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FRAXE Expansion Is Not a Common Etiological Factor among Developmentally Delayed Males

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Summary

Expansion of a $(CGG)_n$ trinucleotide repeat unit at FRAXE, a newly defined fragile site distal to FRAXA, at Xq28, is reported to be associated with mild mental retardation. Three hundred developmentally delayed male patients referred for fragile X testing but negative for the FMR-1 gene trinucleotide expansion were screened for the FRAXE expansion. This group of patients had a wide range of intellectual or behavioral problems and included 19 patients who had low-level fragile site expression detected cytogenetically at Xq27-q28. None of the patients tested positive for the FRAXE expansion. These results suggest that FRAXE is not a common etiological factor among this group of patients. The data support the hypothesis that FRAXE is either very rare or a benign fragile site that is not associated with any clinical phenotype, similar to the FRAXF and FRA16A sites.

Introduction

Fragile X syndrome, the most common form of mental retardation in males, has an incidence of ~1 in 1,250 males and ~1 in 2,500 females (Sherman 1991). The carrier frequency in females has been estimated to be ~1 in 700, although frequencies as high as 1 in 354 have been reported (Rousseau et al. 1993). The fragile X phenotype includes elongated facies, prognathism, protruding ears and macro-orchidism as well as moderate to severe mental retardation (Hagerman 1991) and is caused in nearly all cases by expansion of an unstable trinucleotide (CGG) repeat in the 5′ UTR of the FMR-1 gene (Fu et al. 1991). FMR-1 encodes a putative RNA-binding protein, FMRP, that interacts with ~4% of human brain RNA. It has been suggested that the absence of interaction between FMRP and the appropriate RNA transcripts may be responsible for the phenotypic variability observed in fragile X syndrome (Ashley et al. 1993). Cytogenetically, fragile X syndrome is characterized by the presence of a folate-sensitive fragile site at Xq27.3 (FRAXA) in the lymphocytes of affected individuals (Lubs 1969; Sutherland 1977).

Recently, two other rare folate-sensitive fragile sites distal to FRAXA at Xq28 have been described, FRAXE and FRAXF (Sutherland and Baker 1992; Parrish et al. 1994). Both of these fragile sites are caused by the expansion and subsequent methylation of a $(CGG)_n$ trinucleotide repeat unit (Sutherland and Baker 1992; Parrish et al. 1994). The pathological consequences of expansion of the trinucleotide repeats at these loci are unclear. In the original studies, the expression of the FRAXE fragile site did not correlate with the mental retardation found in the family examined (Sutherland and Baker 1992). However, subsequent studies suggested that some individuals expressing FRAXE may be mildly mentally retarded (Flynn et al. 1993). Thus, it is not clear whether the association between mild mental retardation and expression of FRAXE is causative or merely coincidental. FRAXF has not been associated with any clinical phenotype, although like FRAXE it was ascertained through putative fragile X families (Parrish et al. 1994). Cloning of the FRAXE and FRAXF regions has revealed, in both cases, the presence of an unstable trinucleotide $(CGG)_n$ repeat region adjacent to a CpG island (Knight et al. 1993; Parrish et al. 1994). To date, no genes related to the CpG islands adjacent to either the FRAXE or FRAXF loci have been isolated.

In addition to the 3 X-linked fragile sites described above, 23 other folate-sensitive fragile sites have been identified on human autosomes. These fragile sites occur in ~5% of the population and have not been associated with any phenotype (Sutherland 1991). One of these, FRA16A, has been cloned and shown to consist of a $(CGG)_n$ trinucleotide expansion (Nancarrow et al. 1994). Like FRAXA, FRAXE, and FRAXF, the $(CGG)_n$ region is immediately adjacent to a CpG island that is methylated in fragile site–positive individuals. However, no gene has been identified near this CpG island, and FRA16A has no recognized clinical phenotype. Thus, the only fragile site for which there is convincing evidence of an associated phenotype is FRAXA, which

