A rapid, non-radioactive screening test for fragile X mutations at the FRAXA and FRAXE loci

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Abstract

Screening of referrals for the mutations associated with the fragile X syndrome constitutes a significant workload in many genetics laboratories. Since the great majority of these referrals will be negative, there is a need for a rapid and inexpensive screening test. We have developed an assay which allows simultaneous amplification of the triplet repeat sequences at the FRAXA and FRAXE loci by polymerase chain reaction, and detection of the products on non-denaturing gels stained with ethidium bromide. Alleles of normal size are detected, leaving a small minority of samples to be tested by Southern blotting. A PCR based assay for detection of methylation at the CpG island upstream of the FMR-1 gene has also been devised.

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The fragile X syndrome is the most common inherited cause of mental retardation, affecting approximately 1/1250 males and 1/2000 females.^{1,2} The fragile site at Xq27.3, FRAXA, has been shown to be caused by expansion of an unstable CGG repeat at the 5' end of the FMR-1 gene.^{3,4} Normal subjects have two to 50 copies of the repeat, premutations in normal transmitting males and female carriers range from 50 to 200 copies, and affected subjects have more than 200 copies.^{3,4} Expanded alleles are methylated at a CpG island just proximal to FMR-1 and are not expressed.^{5,6} A more distal fragile site, FRAXE, has recently been identified through screening for the fragile X syndrome.^{7,8} The cloning of the FRAXE site has shown that it is associated with amplification of a GCC repeat which is 600 kb distal to FRAXA.⁹ Normal subjects have six to 25 copies of this repeat, whereas subjects with more than 200 copies of the GCC repeat express the FRAXE site, and most of them suffer from mental impairment.^{9,10}

Direct analysis of the CGG expansion mutation by Southern blotting has begun to replace cytogenetic analysis for the laboratory diagnosis of the fragile X syndrome.¹¹ However, blotting is a relatively expensive and labour intensive procedure, particularly in the context of screening routine referrals for the fragile X syndrome, the great majority of which will be negative. We therefore developed a two stage protocol¹² in which samples are first screened for the presence of a normal allele by amplification

across the CGG repeat using the polymerase chain reaction.¹³ Males in whom an allele of normal size is detected, and females in whom two normal alleles are detected, can be scored as unaffected, and the few remaining samples then analysed by Southern blotting.

Current methods for PCR analysis of the FRAXA mutation involve the use of ³²P to label the PCR product,¹³ or blotting of the products onto a membrane and hybridisation with a biotinylated probe.¹⁴ Furthermore, the fact that up to 10% of cytogenetically diagnosed fragile X positive cases have the FRAXE mutation^{12,15} suggests that this mutation should also be tested for in routine referrals. We have therefore developed a rapid, non-radioactive PCR method which screens for both the FRAXA and FRAXE mutations simultaneously. Males who do not have an expansion mutation at either of these loci are identified by the presence of FRAXA and FRAXE alleles of normal size on non-denaturing gels stained with ethidium bromide. The majority of females who do not have an expansion at the FRAXA locus can also be identified, since about two-thirds of females are heterozygous for the polymorphic CGG repeat,^{13,14} and two alleles of normal size will be detected on the gel. We have also devised a PCR based assay to detect methylation of the CpG island upstream of the FMR-1 gene, since this might provide confirmation of the presence of an expanded FRAXA allele in affected males.

Materials and methods

DUPLEX AMPLIFICATION OF THE FRAXA AND FRAXE TRIPLET REPEATS

DNA (100 ng) was mixed with 20 pmol of the primers FXD (5'TGACGGAGGCGCCGCTGCCAGGGGGCGTGC3') and FXE (5'GAGAGGTGGGCTGCGGGCGCTCGAGGCCA3') for amplification of the FRAXA CGG repeat,¹³ and 35 pmol of each of the primers 598 (5'GCGAGGAAGCGGCGGCAGTGGCACTGGG3') and 603 (5'CCTGTGAGTGTGTAAGTGTGTGATGCTGCCG3') for PCR of the FRAXE⁹ CCG repeat in a total volume of 50 µl containing *Taq* DNA polymerase buffer (50.25 mmol/l Tris-HCl, pH 8.8, 12.45 mmol/l (NH₄)₂SO₄, 1 mmol/l MgCl₂ and 127.5 µg/ml BSA) and 200 µmol/l each of dATP, dCTP, and dTTP, 150 µmol/l dGTP, 50 µmol/l 7-deaza-2'dGTP (C⁷dGTP), 10% dimethylsulphoxide. After denaturation of the DNA for 10 minutes at 95°C, 2.5 U of *Taq* DNA polymerase was added to each tube while maintaining the temperature at 95°C ("hot

