Genotype prediction in the fragile X syndrome

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Abstract
Fragile X positive, mentally retarded males have been shown to have an insertion or amplification of DNA sequences at, or close to, the site of expression of the fragile site. We show here the application of the detection of such changes to the diagnosis of affected males and female carriers and the identification of normal transmitting males. One fragile X negative male with the clinical features of the Martin-Bell syndrome also possesses an inserted/amplified DNA sequence. The implications of these results for screening for the fragile X syndrome are discussed.

The fragile X syndrome is the commonest inherited cause of mental impairment, affecting 1 in 500 males.1 Fifty-six percent of female carriers of the mutation are not affected while 20% of males are phenotypically normal transmitters of the disorder (so-called normal transmitting males, NTMs).2 The disease is associated with the expression of a folate sensitive fragile site at Xq27.3 although the level of expression can vary from a few percent to as high as 50%.3 Typically, daughters of normal transmitting males are not retarded and either do not express the fragile site or only express the fragile site at a low level. This can make genetic counselling particularly difficult. Although closely linked genetic markers have been developed,4-8 their use is sometimes limited by the high recombination frequency, and in some cases the mode of inheritance (through the male or female line) is unclear.

The unusual inheritance pattern observed in the fragile X syndrome has led to the hypothesis that the development of the full phenotype involves a two step process.9-11 The first non-phenotypic, or premutation, event is converted into the full mutation only after passing through oogenesis. Several hypotheses have been proposed as to the nature of this second mutation event, including genomic imprinting and amplification of DNA sequences at the fragile site. Recently evidence has accumulated for the possible role of both of these in the progression to the fragile X phenotype. We and others have shown that fragile X patients are hypermethylated at a CpG island close to the fragile site.12-13 These CpG dinucleotides are not generally methylated in normal transmitting males or in their daughters but do become methylated in the affected grandsons, suggesting that genomic imprinting could be occurring through methylation in early oogenesis. More recently several workers have reported the insertion/amplification of DNA sequences adjacent to these CpG residues in fragile X patients and normal transmitting males.14-16 This insertion/amplification event can be readily observed on Southern blots by a change of fragment size. In this paper we report the use of this sequence change for the determination of genotype in fragile X positive families and in the diagnosis of fragile X negative males.

Materials and methods
SOUTHERN BLOT AND HYBRIDISATION ANALYSIS
DNA from the patients was restricted with the enzymes EcoRI, BglII, or HindIII, subjected to electrophoresis, and transferred onto membranes using standard methods. DNA probe Ox1.9 was isolated from a cosmid crossing the CpG region.17 The 1.9 kb insert was radiolabelled by the random primed method as previously described.13 Filters were washed to a stringency of 1 × SSC and

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exposed against Kodak X-AR5 film at −70°C for one to five days.

**Results**

We have isolated a genomic DNA fragment, Ox1.9, which lies immediately distal to the CpG island which is hypermethylated in fragile X positive, mentally retarded males (fig 1A). Analysis of patient DNA with restriction enzymes having recognition sites on either side of the CpG island showed alterations indicating the occurrence of hypervariation of fragment length in this region. This is shown in fig 1B with EcoRI digests of DNA from members of a family segregating for the fragile X syndrome together with an unrelated affected male. Normal subjects show an EcoRI fragment of 5.1 kb (lanes 2, 4, and 6) whereas affected males show either a faint smear of fragments or a discrete fragment of higher molecular weight (lanes 1, 3, and 7). The affected female (lane 5) in the family shows a 5.1 kb fragment corresponding to her normal X chromosome and additional fragments of higher molecular weight corresponding to the mutated X chromosome. This multi-fragment pattern of the fragile X chromosome is a common feature of the mutation and probably corresponds to somatic heterogeneity in the length of this fragment.

In some subjects the pattern seen with Ox1.9 is so heterogeneous that only a smear of hybridisation is seen. This allows diagnosis of the fragile X chromosome in males but can make the genotyping of females difficult. For example, in fig 2A the two affected males in lanes 2 and 3 clearly have the mutated X chromosome but the smear seen in their mother in lane 4 is only just visible. This female is cytogenetically positive and mildly affected. Samples in lanes 1 and 6 are from females predicted to be normal from linkage analysis. In fig 2B, an affected female (lane 2) clearly shows a fragment of increased size similar to her affected brother (lane 3). The clinically normal, cytogenetically negative mother in lane 4 shows no abnormal pattern.

The occurrence of these fragment changes and their relationship to the expression of the disorder was studied in more detail through the analysis of an extended family segregating for the fragile X syndrome. Fig 3 shows the analysis of samples from such a family by HindIII digestion. Two phenotypically normal carrier females (lanes 3 and 11) show a larger sized fragment corresponding to the inheritance of the mutated X chromosome. Neither of these females is fragile X positive but they are obligate carriers because they have affected children. There are two additional equivalent female carriers in this pedigree, one of whom showed an altered fragment.
on her X chromosome and the other of whom showed a very small shift as a tight doublet (data not shown). These phenotypically normal females have a smaller increase in molecular weight of their fragments compared with the affected female in fig 2B. A sample from an affected female in this pedigree is shown in lane 1 where an extended smear of fragments is barely visible. The normal transmitting male in this pedigree (lane 5) shows a characteristic shift in molecular weight of the HindIII fragment. Normal transmitting males generally show a much smaller shift in fragment size compared to affected males (compare lanes 5 and 8).

