Inheritance of the fragile X syndrome: size of the fragile X premutation is a major determinant of the transition to full mutation

Dominique Heitz, Didier Devys, Georges Imbert, Christine Kretz, Jean-Louis Mandel

Abstract
The fragile X mental retardation syndrome is caused by unstable expansion of a CGG repeat. Two main types of mutation have been categorised. Clinical expression is associated with the presence of the full mutation, while subjects who carry only a premutation do not have mental retardation. Premutations have a high risk of transition to full mutation when transmitted by a female. We have used direct detection of the mutations to characterise large families who illustrate the wide variation in penetrance which has been observed in different sibships (a feature often called the Sherman paradox). A family originally found to show tight genetic linkage between the factor 9 gene and the fragile X locus was reanalysed, confirming the original genotype assignments and the observed linkage. The size of premutations was measured by Southern blotting and by using a PCR based test in 102 carrier mothers and this was correlated with the type of mutation found in their offspring. The risk of transition to full mutation was found to be very low for premutations with a size increase (Δ) of about 100 bp, increasing up to 100% when the size of premutation was larger than about 200 bp, even after taking into account (at least partially) ascertainment bias. These results confirm and extend those reported by Fu et al (1991) and Yu et al (1992) and explain the Sherman paradox. The low risk of transition to full mutation of small premutations leads to the prediction that carriers of such alleles may be more frequent in the population than was previously expected for fragile X carriers, and we have indeed observed a premutation in a man with no a priori risk. Possible mechanisms that could account for the sex biased expansion of the CGG repeat are discussed in relation to the absence of such bias in expansion at the myotonic dystrophy locus.

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The fragile X syndrome is the most common inherited cause of mental retardation. Segregation studies have previously shown the unique characteristics of its mode of inheritance. It was estimated that 20% of males carrying the mutation have no phenotypic expression; these were called normal transmitting males. Among carrier females, 55% were found to express the fragile site, and one third to have mental impairment in addition. However, daughters of normal transmitting males appeared to have little or no phenotypic expression. Even more curious was the observation that penetrance was low (~18%) in brothers of NTMs, and high (>80%) in brothers of affected males, a phenomenon often called the Sherman paradox. This led to a clustering of NTMs in certain sibships and of affected patients in others, even within the same family. It has recently been shown that the fragile X mental retardation syndrome is caused by elongation of a small target DNA fragment, containing a repeat of the trinucleotide CGG3-4 located in a 5’ exon of the FMR-1 gene6 in Xq27.3. We have proposed that mutations can be classified as two main types, according to the size of the elongation (Δ), its stability in somatic cells, and the methylation status of nearby restriction sites. Premutations are characterised by an elongation of 70 to about 500 bp, show little or no somatic heterogeneity, and the nearby transmitting sites are not methylated in males or on the active X chromosomes of females (but are methylated on the inactive X chromosome). Premutations do not appear to cause mental retardation, and their carriers show very low or absent expression of the fragile site. They are typically found in normal transmitting males and in their daughters (although some daughters of a carrier female may also have a premutation). The full fragile X mutation is characterised by an elongation (Δ) of more than 600 to 700 bp, often shows important somatic heterogeneity, and nearby sites are methylated on both active and inactive X chromosomes.

In a study of 63 fragile X families, all males and 53% of females carrying a full mutation were found to have mental retardation. Although the distinction between premutation and full mutation appears clear at the cellular level, we have found that about 15% of subjects who have a full mutation are in fact mosaic and also have cells with a premutation. Such mosaic subjects may be more mildly affected than persons with a full mutation only. This is supported by the finding that FMR-1 mRNA is present in leucocytes of mosaic patients but not of patients with fully methylated mutations. The transition from premutation to full mutation (or to mosaic status) appears to occur.
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only after transmission through the female germline, which fits with the pattern of inheritance of phenotypic traits. In previous studies we found an 80% overall risk of transition. However, this was uncorrected for ascertainment biases. A more accurate study of this risk and of the parameters affecting it is of importance both for genetic counselling purposes and as a test of the main hypotheses proposed to account for fragile X inheritance, and in particular for the Sherman paradox. Laird proposed that the fragile X mutation locally blocks the reactivation, before oogenesis, of a previously inactive X, and the failure to erase the imprint specific to an inactive X chromosome (most likely DNA methylation) would account for the transition from a mutation with no phenotypic consequence (such as that found in normal transmitting males or in their daughters) to a mutation causing disease.