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start" procedure).¹⁶ The samples were then subjected to 30 cycles of amplification (95°C, one minute; 64°C, 1.5 minutes; 72°C, two minutes; with a final extension of five minutes at 72°C). A total of 10 µl of product was analysed by electrophoresis in a 2% agarose gel (1% agarose, 1% Nusieve) containing ethidium bromide. The presence of two bands in the correct size range in a male, or heterozygosity for alleles of normal size in a female, excludes the presence of an expansion mutation at either the FRAXA or the FRAXE locus.

DETECTION OF HETEROZYGOSITY FOR NORMAL FRAXA ALLELES IN FEMALES

In order to detect females who were heterozygous for normal alleles at the FRAXA locus, improved resolution was obtained on a larger polyacrylamide gel, which allowed resolution of differences of one repeat unit (3 base pairs) between alleles. The residue of the PCR products from the analysis on the minigel were used for this assay. The intensity of the bands observed on the minigel was used to adjust the volume of PCR product loaded on the larger gel. PCR product (10 to 20 µl) was separated on a 20 cm non-denaturing 10% polyacrylamide gel (Biorad Protean) containing 10% glycerol by electrophoresis for 18 hours at 250 V in 0.5 × Tris-borate-EDTA buffer.¹⁷ Bands were visualised under ultraviolet light after staining the gel with ethidium bromide.

PCR ASSAY FOR FRAXA METHYLATION AT THE FMR-1 GENE

A new PCR test for methylation of the CpG island upstream of the FMR-1 gene, which occurs in the presence of a FRAXA expansion mutation, was developed. Genomic DNA samples were first digested with a methylation sensitive enzyme. This was followed by amplification of the methylated site upstream of the gene. The PCR should only produce a product if the site was methylated, and hence protected from cleavage with the enzyme.

Two tubes were set up for each person. Each tube contained 1 µg of genomic DNA, 2 µl of 10 × restriction endonuclease buffer (Promega), and 100 µl/ml BSA, in a total volume of 20 µl (made up with distilled water). A total of 12 U of the methylation sensitive enzymes *Bst*ZI or *Eag*I were added to one of the tubes, while the other contained no enzyme. The tubes were then incubated at 55°C (*Bst*ZI) or 37°C (*Eag*I) for 16 hours as recommended by the manufacturers. A PCR was then set up for each of the two tubes, which contained 2 µl of the digestion reaction, 20 pmol each of the primers FXG (5'AGTGCGACCTGTCACCGCCCTTC3') and FXH (5'GAAACCA-CGTACGTGATCAACGCTGTTCC3'),¹³ *Taq* DNA polymerase buffer (50.25 mmol/l Tris-HCl, pH 8.8, 12.45 mmol/l (NH₄)₂SO₄, 1.5 mmol/l MgCl₂ and 127.5 µg/ml BSA), 200 µmol/l each of dATP, dCTP, dGTP, and dTTP, and 10% dimethylsulphoxide, in a final volume of 25 µl. PCR conditions were the same as for the FRAXA/FRAXE PCR except that

the annealing temperature was 65°C. PCR products were analysed on a 2% Nusieve agarose minigel.

FRAXA AND FRAXE ANALYSIS BY SOUTHERN BLOTTING

Analysis of the FRAXA expansion mutation was carried out by double digestion of genomic DNA with both *Eco*RI and *Eag*I, blotting, and hybridisation with probe StB12.3.⁵ The FRAXE mutation was analysed by double digestion with *Hind*III and *Not*I, and probed with OXE2.0.¹⁰

Results and discussion

PCR TEST FOR FRAXA AND FRAXE IN MALES

The main purpose of this study was to develop a simple PCR test to screen for both FRAXA and FRAXE mutations in large numbers of routine referrals for diagnosis of the fragile X syndrome. The test is based on the simultaneous detection of FRAXA and FRAXE alleles with a triplet repeat size which is within the normal range. In order to do this, primers which would generate a PCR product 74 to 224 bp in length (corresponding to a normal range of two to 54 CGG repeats¹³) were used to amplify the FRAXA repeat. This product does not overlap in size with the FRAXE product, which has a minimum size of 306 bp for an allele with six repeats. The only exception to this would be the presence of a deletion of DNA between the position of the FRAXE primer pairs. We have not detected a deletion at this position in 689 chromosomes which we have analysed by sizing FRAXE PCR products on fluorescent DNA sequencing gels (Wang *et al*, manuscript in preparation).