We have observed this insertion/amplification event in 56 out of 59 unrelated fragile X positive, mentally retarded males and in seven normal transmitting males. No insertion was observed in 43 normal X chromosomes. In the three cases that do not show an insertion, it is possible that these subjects are mosaics\textsuperscript{16,17} or that they are cases of misdiagnosis.

One mentally retarded subject who was fragile X negative on two occasions was a member of a family who had distant relatives suffering from the fragile X syndrome (see pedigree in fig 4, F1, subject 6). He has bilateral epicanthic folds, normal testes, and is in a special education class with an IQ of 58. \textit{BglII} digests were analysed on this occasion as these were already available from previous RFLP typings with \textit{DXS15}. The digest covers the same region of the fragile site as the \textit{EcoRI} and \textit{HindIII} digestion, but on a larger 12 kb fragment. All the fragile X positive males in this pedigree showed a characteristic smear of gel fragments similar to that in the affected male (subject 3, fig 4) in family F2. The fragile X negative male also showed a small shift in the size of the \textit{BglII} fragment (fig 4, lane 6). This suggests that his phenotype is indeed the result of a mutation at the fragile X locus even though he is fragile X negative. Why this affected male does not express the fragile site warrants further investigation. None of the other males in this branch of the pedigree shows

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\textbf{Figure 2} (A) Hybridisation of DNA probe Oxl.9 to \textit{HindIII} digests of DNA from normal males (lanes 5 and 7), normal females (lanes 1 and 6), affected males (lanes 2 and 3), and their mother (lane 4). (B) Hybridisation of the DNA probe Oxl.9 to \textit{HindIII} digests of DNA from an affected male (lane 3) and his affected sister (lane 2) in a family segregating for the fragile X syndrome. Lane 1 is the normal sister of these subjects, lane 4 the mother, and lane 5 the father. Lane 6 is an unrelated normal male.
We have shown that carriers and affected subjects possess an insertion/amplification of DNA sequence adjacent to the CpG island which we previously showed to be abnormally methylated in patients. The data presented here, together with those already published, suggest that this is a good test for the presence of the fragile X mutation. Although some affected males do show the normal sized band, owing to mosaicism or molecular heterogeneity, more than 95% show an altered fragment or smear of fragments. The size of the DNA fragment close to the fragile site increases even in normal transmitting males permitting this assay to be used to determine the mode of inheritance of the syndrome in many cases. This DNA analysis therefore provides information that cytogenetic analysis does not, since normal transmitting males are generally fragile X negative. As this insertion/amplification can be seen in BglII, EcoRI, and HindIII digests of DNA samples, Ox1.9 may be used to analyse Southern blots previously used for RFLP typings of VK23 and DX13. This may prove useful for the analysis of subjects from whom DNA samples are limited.

It has been suggested that larger fragment size changes are observed for affected subjects compared to phenotypically normal carriers and this may be useful for the diagnosis of the mental impairment of a carrier female. Our analyses support this view although, as noted previously, the correlation is not absolute. The detection of changed fragments in females appears to be more variable. We find no obvious correlation between the fragment size observed and the degree of expression of the fragile site, nor is there an apparent association with inheritance of the mutant allele from an NTM or female carrier parent.

It is now possible to screen for the fragile X mutation in males by assaying for an increase in fragment size in the region of the fragile site. This analysis is both simple and rapid. The increase in size of the BglII fragment in a fragile X negative, mentally retarded male suggests that this assay may be more sensitive than the cytogenetic expression of the fragile site. As a primary diagnosis, it may therefore be worth screening all fragile X negative males presenting with the Martin-Bell phenotype for fragment changes.

The insertion/amplification event occurs immediately adjacent to the CpG island we have shown to be imprinted in fragile X males and thus may be influencing the change in methylation status of these CpG residues. As we can detect altered fragments in normal transmitting males, this indicates that the insertion/amplification event precedes the methylation changes. This order of events may therefore represent the two stages in the development of the Martin-Bell phenotype. More detailed analysis is in progress to define completely the genetic alterations detected in the fragile X population. A gene has
Figure 4  (A) Hybridisation of DNA probe Ox1.9 to BglII digests of DNA samples of subjects from fragile X families F1 (B) and F2 (C) including a fragile X negative, mentally retarded boy (F1, 6), fragile X positive, mentally retarded males (F2, 3 and 9), and a fragile X negative, phenotypically normal male (F2, 5). The lane numbers correspond to the members of the pedigrees. Boxes indicate males discussed in the text. Filled boxes are affected subjects. Fragile site expression is scored as present (+) or absent (−) above key affected males (filled box) and an unaffected NTM (box) as discussed in the text.
recently been identified associated with this CpG island and the insertion/amplification event appears to occur near the 5' end of this gene. Hopefully, the mysteries of this fascinating disorder will soon begin to become unravelled.

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