Identification of the\textit{ FRAXE} fragile site in two families ascertained for X linked mental retardation

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Abstract

Chromosome fragility in two families not exhibiting amplification of the CGG trinucleotide associated with the fragile X site has been examined. Fluorescence in situ hybridisation with cosmid DNA from loci immediately flanking \textit{FRAXA} and other distal loci have confirmed that cytogenetic fragility in these subjects is the result of expression of a new folate sensitive fragile X site, \textit{FRAXE}. (J Med Genet 1993;30:97-100)

Fragile X, or Martin-Bell, syndrome is characterised by X linked mental retardation and dysmorphic clinical features with a wide variation in the phenotype seen in affected subjects.\textsuperscript{1} It is associated with the expression of a rare folate sensitive fragile site at Xq27.3 (\textit{FRAXA}) which can be induced in lymphocytes cultured under conditions of folate depletion.\textsuperscript{2} Expression of the \textit{FRAXA} site has been taken as diagnostic of the fragile X syndrome, although several authors have reported the discordance of mental retardation and cytogenetic expression.\textsuperscript{3,4}

Cytogenetic, genetic, and physical analysis of Xq27/q28 has led to the localisation of the \textit{FRAXA} site and the identification of a gene exhibiting unusual properties on the fragile X chromosome.\textsuperscript{5-13} This gene, FMR-1, contains a CGG trinucleotide repeat sequence, the length of which becomes increased and hypervariable on the fragile X chromosome. This triplet element appears to be coincident with the position of the site of cytogenetic fragility. An increase in size of the trinucleotide repeat sequence, which appears to be located in the first exon of the gene, is now held to be diagnostic of the syndrome. The amplification status of this repeat shows good correlation with phenotype, which makes possible prenatal diagnosis and carrier detection by molecular analysis.\textsuperscript{14-16}

Exceptions to the usual pattern of molecular findings have been identified. A fragile X negative, mentally retarded male with the Martin-Bell phenotype has been identified with a large de novo deletion removing the proximal portion of the FMR-1 gene, the FMR-1 HTF island, and the CGG trinucleotide region.\textsuperscript{17} This provides evidence that the gene is critical in the development of the fragile X syndrome and strengthens the suggestion that the trinucleotide repeat or elements in the immediate vicinity play a role in cytogenetic expression of fragility. Several affected subjects are mosaic for the amplification and do show some expression of the FMR-1 gene which most likely accounts for the milder phenotype observed.\textsuperscript{18}

In addition to the patients mentioned above, several fragile X site positive families do not show amplification of the repeat sequence. The observed fragility in one such family has been shown to result from a newly identified folate sensitive fragile site \textit{FRAXE}, lying distal to \textit{FRAXA}.\textsuperscript{19} We have previously reported two such fragile site positive, insertion negative families.\textsuperscript{20} Using FISH probes we show that these two families also possess a folate sensitive fragile site mapping to the \textit{FRAXE} region.

Materials and methods

CLINICAL DETAILS

The clinical phenotypes of the probands and other fragile X positive subjects in these two families have been described in detail,\textsuperscript{21} and are not thought to be characteristic of the Martin-Bell syndrome: the mental impairment is in all cases rather mild and there is no obvious macro-orchidism or facial dysmorphism.

CYTOGENETICS

Blood samples were obtained from one expressing male of each family (III-21 from family 1 and I-2 from family 2 in reference 20). Expression of the fragile site was induced in peripheral blood lymphocytes using folate depleted culture medium and chromosome spreads were prepared by standard techniques.