The presence of a high G + C content in the target DNA template presents difficulties for in vitro DNA amplification across CG rich triplet repeats. In our experiments the G + C content in the FRAXA fragment was between 81% (two CGG repeats) and 93% (52 CGG repeats). The incorporation of C⁷dGTP to destabilise secondary DNA structure allows successful amplification of CG rich segments,¹⁸ which led to the use of a 3:1 ratio of C⁷dGTP:dGTP in the original FRAXA PCR test.¹³ However, since PCR products with a high content of C⁷dGTP do not stain efficiently with ethidium bromide,¹⁹ current methods for detecting the amplified fragment involve radioisotope labelling¹³ or blotting¹¹ and detection with biotinylated or chemically modified nucleotides.¹⁴ Several different ratios of C⁷dGTP:dGTP were tested, and a ratio of 1:3 was found to allow amplification of FRAXE and FRAXA alleles within the normal range, and efficient staining of the PCR products with ethidium bromide. The largest FRAXA allele amplified with this protocol contained 53 repeats, whereas alleles of 62 repeats (as measured by a radioactive PCR assay and sized on a sequencing gel¹²) or more failed to amplify. The largest FRAXE allele amplified with this protocol from control DNA samples was 48 repeats. Thus males who do not have full mut-

Figure 1 (A) Amplification products from the FRAAXE (*fxe*) and FRAXA (*fxa*) loci analysed on a 2% agarose minigel. Lanes 1–5 are normal males, 6–9 normal females, 10 is a male with a FRAXA expansion mutation, and 11 a male with a FRAAXE mutation. Lane 12 is a negative control for PCR contamination. (B) Resolution of one repeat unit differences at the FRAXA locus on a 20 cm 10% polyacrylamide gel using primers directly adjacent to the CGG repeat. The samples in lanes 1 and 4 are from females who are homozygous on the sequencing gel assay; those in lanes 2, 5, and 6 differ by one repeat unit; and the sample in lane 3 has alleles which differ by several repeats.

ations at either of these loci can readily be identified by electrophoresis of the PCR products on a minigel (fig 1A, lanes 1–5). Male samples in which a band is not present either in the FRAXA or the FRAAXE position (fig 1A, lanes 10–11) are likely to have a premutation or a full mutation, neither of which will be amplified using this protocol. Such samples should be analysed further by Southern blotting. All samples will produce a product from either the FRAXA or the FRAAXE loci, thus controlling for failure of the PCR.

The reliability of the PCR assay was examined in a blind test with 125 coded samples, which included 43 males with FRAXA mutations, eight males with FRAAXE mutations, and 74 normal males. All samples were correctly identified, as compared to results obtained by Southern blotting with the appropriate probes (see Materials and methods).

DETECTION OF HETEROZYGOSITY FOR NORMAL ALLELES IN FEMALES

Female samples in which two FRAXA alleles of normal size are detected (fig 1A, lanes 6–8) can also be scored as normal for FRAAXE, since an allele with a premutation or a full mutation will not be amplified (with the exception of a deletion at the FRAAXE locus, see below). The heterozygosity rate for females is only about

20% with the resolving power of the minigel which was used to screen male samples. However, the shorter FRAXA PCR product generated in our assay had the additional advantage that alleles which differed by only one repeat unit could be resolved on a 20 cm non-denaturing 10% polyacrylamide gel. Lanes 1 and 4 in fig 1B are products from females previously shown to be homozygous for a normal allele by PCR analysis on a denaturing gel and Southern blotting, and samples in lanes 2, 5, and 6 had alleles which differed by one repeat unit. Also, the residue of the PCR products from the amplification for the minigel can be used for the larger gel, so the PCR does not have to be repeated. We have tested 178 normal females and found 66% to be heterozygous. This is comparable with other reported rates of 63%¹³ and 80%.¹⁴ The size of the FRAAXE PCR product using the published primers⁹ is too large to resolve alleles which differ by less than three to four repeats.