Laird's hypothesis predicted that the observed probability of such a transition should be only 50% and it was proposed that the higher value, as well as the Sherman paradox, could be accounted for by ascertainment biases, and by a small number of oogonial precursors at the time of X inactivation. The finding of abnormally methylated linked and the the mutation causing disease appeared to support Laird's hypothesis. When the true nature of the mutation was shown, we suggested as an alternative possibility that the sequence of the premutation could dictate the risk of transition to the full mutation. Results were first presented by Sutherland et al. at the 5th X Linked Mental Retardation Workshop (August 1991) suggesting a strong positive correlation between the size of the premutation in the female and the risk of a full mutation in the offspring. Similar results were reported by Fu et al while this work was in preparation. We present here the molecular findings in families which illustrate well the Sherman paradox. Our results confirm and extend those of Fu et al and Yu et al, as they are based on a larger population and have been, at least in part, corrected for ascertainment biases. The finding that small premutations have a low risk of transition to full mutation suggests that carriers of premutations may be more frequent in the population than previously expected, and we have indeed detected one in a person with no a priori risk.

Material and methods

The families were those analysed previously with some new families included. Mutations were analysed in EcoRI + EagI digests or, for more accurate sizing, in PstI digests separated on 1.2% agarose gels and hybridised to probe StB12XX. The Δ values are given by reference to a base level corresponding to the most common normal allele (29 repeats).

PCR across the CGG repeats was performed in a total volume of 50 μl with about 100 ng of DNA, in the presence of 10 mmol/l Tris-HCl pH 8.3, 50 mmol/l KCl, 20 mmol/l MgCl₂, 100 μmol/l dATP, 100 μmol/l dCTP, 100 μmol/l dTTP, 100 μmol/l 7-deaza-dGTP, and 2.5 U of Ampli-Taq DNA polymerase.

Primers PF199: 5'ATCTTCTCTTCAGCC-CTGCTAGCGG' and PE138: 5'GATGGAATTCGAGCTTTGATTTCACCCAGC-CACG' were added at a final concentration of 0.5 μmol/l. These primers allow amplification of a 600 bp fragment for an abnormal allele with 29 CGG repeats. The reactions were heated at 96°C for five minutes, followed by 35 cycles of 0.5 minutes denaturation at 96°C, one minute annealing at 60°C, four minutes of elongation at 72°C with a second extension at each cycle, and a final elongation of 10 minutes at 72°C. Addition of 2% deionised formamide usually helped the amplification of DNA that was refractory to PCR across this region. A 5 μl aliquot of the PCR reaction was loaded on a 2% agarose gel in buffer containing 5 to 10 ng of the 0.9 kb EagI-PstI fragment corresponding to the StB12.5 probe, and which serves as internal marker for accurate size determination. Another size marker (MspI digest of pBR322) was added separately to each gel. After migration, the gel was treated for 20 minutes in 1.5 mol/l NaCl, 0.5 mol/l NaOH, neutralised in 1.5 mol/l NaCl, 0.5 mol/l Tris-HCl pH 7.2, 1 mmol/l EDTA, and blotted onto Hybond N+ for one or two hours. The membrane was hybridised for six hours or more at 42°C in the presence of 50% formamide to probe StB12.5 (labelled by random priming), showing the PCR products and the 0.9 kb reference band. Small premutations were also sized on sequencing gels after PCR in the presence of 3′2P-dCTP, as described by Fu et al.