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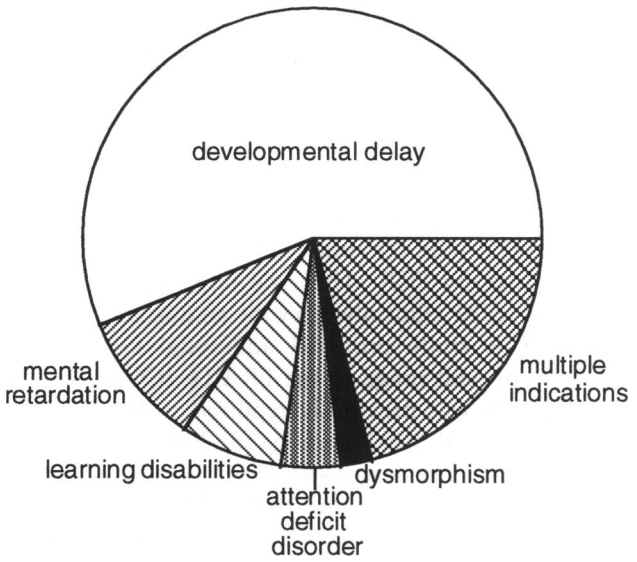

Figure 1 Reasons for referral for fragile X testing. Patients referred with multiple indications (i.e., developmental delay plus learning disabilities) are indicated separately.

is associated with the fragile X syndrome (Lubs 1969; Sutherland 1977). The purpose of the present study was to investigate the frequency of the FRAXE expansion among male patients presenting with mild to moderate mental retardation, developmental delay, and/or learning disabilities.

Material and Methods

For Southern blot analysis, 1.5 μg of genomic DNA extracted from peripheral blood lymphocytes was digested with *Hin*dIII overnight at 37°C. The digested DNA was run on a 0.8% agarose (Biorad) gel overnight at 35 V, blotted onto HyBond N+, and hybridized overnight at 42°C to the *Eco*RI/*Eag*I fragment of the OxE20 (Knight et al. 1993) plasmid. Both 1- and 3-d exposures were done to ensure that there was no mosaicism in the patient samples.

PCR amplification was performed as follows: 300 ng of DNA was added to 14.2 μl of a PCR cocktail containing 1.5 mM $MgCl_2$; 10 mM Tris; 50 mM EDTA; 10% DMSO; 235 μM each of dATP, dCTP, dTTP, and 7-deaza-dGTP (Pharmacia); 2.5 U AmpliTaq (Cetus); and 0.5 μl each of FRAXE oligos A (5′-GCG AGG AAG CGG CGG CAG TGG CAC TGG G-3′; 25 pM/μl; fluorescent-labeled with 5′-carboxy-fluorocein [ABI]) and B (5′-CCT GTG AGT GTG TAA GTG TGT GAT GCT GCC G-3′; 50 pM/μl; unlabeled) (Knight et al. 1993). The template was denatured for 10 min at 95°C followed by 23 cycles of 95° for 1.5 min, 65° for 1 min, and 72° for 2 min. A final elongation step of 10 min at 72° was used.

For PAGE analysis, 2.5 μl of the PCR product, 3.5 μl of 80% formamide-loading buffer, and 1 μl of Genescan 1,000-molecular-weight standard fluorescent-labeled with ROX (ABI) were loaded onto a 6% denaturing gel (BRL) and run for 3 h at 850 V on an ABI 373A automated sequencer. The size of the PCR product was calculated by comparison with the standard bands using Genescan Analysis 1.1 software (ABI). The size of the CGG repeat was calculated by subtracting the size of the nonrepeat sequence (288 nt) and dividing by 3.

Results

Patients

A linear series of 300 male patients referred for fragile X testing but found to be negative for the $(CGG)_n$ expansion in the FMR-1 gene were tested for the presence of the FRAXE expansion. Clinical information was compiled for approximately one-third of the patients studied, and 68.8% were described as developmentally delayed, 12.9% were learning disabled, and 9.7% were described as mentally retarded (fig. 1). Patients were also referred because of attention deficit disorder (6.5%), autism (5.4%), dysmorphism (4.3%), hyperactivity (3.2%), and behavioral problems (2.2%). No patients were described as severely or profoundly mentally retarded. Thirteen patients were referred for more than one indication (i.e., developmental delay plus hyperactivity, or autism plus dysmorphism), and these patients are indicated separately in figure 1.

