

# Selection in blood cells from female carriers of the fragile X syndrome: inverse correlation between age and proportion of active X chromosomes carrying the full mutation

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## Abstract

**We have studied the patterns of mutation and X inactivation in female carriers of a fragile X mutation, to try to correlate them with various phenotypic features. We used a simple assay, which shows simultaneously the size of the mutation, its methylation status, and DNA fragments that represent the normal active and inactive X chromosomes. We have observed an age dependent process, whereby the 'full' fragile X mutation is found preferentially on the inactive X in leucocytes in adult females, but not in younger ones. This phenomenon was not observed in female carriers of a 'premutation', who have little phenotypic expression. Preliminary data suggest that young females who show preferential presence of a full mutation on the active X in leucocytes may be at increased risk for mental retardation. We have also obtained preliminary evidence for an age dependent decrease in the somatic heterogeneity of full mutations, possibly owing to selection for smaller mutated fragments. If confirmed, the latter phenomenon might account for the known decrease with age of the expression of the fragile site. Our observations suggest that a gene whose expression is affected by the presence of a full mutation (possibly the FMR-1 gene) has a cell autonomous function in leucocytes, leading to**

**a slowly progressive selection for cells where the mutation is on the inactive X chromosome.**

Abnormal DNA methylation at a single CpG island has been found to be associated with phenotypic expression of the fragile X syndrome.<sup>1-3</sup> Cloning of sequences around this CpG island generated probes that detect both the fragile X mutations, as a size increase of a small target DNA fragment, and the abnormal methylation pattern.<sup>4,7</sup> We have distinguished two main types of mutation which can be found both in males and females.<sup>4,7</sup> When the size increase ( $\Delta$ ) is 100 bp to 500 bp, the CpG island is unmethylated on the active X chromosome and there is no significant mental impairment. This may be equated with the 'premutation' postulated by Pembrey *et al*,<sup>8</sup> and is notably found in normal transmitting males and in their non-expressing carrier daughters. Upon transmission by a female, the premutation has a high risk of changing to a 'full mutation' characterised by a much greater increase in size ( $\Delta > 600$  bp) and abnormal methylation. As presence of the full mutation generates somatic instability, it often appears in Southern blot analysis with a heterogeneous pattern of bands or even as a smear.<sup>4,5</sup> A third mutation pattern is encountered in a minority of subjects, whom we term 'mosaics'. They show the presence of both small unmethylated mutated fragments and large methylated ones.<sup>4,7</sup> Their phenotypic expression is quite similar to that of carriers of a full mutation.<sup>7</sup>

The fragile X syndrome is characterised by a high rate of phenotypic expression in heterozygous carriers. It has been estimated that about 30% of all female carriers, and 50% of the fragile X positive ones, show some mental impairment.<sup>9,10</sup> This disease is thus a major cause of mental retardation in females. Partial expression of the characteristic facial features was found in about 15% of mentally normal carriers.<sup>10</sup> It has been proposed that differences in the phenotype might reflect variations in the ratio of active and inactive X chromosomes carrying the

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Received for publication 13 September 1991.

Accepted for publication 16 September 1991.

mutation. Several studies used cytogenetic methods to estimate the proportion of lymphocytes which carry the fragile site on early or late replicating X chromosomes, and tried to establish a correlation with the mental status.<sup>11</sup> The results were somewhat inconsistent, probably owing to methodological difficulties inherent in the use of BrdU for inactivation studies,<sup>12</sup> as BrdU also inhibits expression of the fragile X. In the assay we recently developed for typing the fragile X mutations, DNA fragments corresponding to the normal active and normal inactive X can also be seen in females.<sup>7</sup> We have now taken advantage of this feature to study the X inactivation pattern in leucocytes of females carrying the 'premutation' or the 'full mutation', and to correlate it with phenotypic expression. We have observed an age dependent non-random X inactivation pattern and differences in the mutation pattern which can be best explained by a cell selection process.

#### Material and methods

DNA was extracted from leucocytes (or in seven cases from lymphoblastoid cell lines) of 72 carrier females: 19 were fragile X negative, 17 had more than 5% of fragile sites, and 24 have not been cytogenetically tested. We classified the women into three age classes (0 to 20 years, 21 to 40 years, and more than 40 years). When the exact year of birth was not known, we inferred the age class of a woman from her position within the pedigree. The mutation pattern was scored according to the size increase ( $\Delta$ ) observed and the methylation pattern<sup>7</sup>: S (or 'premutation') = 100 bp <  $\Delta$  < 600 bp and presence of unmethylated mutated fragments, and L (or 'full mutation') =  $\Delta$  > 600 bp and presence of methylated fragments. The results of the analysis of the six women with a mosaic pattern were pooled with those of the L category. After hybridisation of *EagI*+*EcoRI* digests with probe StB12.3,<sup>7</sup> densitometry was used to measure the respective intensity of the restriction fragments corresponding to the normal active X (NA) and to the normal inactive X (NI). When the signal of the NI fragment was too close to the mutated inactive one (MI) (in some carriers of a premutation), we considered the mutated active (MA) as the equivalent. (As  $NA + NI = MA + MI$ , and  $NA + MA = NI + MI$ , it follows that  $NA = MI$  and  $NI = MA$ ). The ratio  $NA/NA + NI$  gave us the proportion of cells with their mutated X inactive. We checked in 27 normal females that the intensity of the band at 2.8 kb (NA) was the same as the intensity of the band at 5.2 kb (NI), thus establishing the validity of the assay. Scanning was performed by video-image analysis of autoradiograms using the Elphor software (Biocom®). Statistical

analyses were performed using the StatViewII® software.