Results

We have systematically analysed fragile X families for the type of mutation, using the strategy described by Rousseau et al. In several large families, the Sherman paradox was well illustrated by the clustering of premutations in some sibships and the clustering of full mutations in others. In family A a woman with a rather small premutation (Δ = 120 bp), the sister of an affected male (with a full mutation), had seven normal children who inherited the premutation (with a range of Δ = 130 to 550 bp), and none with a characteristic full mutation (figs 1 and 2). It can be seen that II-7 (lane 5) was one of the rare cases where the diagnosis of premutation was based solely on mutation size (Δ = 550) and not on the presence of an unmethylated premutation, which is in general seen in EcoRI + EagI digests. In this woman, the pattern of X inactivation was extremely biased with very little of the 5.2 kb band indicative of the normal inactive X. This could well account for the failure to see the small expected amount of mutated fragment on the active X chromosome (especially since unmethylated premutations in this range tend to give fuzzy bands owing to some degree of somatic instability). However, it cannot be eliminated that she had in fact a small full mutation, as a homogeneous pattern with preferential inactivation of the mutated X...
is not infrequent in adult carriers of a full mutation. This woman twice passed a full mutation to her offspring (and no premutation). Another example of such clustering was that of family B (fig 1) with four normal transmitting males known from pedigree or DNA analysis. A fifth brother had a Δ of ~60 bp (tested by the more accurate PCR test) (fig 3) and was later verified to correspond to 48 repeats at the limit between premutation and normal. He was tested for two microsatellite markers within 150 kb of the fragile X locus (DXS458 and FRAXAC2) and was found to be identical to his NTM brothers, suggesting that he had in fact inherited the premutated allele from his dead mother (unless she was homozygous for both polymorphic markers). Eight daughters in generation II all had a premutation (in the Δ = 150 to 250 bp range); in the next generation, five children had inherited a full mutation (in two cases with the smallest fragment partially unmethylated) and none had a premutation. One child (III-5) with mild mental retardation was normal at the fragile X locus. The clustering was not always absolute, however, as shown by family C (fig 4), where two sibships showed a mixture of...
Figure 3 PCR based assay for detection and sizing of premutations. The assay was performed as described in Material and methods. The 0-9 kb band corresponds to the internal control fragment loaded with the PCR product. The 0-6 kb fragment corresponds to the amplified product for a common allele with ~30 CGG repeats. Lanes 1 to 3 correspond to a branch of a fragile X family who had no a priori risk of having mutation. The abnormal band has a Δ of 80 to 90 bp (54 and 58 repeats respectively as measured on sequencing gels). Lanes 4 to 7 correspond to part of a family also shown in fig 7B. Lanes 9, 17, 19, and 23 correspond to subjects in family B (fig 1). In lane 17, the male I 1 had a ~60 bp Δ (48 repeats measured on sequencing gels) and may correspond to a high normal allele or a small premutation (see text). Subjects in lanes 1, 5, and 20 have only normal alleles.

Family C

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<th>3</th>
<th>4</th>
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<th>6</th>
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<td>+</td>
<td>Nor</td>
<td>Pre 0-16</td>
<td>Nor</td>
</tr>
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<td>Nor</td>
<td>Full 2-5</td>
<td>Mos 0-25-3</td>
<td>Nor</td>
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<td>2</td>
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<td>DX: Δ(kb):</td>
<td>Nor</td>
<td>Full 2-3</td>
<td>Nor</td>
<td>2-3</td>
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</tr>
</tbody>
</table>

Figure 4 A pedigree showing partial clustering of premutations. Same symbols as in fig 1. A fragile X site had been observed in 1% of cells in II-10, who was later found to be normal.

subjects with a premutation or a full mutation (or a mosaic pattern). It can be noted in this family that the woman who had three children with a premutation and one with a full mutation had a small (Δ = 110 bp) premutation. II-10, who was originally found to have 1% of fragile X site, had inherited a normal haplotype for flanking RFLPs, and was later confirmed to have no fragile X mutation. Finally the family who had originally shown close linkage between an RFLP at the coagulation factor 9 locus and the fragile X locus was also reanalysed (fig 5). All the seven daughters of a normal transmitting male (Δ = 230 bp) had inherited premutations in the Δ = 230 to 310 bp range (fig 5B), while in the next generation direct DNA analysis confirmed the former genotype assignments, that is, all the phenotypically normal children had no mutation and all the 10 affected children, who had inherited the grandpaternal F9 allele, had a full mutation or were mosaics. No DNA was available for direct mutation analysis for five chorionic villi samples, but their status was deduced either from cytogenetic results (III-18 and III-22) or from the F9-DXS52 (St14) haplotype as they did not show recombination between these markers that flank the fragile X locus. It is of interest to note that in this family there are no recombinants between F9 and Frax in 21 meiotic events (or 18 if one takes only those who have a status established by direct DNA analysis or by cytogenetics). At the time of the original description there were only 15 non-recombinants. This confirms the striking tight linkage, contrasting with the ~20% recombination found between the two loci in other families, the probability of observing this by chance is 0.8%, about 1 in 100. In fact this family was one of the three which accounted for most of the heterogeneity of recombination reported by Brown et al.