The folate sensitivity of the fragile site in family 1 was assessed by culturing lymphocytes from III-21 in folic acid depleted medium without serum (FX-1 Biological Industries) and comparing the frequency of fragile sites with the frequency found when folic acid (Sigma) at a final concentration of 1 mg/ml was added for the last 24 hours to a parallel culture in the same medium.\textsuperscript{22} Slides from these cultures, other parallel cultures from the same subject, and a fragile X negative control were coded and scored blind. A repeat blood sample could not be obtained from subject I-2 of family 2, so folate sensitivity was assessed by using a lymphoblastoid cell line established from III-1. Cultures were incubated for 48 hours in FX-1 medium with 5% serum. The fragile site was induced by adding FUDR (Sigma) at a final concentration of 10\textsuperscript{-7} mol/l for the last 24 hours of culture\textsuperscript{23} and the effect on expression was examined in parallel cultures where folic acid was added at a final
concentration of 1 mg/ml in addition to the FUdR. The slides were coded and scored blind.

PROBES
Cosmids corresponding to the loci 9L (G9L, ICRFc104D08104), 141R (E2165, ICRFc100E2165),23 and VK21 (G3)24 were isolated from genome libraries by hybridisation screening and DNA prepared by standard methods. The locus 9L lies approximately 70 kb proximal, 141R 120 kb distal, and VK21 up to 650 kb distal to the FMR-1 HTF island. The cosmid E2165 also spans the DNA marker D033.

IN SITU HYBRIDISATION
Cosmid DNA for the loci 9L, 141R, and VK21 were labelled with biotin by nick translation. The DNA fragment size upon labelling averaged around 300 bp. Hybridisation and detection procedures have been described in detail elsewhere.23 Briefly, 80 ng of labelled cosmid DNA was hybridised overnight in a volume of 10 µl hybridisation mix per slide. Biotinylated alphoid centromere probe specific for the X centromere (DXZ1, Oncor) was also added at 5 ng per slide to identify the X chromosome. Images were visualised and captured on a Biorad MRC600 confocal laser microscope. Metaphases were systematically checked for fragile X expression and the position of the cosmid signal scored as either proximal, mid, or distal to the fragile site.

LINKAGE ANALYSIS
DNA probes p4D8 (DXS98) and RN-1A (DXS369), mapped as proximal to FRAXA and VK21 (DXS296) and U6-2 (DXS304) distal to FRAXA,25 in conjunction with appropriate restriction enzymes, were used in the linkage analysis of these families. Southern transfer, probe labelling, and hybridisation were carried out by standard methods.

Results
CYTOGENETICS
As reported previously20 members of both families expressed a fragile site at Xq27.3, indistinguishable from that in classic FRAXA families having a large CGG amplification in the FMR-1 gene, although no such DNA amplification could be detected in any member of either family.8,20

The addition of folic acid to a folate depleted culture from I11-21 of family 1 reduced the fragile site expression from 28% (14/50 cells) to 0 (0/50 cells). This site is therefore clearly folate dependent. Expression of the fragile site in I11-1 from family 2 was only 6% when induced with FUdR in lymphoblastoid cells; nevertheless the simultaneous addition of FUdR and folic acid reduced expression of this fragile site from 6% (7/123 cells) to 0 (0/91 cells). Thus the fragile sites in both families show sensitivity to folate.

Discussion
The trinucleotide amplification in the FMR-1 gene is now considered diagnostic for the syndrome and is used for the determination of genotype in fragile X positive families. Its use has led to the identification of exceptional families in which the FMR-1 gene showed normal methylation pattern of the CpG island and no detectable change in the length of the trinucleotide CGG repeat. Most of these families were originally designated fragile X syndrome on the basis of mental retardation combined with the expression of a fragile site at Xq27.

The two families described here have been reported previously as lacking amplification and clinically as presenting with features not typical of the fragile X syndrome.20 In particular the mental retardation is mild with most subjects showing only learning difficulties. In family 1 there are high levels of fragile site expression among the females of the family,
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Figure 1  In situ hybridisation of cosmids 141R and VK21 and the X centromere probe DXZ1 to chromosome preparations of patients 1 and 2. (a,b) Hybridisation of cosmid 141R proximal to the fragile site in patients 1 and 2. (c,d) Hybridisation of cosmid VK21 distal to the fragile site in patients 1 and 2.