#### Results

We have recently devised a simple diagnostic test that distinguishes the different types of mutation as well as their methylation status at the CpG island.<sup>7</sup> As the CpG island is normally methylated on the inactive X chromosome, this also allows the visualisation of bands specific for the normal active and normal inactive X chromosomes in females. In brief, probe StB12.3 detects in a double *EagI*+*EcoRI* digest a 2.8 kb *EagI*-*EcoRI* fragment corresponding to the normal active X (NA) in males and females and an additional 5.2 kb *EcoRI*-*EcoRI* fragment corresponding to the normal inactive X (NI) in females, as the *EagI* site cannot be cut when methylated (figs 1 and 2). The mutated active (MA) and mutated inactive (MI) chromosomes appear as additional bands in carriers of the 'premutation', while in carriers of the 'full mutation', the MA and MI fragments cannot be distinguished, as both are methylated. In the latter case the full mutation can appear either as a single band (> 5.8 kb), a few discrete bands, or a mixture of bands and smear.

In carrier women there are two types of cells owing to random X inactivation: those with their mutation on the active X and those with it on the inactive X. The proportion of cells with a normal active X, that is, a mutated inactive X, was measured in blood cells (generally leucocytes) of 45 women carrying a mutation with a small  $\Delta$  (S or premutation) and in 27 women carrying a mutation with a large  $\Delta$  (L or full mutation, including six mosaic cases).

We found no significant deviation from a Gaussian distribution of the proportion of normal active X in females with a small  $\Delta$  ( $\chi^2$  test for goodness of fit  $p=0.76$ ), but the mean of this distribution was 40% (significantly different from the expected 50%:  $p=0.007$ , bilateral  $t$  test) (fig 3). Females with a small  $\Delta$  appear more often to be daughters of normal transmitting males (NTMs) than daughters of carrier females.<sup>7</sup> A similar mean value of the distribution of active X (39%) was found when only the 36 daughters of NTMs were considered. In daughters of carrier females, the expected 50% average proportion was observed, but the number of cases analysed (seven) was too small to ascertain whether this reflects a true difference with the daughters of NTMs. None of the 45 women with a small  $\Delta$  was mentally retarded, including the single woman with more than 5% fragile sites (16%).

In blood cells of female carriers with a large  $\Delta$  (pattern L), the mutation was carried significantly more often on the inactive X chromosomes when