A PCR BASED TEST FOR THE ANALYSIS OF PREMUTATIONS

In order to characterise quickly and accurately the size of premutations, we have developed a PCR based test. Primers were chosen that should amplify a 600 bp normal fragment (for subjects with the most frequent allele containing 29 repeats). The extreme GC richness of the region (100% for the CGG repeat) prompted us to use 7-deaza-dGTP instead of dGTP for the PCR reaction. However, under such conditions, it is not possible to detect the DNA synthesised by fluorescence in the presence of ethidium bromide. The reaction products were thus analysed on agarose gel, blotted, and hybridised to a 900 bp probe (StB12.5) that includes the amplified segment and contains 20 CGG repeats. An aliquot of
the StB12.5 insert was included in the loading buffer to provide an internal control band. The blotting, hybridisation, and exposure steps could be considerably shortened compared to standard genomic blotting and results were obtained in less than two days from the start of the PCR reaction (see Material and methods). While this work was completed, two other PCR based tests were reported. A direct PCR test developed by Fu et al. allows even more accurate sizing of premutations on sequencing gels. A hybrid method similar to ours, but using the CGG repeat itself as probe, was reported by Pergolizzi et al. which also allows detection, in most cases, of full mutations.

Our procedure allows accurate sizing with an estimated precision of 20 bp, that is, seven CGG repeats (fig 3). This was especially applied to the study of small premutations or ambiguous cases. We could thus confirm that an abnormal fragment first observed in EcoRI + EagI digests in a man with no a priori risk and in his daughter had a size typical of a small premutation (Δ = 80 and Δ = 90 bp, fig 3, lanes 2 and 3 corresponding to 54 and 58 repeats when measured as described by Fu et al.). It is also obvious with this assay that large premutations (Δ > 200 bp) are already somatically unstable with a span of ~50 bp (fig 3, lanes 10–12, 19, 23). This can also be detected as fuzzy bands in Southern blots using PstI or EcoRI + EagI digests.

RELATIONSHIP BETWEEN SIZE OF PREMUTATION IN THE MOTHER AND RISK OF TRANSITION TO FULL MUTATION

A correlation between the size of amplification in a mother and the risk of inheritance of a large and somatically unstable mutation in the offspring has recently been reported by Yu et al. and Fu et al. while our large data set was being analysed. We measured size of premutations in mothers using the EcoRI + EagI digest described by Rousseau et al. (fig 2) and in most cases also in PstI digests (fig 5B). The majority of these mothers were also analysed in a PCR assay, which is more accurate for measuring small premutations as the normal fragment is only 600 bp (compared to 28 kb in the EcoRI + EagI digest, and 1 kb in the PstI digest (fig 3)). In fact, except for the smallest premutations, we found that measurement in the EcoRI + EagI digest gave values in very good agreement with those obtained with the other assays (as noted previously, a Δ value of 200 bp corresponds to a differential migration of ~5 mm in an EcoRI + EagI digest and
Offspring of mothers with premutations. Mothers have been grouped into classes according to the size of their premutation. The number of children with a premutation, full mutation, or who inherited the normal allele (as controlled by direct DNA analysis) is indicated. Untested children are classified as normal or mentally retarded.

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<table>
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<th>Δ bp in mothers</th>
<th>Tested offspring</th>
<th>Untested offspring</th>
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<tr>
<td></td>
<td>PM</td>
<td>FM</td>
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<td>125-175</td>
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<tr>
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<tr>
<td>Total</td>
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<td>145</td>
</tr>
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</table>