Localisation of hybridisation signal for probes in relation to the fragile sites FRAXA and FRAXE.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Locus</th>
<th>Proximal</th>
<th>Mid</th>
<th>Distal</th>
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<td>23</td>
<td>16</td>
<td>0</td>
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<tr>
<td></td>
<td>141R (5)</td>
<td>0</td>
<td>5</td>
<td>56</td>
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<tr>
<td></td>
<td>VK21 (5)</td>
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<td>15</td>
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<tr>
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<td>20</td>
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<td>0</td>
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<tr>
<td></td>
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<td>9</td>
<td>18</td>
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<tr>
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<tr>
<td></td>
<td>VK21</td>
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Scoring for FRAXA patients is combined data obtained from mapping on several subjects (number of patients in brackets). Data for cosmid G9L from three of these subjects has been published previously.

who are clinically normal. In family 2 a fragile X positive male failed to transmit the fragile site to his daughter who is phenotypically normal but the fragile site reappears in the grandson who presents with learning difficulties. This is an unusual situation, as normal transmitting males are usually fragile X negative. In both these families the site of chromosome fragility is not at FRAXA, but at a more distal locus, FRAXE, which has been shown in both families to be folate sensitive. The FRAXE site was first reported by Sutherland and Baker in a family with three fragile X positive brothers. In that family, only one of the sibs was mentally impaired, suggesting that fragile site expression and mental impairment are not associated and this may be the case in the two families reported here.

Two other families have been reported where a folate sensitive fragile site shows no association with mental retardation. Within the Voelckel pedigree the fragile X positive male was mentally normal and the fragile site failed to be transmitted from father to daughter in the single observed case. This is similar to the observation in family 2, where fragility then reappears in the grandson. In the Voelckel case the grandson was not tested for fragility.
In the family of Romain et al., where the fragile site segregates through the pedigree, there is no evidence of mental retardation.

Two cases of a folate insensitive fragile site at Xq27-28 have been reported in subjects with mild mental impairment but no amplification of FMR-1 sequences. This is unlikely to be the common fragile site at Xq27.2 as the expression levels are high and may represent yet another rare fragile site within the region. In a family reported by Oberle et al., a folate insensitive fragile site is associated with Robin sequence in a mildly mentally retarded boy. He does not have the typical Martin-Bell phenotype and his speech problems are thought to be the result of Robin sequence complications. The association of Robin sequence with expression of a fragile site at Xq27-28 has been observed in a further four patients for whom no analysis of the amplification status has yet been reported. In one of the four amplification negative families reported by Rousseau et al., a mildly mentally retarded boy expressed a folate insensitive fragile site at high levels. This patient does not have the Martin-Bell phenotype. In other families it may be difficult to assess the relationship between phenotype and the expression of a fragile site when other abnormalities are also present. The behaviour of a fragile X positive, insert negative, mentally retarded boy is thought to be the result, in part, of his 47,XY karyotype.

Other fragile site positive families lacking the CGG trinucleotide amplification may be expressing a FRAXE site, and the nature of the fragile site in these families can now be readily determined by FISH. At present it is unclear whether FRAXE carries any phenotypic effects; it is possible that its association with mentally impaired subjects and families could be because of ascertainment bias, although pedigree analysis in family 1 would suggest X linkage. As more FRAXE subjects are characterised, any relationship with mental impairment will be clarified. It is known, however, that the expression of FRAXE can have an effect on the expression of the Hunter gene. What effect, if any, the expression of FRAXE may have upon FMR-1, MRX3, or other genes in the region is as yet unknown. The expression of the FMR-1 gene in lymphoblastoid cell lines of our two families appears normal (unpublished data). A detailed map of the FRAXE region is now needed to determine whether any DNA abnormality exists at or around the FRAXE site. Our earlier mapping showed the presence of an HTP island within the FRAXE region, which does raise the possibility of another gene in this area associated with a mild form of X linked mental retardation.

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