About 80% of routine referrals to our Genetics Centre for fragile X testing are from male patients, and 3% of all samples are positive.¹² An average of 77 to 78 males out of 100 samples will therefore be scored as normal for both FRAXA and FRAAXE on the minigel, and three to four females will be heterozygous for normal FRAXA alleles. A further nine to ten females will be shown to be heterozygous for FRAXA on the 20 cm gel. Thus about 90% of routine referrals can be eliminated from testing by Southern blotting with the PCR assay, with the limitation that female carriers of FRAAXE mutations will not be identified. A false negative diagnosis would be obtained in an affected male who was a mosaic for a normal allele and a full mutation. However, no such cases have been reported to date.²⁰ The usual precautions for the prevention of contamination of the PCR with normal FRAXA or FRAAXE product must, of course, be observed.

PCR ASSAY FOR FRAXA METHYLATION AT THE FMR-1 GENE

The CpG island at the 5' end of the FMR-1 gene is known to be methylated if the gene has an expansion of more than about 200 CGG repeats.¹⁹ It is also methylated on the inactive X chromosome in females, but is unmethylated on the active X and in males. We devised a PCR assay to test for methylation of the FMR-1 CpG island in males (see Materials and methods). The purpose of the assay was to establish whether it would be possible to confirm the diagnosis of a FRAXA expansion mutation in affected males in whom no CGG allele had been detected on the initial PCR, and thus eliminate the need for Southern blotting in laboratories not equipped for radioactive analysis.

When DNA from FRAXA positive males was digested with the methylation sensitive enzymes *EagI* or *BstZI*, and an aliquot of the digest subjected to PCR across the CpG island, an amplification product of the expected size was seen, which was of similar intensity to that from the tube incubated without enzyme (fig

Figure 2 PCR assay for methylation at the FMR-1 gene. Lanes 1-4 are from normal males, and lanes 5-6 from FRAXA affected males. + = digestion with the methylation sensitive restriction enzyme EagI; - = not digested. A minority of sites are resistant to digestion in some normal males (lanes 2 and 3).

2, lanes 5-6). This was expected, since the alleles would be fully methylated and thus not digested by the enzyme. We did not expect to obtain a product in normal males, since the site would be unmethylated and hence fully cleaved by the enzyme (fig 2, lanes 1 and 4). However, a weak signal was observed in the digested track from some normal males (lanes 2 and 3), at a level of about 5 to 10% of that observed in the undigested track. We attempted to exclude partial digestion as the cause of this by re-extraction of samples with phenol-chloroform, and by digestion with a large excess of either *Bst*ZI or *Eag*I, but the weak signal in some samples was retained. *Bsh*III, which has two methylated sites within the amplified region, provides better discrimination. We conclude that either some molecules are resistant to digestion because of secondary structure of the DNA in this region, or that a small proportion of FMR-1 genes with normal CGG alleles are methylated. The fact that methylated normal alleles have not been observed by Southern blotting could be explained by the lower sensitivity of the blotting technique.

The methylation assay was tested for its ability to detect affected FRAXA males in a blind test of 40 coded samples which included 20 normal and 20 affected males. All samples were correctly identified, as compared with results obtained by Southern blotting. The fact that a low level of amplification was seen in digested DNA from some normal males suggests that particular care should be exercised in the interpretation of the results of this assay. We have observed this in a total of 13 out of 52 (25%) normal males tested with the assay, and although all were clearly distinguishable from the equivalent signals observed in digested and undigested DNA from affected males, we suggest that extensive blind testing should be carried out in individual laboratories before using the assay for diagnostic purposes.

In conclusion, the non-radioactive PCR assay that we have described appears to be a reliable, cheap, and efficient test for initial screening of samples for the presence of

FRAXA and FRAXE mutations. This should be particularly useful for laboratories which receive a large number of referrals, of which the great majority do not have a fragile X mutation. The fact that the test is non-radioactive and simple to perform will bring fragile X screening within the range of the many laboratories outside Western Europe and North America which do not have access to or experience of radioisotope labelling techniques. Positive confirmation of the presence of an expanded allele in affected males by Southern blot analysis should, however, always be carried out if facilities for radioactive analysis are available, and is essential for the diagnosis of female carriers.

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