The size of the full mutation was estimated by direct DNA digestion. The number of premutations found in 30 cases, classified as normal or mentally retarded, were 0-3-5-9 mm in a Pst digest). On the basis of both size and methylation analysis, we divided offspring into two classes: those with premutation and those with full mutation (table). All 30 children with premutations had mothers with a premutation of Δ < 225, and for 18 of these, the mother's Δ was < 125 (in only two of these 30 cases did the size of the premutation decrease). In contrast, in mothers with a Δ > 225 bp, the transition to full mutation (or mosaic) occurred in all 62 children analysed. However, it was evident that our data suffered from ascertainment bias in favour of children with a full mutation, as indicated by the fact that in the sibs analysed 175 children had inherited a mutated chromosome and only 99 inherited the normal one (ratio 1:77 to 1). To try to correct for this bias we subtracted from each family a single affected proband (assuming single ascertainment). Under these conditions, the mutated to normal ratio decreased to 1:32 and the ratio of premutated to full mutated was 0:3. This apparently incomplete correction reflects in part the fact that 46 sibs and 208 probands were studied (of whom only 11 were mentally retarded). Assuming that the proportion of premutations in the untested normal sibs is the same as in the tested ones, the mutated to normal ratio would be 1:19. The difference from the expected ratio of 1 is most probably the result of multiple ascertainment for some families. The effect of size of the premutation on the type of mutation inherited by the offspring (after this still partial correction for ascertainment bias) is presented in fig 6. It shows that for Δs of 75 to 125 bp, about 90% of children inherit a premutation. Rare examples of small premutations leading to full mutation in the offspring are shown in fig 7. The smallest of these (lane 1, left panel) has ~60 repeats as measured on a sequencing gel after PCR. An about even risk was found for Δs of 125 to 175 bp while, as stated above, premutations larger than 225 bp always led to a full mutation. It should be noted that very few premutations were found above 325 bp, which is probably because, past 175 bp, a premutation is more likely to pass to a full mutation than to have a moderate increase in size within the range of premutation.

Discussion

Our results confirm that the size of the premutation is a major (and perhaps the only) determinant of the risk of transition from premutation to full mutation. We have analysed 102 sibships from fragile X families whose mothers had a premutation, a sample size larger than that of Fu et al 8 (30 sibships) or Yu et al 9 (60 sibships). We have tried to correct for ascertainment bias, which favours affected children and which was not taken into account in the previous reports. Although our correction appears still incomplete, it should result in a more accurate estimation of the risk of transition from premutation to full mutation. This risk would be about 10% for premutations

Figure 6 Size of premutation in bp (number of mothers in each size class)

Figure 7 Small premutations that had directly or indirectly led to a full mutation as visualised in an EcoRI + EagI digest. In the family on the left, the mother (lane 1) had a premutation (measured in PstI digests) with a Δ of 90 bp (60 repeats were measured in the direct PCR assay of Fu et al 8) while the daughters in lanes 2 and 3 had larger premutations (Δ = 200 to 300 bp; in lane 3, the abnormal allele is mostly on the inactive X), and the third daughter had a heterogeneous full mutation. In the right panel, lanes 7, 11, 8 and 6 correspond to lanes 4, 5, 6 and 7 respectively in fig 5. The premutation appears smaller in lane 6 because most of it is on the inactive X (slightly above the 5-2 kb normal band), which does not allow accurate sizing, and because this woman inherited from her father a high normal allele (see fig 5, lane 7). Premutations have a Δ of 120, 115, 125, and 200 bp in lanes 6, 7, 8, and 10 respectively.
with amplification (Δs) of 75 to 125 bp (corresponding to 55 to 70 repeats), about 50% between 125 and 175 bp (70 to 90 repeats), and >98% above 225 bp (105 repeats) which fits remarkably well with the data of Fu et al.15 Although this has important consequences in genetic counselling, since the risk of having affected children can vary considerably depending on premutation size, for a situation with a low but measurable risk it will probably be necessary to consider prenatal DNA diagnosis. It remains to be seen, however, whether different patterns of interspersed AGG units exist within the CGG repeat, which might influence the risk of transition to full mutation. It has been shown for (CA)n microsatellites that imperfect tandem repeats are less polymorphic (that is, more stable) than perfect ones.25