Southern Blot Analysis

Southern blot analysis was done on a positive control sample from a FRAXE-positive male and samples from

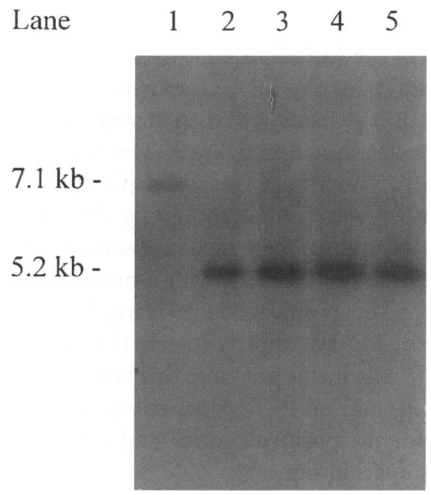

Figure 2 Southern blot of the FRAXE positive control (lane 1), a negative control (lane 5) and three patient samples (lanes 2–4) probed with the *Eco*RI/*Eag*I fragment of the OxE20 probe.

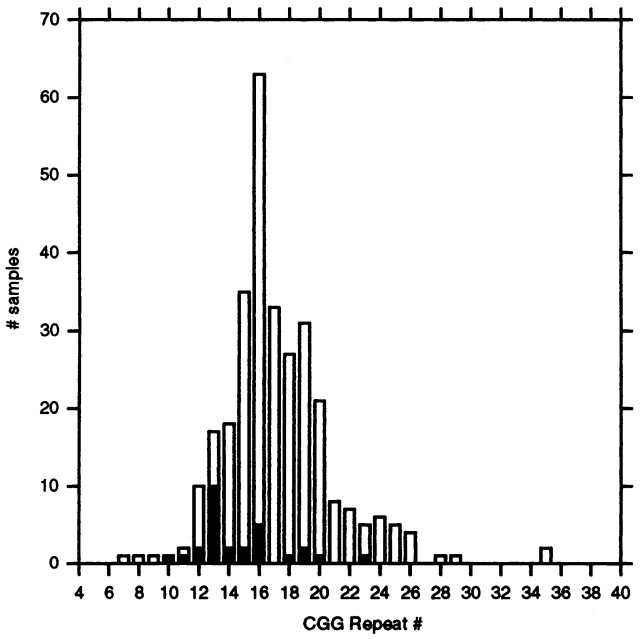

Figure 3 FRAXE $(CGG)_n$ repeat lengths for control and patient samples. Control samples are represented by the blackened bars, and patient samples are represented by the unblackened bars.

three of the patients to determine the size of the expansion in the control sample and to look for expansions in the patients. As can be seen in figure 2, the *Eco*RI/*Eag*I fragment of the OxE20 probe detects a band of ~7.1 kb in *Hin*dIII-digested DNA from the positive control, suggesting an amplification of 600–650 CGG repeats, while in the three patients the normal 5.2-kb band is detected (Knight et al. 1993).

PCR Analysis

The remaining patients were screened by PCR amplification of the FRAXE $(CGG)_n$ region by using a fluorescent-labeled primer and the automated DNA fragment analysis application of the ABI 373A automated sequencer. No individuals positive for the FRAXE expansion were identified among the 300 patients tested. A histogram of the repeat sizes observed is shown in figure 3. The smallest repeat size was $(CGG)_7$, while the largest was $(CGG)_{35}$. The modal repeat number was $(CGG)_{16}$. Among 28 normal control samples tested the smallest repeat observed was $(CGG)_{10}$, and the largest was $(CGG)_{23}$, with a modal repeat number of $(CGG)_{13}$. The positive control sample was shown by Southern blot to have approximately $(CGG)_{600-650}$. As expected, PCR was inefficient over this large expanded CGG repeat region, and no PCR product was seen. Electropherograms for the positive control and one of the patients tested are shown in figure 4.

