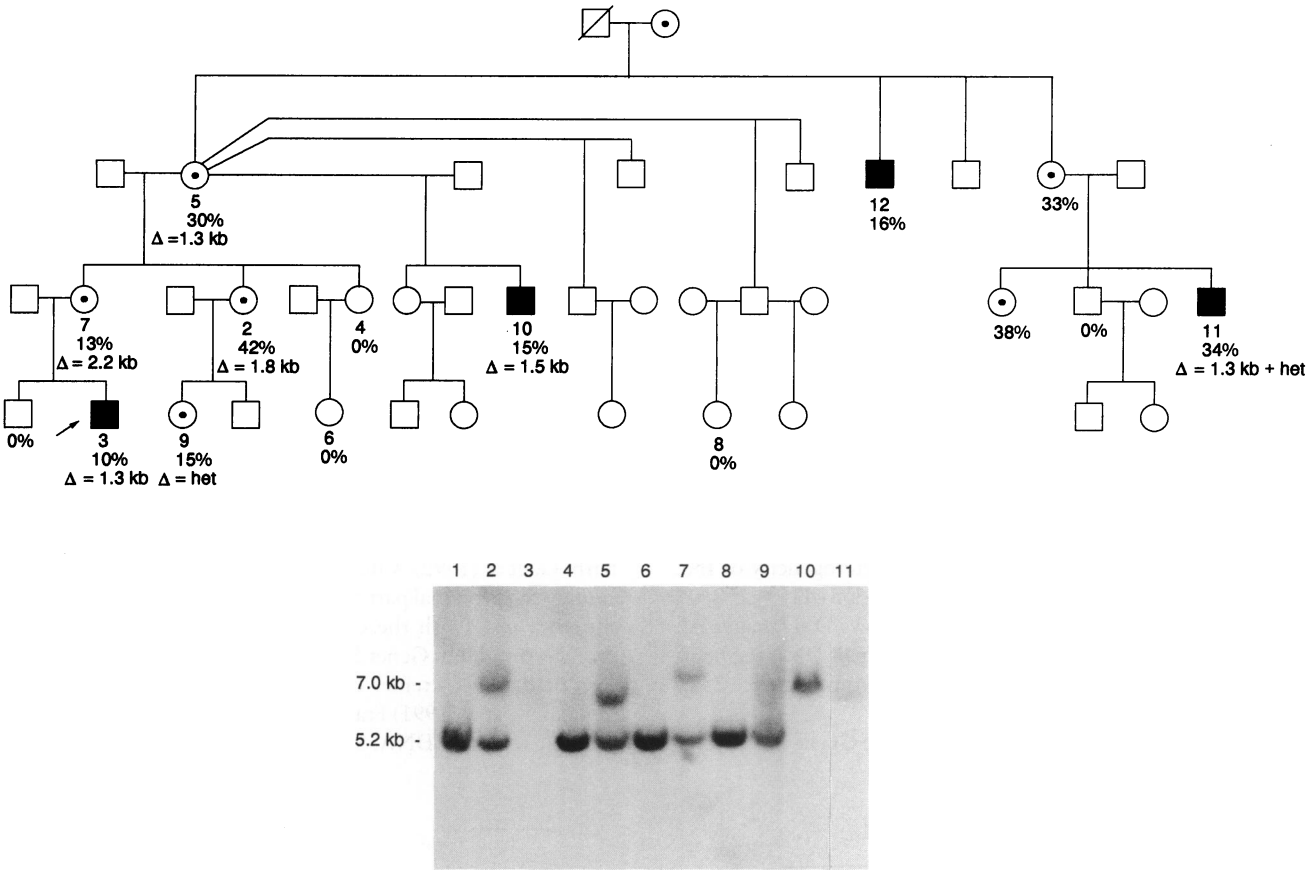

Figure 4 Analysis of *FRAXE* family 2 with probe OxE20. DNA samples derived from lymphoblastoid cell lines were digested with *Hin*dIII. The numbers above the lanes correspond to the individual numbers on the pedigree. Lane 1, Normal male control. □ = Normal male; ○ = normal female; ⊙ = obligate carrier female; ■ = affected male; and ⊠ = deceased male.

domain of late replication caused by the *FRAXA* mutation may explain the transcriptional inhibition reported at the IDS locus of *FRAXA* individuals (Clarke et al. 1992).

Genotype-phenotype studies have been limited by a lack of comprehensive cognitive and psychometric testing in *FRAXE* families. These will be necessary to establish whether there is indeed an association between *FRAXE* and mental impairment. Although the *FRAXE* locus shows many similarities to the *FRAXA* locus, individuals with the *FRAXE* mutation may escape ascertainment through screening programs designed to detect the fragile X syndrome (i.e., *FRAXA*), because they have a generally milder phenotype. If this is the case, it will be important to screen children with learning difficulties who are attending special schools and to perform population screens to assess both the frequency of this mutation and the clinical spectrum of the disease. These studies are currently in progress.

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