As already discussed by Fu et al.15 these findings account very well for the Sherman paradox and the clustering within sibships of either normal transmitting males (or unaffected carriers) or of males or females with phenotypic expression. After correction for ascertainment bias we found a 77% overall risk for transition to full mutation, which fits well with the 80% penetrance value found by Sherman et al.8 in fragile X families. The fact that about 15% of carriers with premutations (or about 10% of all carriers) in fragile X families have a small amplification associated with a low risk of having affected children strongly suggests that their frequency in the general population might be as high as that of carriers with a Δ of 150 to 300, who make up the majority of premutation carriers in fragile X families. Assuming that one male in 1500 has clinical expression of the fragile X syndrome, the frequency of carrier females would be about 1 in 600, assuming an 80% average risk of transition to full mutation, but that figure may be considerably increased, to perhaps 1 in 400 or more, if one takes into account women with a small risk who do not belong (yet) to fragile X families. This is supported by the observations of abnormal fragments of premutation size in spouses26 (fig 3, lanes 1, 2, 3) and in one CEPH family, and raises the possibility of systematic screening for such mutations in women of child bearing age in the general population.

The 100% risk of transition of premutations with >100 repeats to a disease causing full mutation during female transmission is very difficult to reconcile with Laird's hypothesis of a correlation with the X inactivation status, as the latter would predict a maximum risk of 50%.90 Laird's hypothesis would still be tenable, however, if in oogonial precursors a selection was occurring which would result in preferential survival of cells with the premutation on the inactive X. We have observed such a selection (albeit with an extended course of time) in leucocytes of female carriers of a full mutation, but not in carriers of a premutation.17 However, it is formally possible that oogonal precursors would be critically dependent on FMR-1 function and that expansion of about 100 repeats could impair function of the FMR-1 gene, either through modification of the protein (if the repeat is translated) or by decreasing stability of the mRNA.

This obligatory and sex biased expansion, with catastrophic consequences, appears to show a major difference from myotonic dystrophy, where expansions of similar size can occur in both sexes.27-29 As the full mutation in fragile X is correlated with abnormal methylation of nearby sites, and the CGG repeat itself is able to be methylated (although it has not been experimentally shown that it is indeed methylated in a full mutation), while the CTG repeat is unable to be methylated, it is tempting to propose that methylation could be the basis for the difference between the two diseases, if it were a cause rather than a consequence of the large expansion. We have proposed earlier that methylation could modify the secondary structure of the CGG repeat, interfere with replication processes, and induce a greater instability. A second unsolved question is whether large expansion really occurs during female meiosis or very early in embryogenesis. In the latter case, one would have to suppose a sex imprint (not necessarily linked to female X inactivation) in the FMR-1 region which would distinguish between a premutation on a paternally or maternally derived X. This question is raised by the puzzling case of 'mosaics' who have cells with either a full or premutation.378 In the first alternative (meiotic expansion) some postconception processes (sister chromatid exchange for instance) could lead both to shortening of the repeat and loss of methylation. However, this is in apparent contradiction to the very rare occurrence of extensive shortening in offspring from females with a full mutation' (and in the rare event recorded, one cannot conclude that the mother was a 'mosaic' and the offspring with a premutation had inherited an X chromosome that originally carried a premutation). Thus it seems that the second alternative (expansion being mostly a postconceptional event) is at least as attractive, especially as heterogeneous methylation (by somatic mutation) is already present early in fetal life and further mutation may be much reduced postnataally.30 We suggest as one possibility that abnormal methylation could occur very early in embryogenesis on the maternally derived chromosome carrying a premutation, with a frequency which depends on the size of the premutation (that is, of the methylable target). This would lead to expansion to full mutation and stabilisation of the methylation. That abnormal methylation (of the full mutation) occurs very early is suggested by the finding that in chorionic villi the full mutation is in general (but not always) methylated, whereas the normal inactive X is in general unmethylated at the FMR-1 CpG island.30 Undermethylation of CpG islands on the inactive X in chorionic villi is well known, and is attributed to the later timing of the methylation process with respect to the earlier differentiation of trophoblasts.31,32 Methylation patterns may, however, be obscured by existence in early embryogenesis of both methylation...
and demethylation activity.\textsuperscript{33,34} It is obvious that these questions will be difficult to analyse directly in humans, and solutions may come from comparison with molecular patterns observed at other X linked or autosomal fragile sites, or at loci such as that for myotonic dystrophy, and by trying to find or establish animal (murine) models which mimic the various features of unstable expansion of such repeats.

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