Discussion

No males with an amplification of FRAXE were identified among the 300 patients tested in this study. The lack of PCR product in the positive control sample confirms that the region amplified in the PCR assay was indeed the FRAXE expansion region. No PCR product would be expected in this sample, since the region flanked by the primers is highly repetitive and would be >2 kb in length. The presence of the normal 5.2-kb band in the three patients analyzed by Southern blotting was consistent with the production of bands within the normal range by PCR. Thus, FRAXE expansion was not found to be clinically relevant among the patients studied. This study provided additional information about the normal range of the FRAXE $(CGG)_n$ repeat region, which was previously reported to be 6–25 repeats (Knight et al. 1993). Among the 328 chromosomes analyzed, the range was 7–35 repeats, suggesting that the normal range is broader than originally reported, although only ~2% (7/328) of chromosomes had >25 repeats.

The present study group comprised patients with a wide range of intellectual or behavioral problems who were originally referred for fragile X testing. Although no patients were reported to be severely mentally retarded, a proportion of patients were referred for more than one indication (i.e., developmental delay plus hyperactivity, or autism plus dysmorphism). Thus, this population should include candidates for any form of mild X-linked mental retardation. The data would suggest either that FRAXE is very rare or that it is associated with a phenotype not represented in the present study population. However, since FRAXE amplification was reported to be associated with mild mental retardation (Knight et al. 1993, 1994), it would be expected that the present group of patients was appropriate. In addition, 19 of the patients in the study group were reported to exhibit low level (1%–2%) fragile site expression at Xq27-q28 (I. Teshima, personal communication). It is possible that these patients are expressing FRAXF or another, as yet unidentified, fragile site. Another explanation is that the presence of both mental retardation and FRAXE expression in the families reported (Flynn et al. 1993) was coincidental and unrelated. Although the FRAXE site is adjacent to a CpG island, the identification of a gene in the vicinity has not been reported and there is, as yet, no evidence that a gene for X-linked mental retardation is present in this region. Initially, FRAXE expression was not thought to be associated with mental retardation because, although one of the individuals expressing FRAXE was mentally retarded, his two brothers, who also expressed FRAXE, were not (Sutherland and Baker 1992). A subsequent report of "X-linked mental handicap" in FRAXE-positive males was not well supported by quantitative measures of such a deficiency, and at least one other phenotypically normal male expressing FRAXE was described (Knight et al. 1994). In the few families expressing FRAXE that

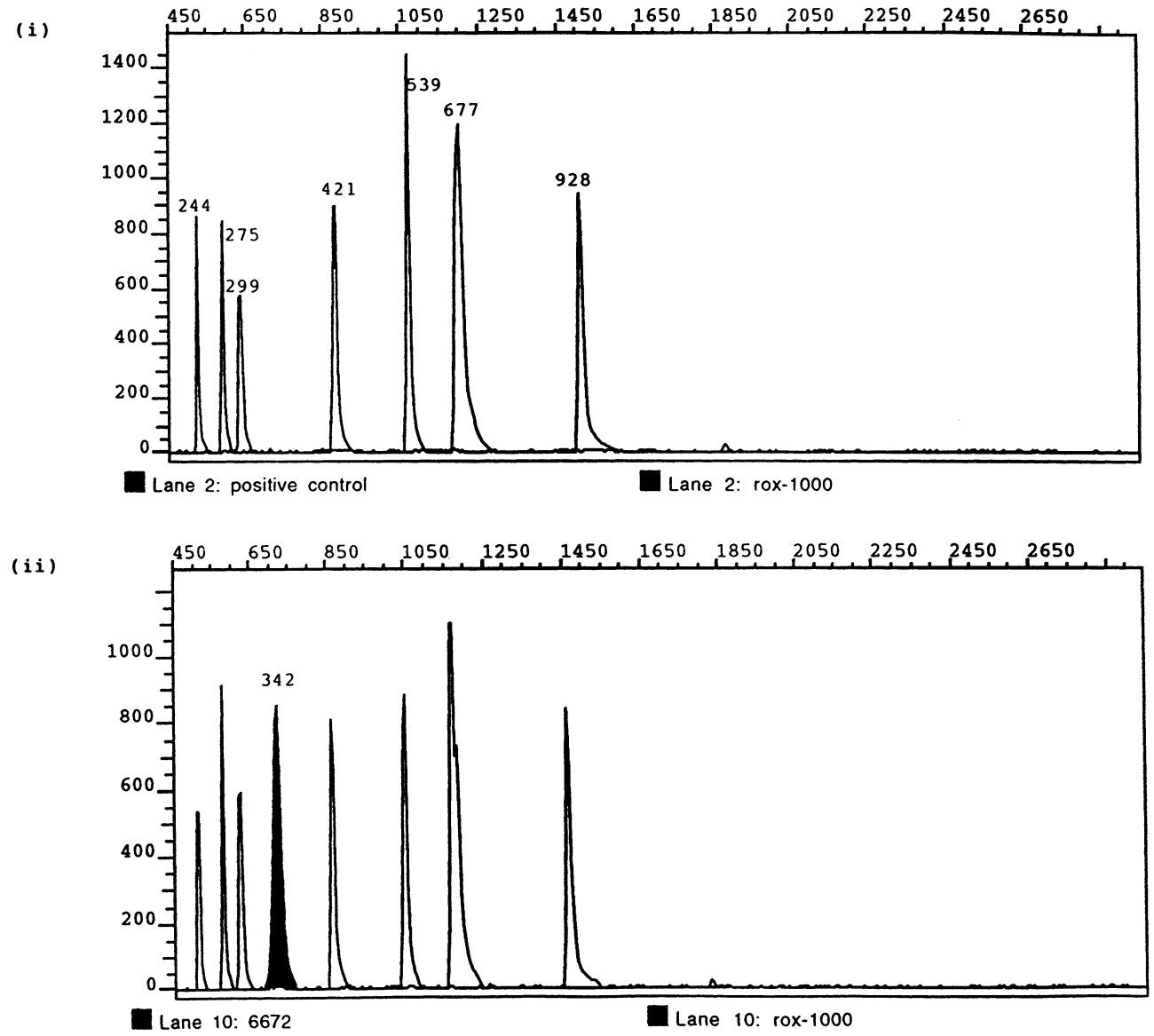

Figure 4 Electrophoretograms. Panel i is for FRAXE-positive control. No PCR band was detected, because of the large size and repetitive nature of the CGG repeat. Panel ii is for one patient sample. The size of the FRAXE peak (342 bp; $(CGG)_{18}$) in the patient sample is estimated from the molecular weight standards (size indicated in base pairs above peak).

have been reported to date, there was definite ascertainment bias since these families were examined because of mental retardation and fragile site expression but absent FMR-1 CGG expansion (Flynn et al. 1992; Knight et al. 1994). Therefore, the presence of the fragile site in these families does not necessarily indicate a causative effect.

To date, 26 folate-sensitive fragile sites, inherited in a Mendelian manner and occurring in ~5% of the population, have been identified in the human genome (Sutherland 1991). In the four cases that have been examined at the molecular level, all involve the expansion of CGG trinucleotide repeats situated near CpG islands. In each case, the expansion is accompanied by methylation of the CpG island. However, only with FRAXA has this methylated island been associated with a change in expression of a specific gene. The characterizations of FRAXF and FRA16A individuals (Nancarrow et al. 1994; Parrish et al. 1994) suggest that these expansions, unlike FRAXA, are not associated with an obvious phenotype. This would suggest that the methylation of the CpG island at these loci either does not affect expression of nearby genes or that these genes are nonessential and loss of expression does not produce a phenotype. Similarly, the dissociation of FRAXE expansion and mental deficit in some FRAXE families (Sutherland and Baker

1992; Knight et al. 1994) suggests that the two phenomena may be unrelated and that FRAXE expansion does not result in a identifiable phenotype. On the other hand, if FRAXE expansion is associated with a mild mental handicap, this phenotype may be extremely variable, ranging from learning disability or developmental delay in some individuals, to normal or nonpenetrant in other individuals in the same family. The elucidation of these questions will require the unbiased identification of a larger population of individuals with expansion of these trinucleotide repeats for detailed clinical evaluation. This would ideally be achieved through population screening. At the molecular level, the identification and characterization of the genes surrounding these loci will be important in determining the effect, if any, of trinucleotide expansion.

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