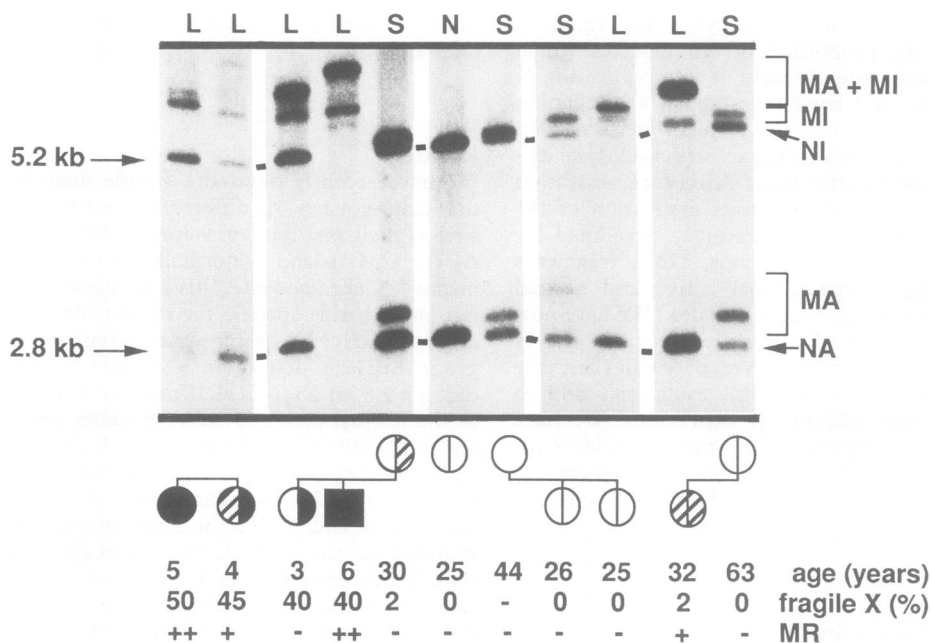


Figure 1 Examples of actual patterns detected in EcoRI + EagI double digests. Digestion, electrophoresis on 0.9% agarose gels, and hybridisation to the StB12.3 probe were as described previously. The sizes (in kb) of normal active (NA) and normal inactive (NI) X chromosomes are indicated on the left. At the top, mutation patterns are scored according to the size increase ( $\Delta$ ) of additional mutated active (MA) or inactive (MI) fragments: S = 100 bp <  $\Delta$  < 600 bp, L =  $\Delta$  > 600 bp, N = normal. At the bottom, age and percentage of fragile sites of the subjects whose DNA was analysed are given. Their mental retardation (MR) is also indicated as follows: - no mental retardation, + mild mental retardation, ++ mental retardation.

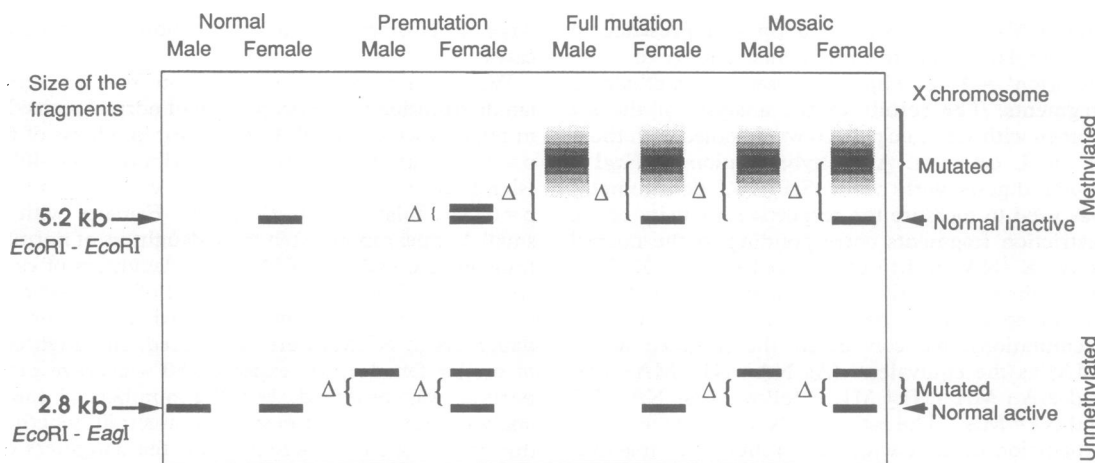


Figure 2 Schematic representation of the hybridisation patterns detected by probe StB12.3 in EcoRI + EagI double digests. The patterns presented correspond to those observed in a Southern blot assay for normal subjects, carriers of a 'premutation', carriers of a 'full mutation', and 'mosaics'. The normal 2.8 kb and 5.2 kb fragments detected by probe StB12.3 are indicated on the left (plain horizontal bands). Mutated fragments are detected as additional bands with a size increase indicated by  $\Delta$ . A heterogeneous pattern of mutated fragments may appear as a smear (gradual range of greys). Interpretation in terms of mutation and methylation is indicated on the right.

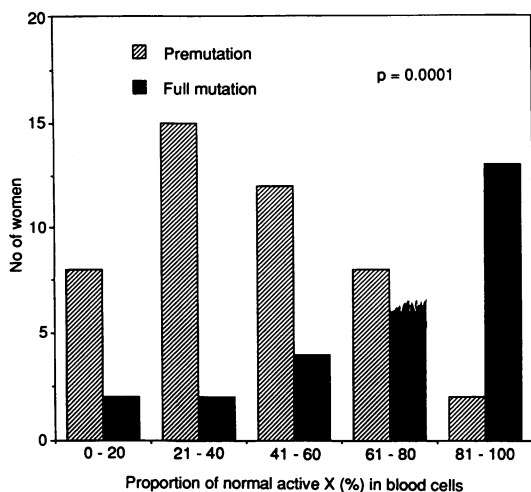


Figure 3 Proportion of normal active X chromosomes in blood cells of carrier females. The NA/NA+NI ratio was measured as described in Material and methods. The height of bars represents the number of women in each category. Hatched bars: women with a small  $\Delta$  (premutation). Black bars: women with a large  $\Delta$  and mosaics (full mutation). The distribution of normal active X chromosomes in the two classes of female carriers was compared with a  $\chi^2$  test.

compared to the group of females with a small  $\Delta$  (fig 3,  $p=0.0001$ ). In 13 of 27 of these females, the 5.2 kb band was very weak compared to the 2.8 kb band. The reverse pattern was observed in only two cases. Such a non-random inactivation pattern might best be explained by a selection process against cells carrying the full mutation on the active X chromosomes.

To ascertain whether this pattern is established early in life, or appears gradually in female carriers of a full mutation, we analysed it as a function of age. While females in the younger age group (0 to 20 years) had on average 51% of normal active X chromosomes, a value of 79% was found in women in the 21 to 40 years class (fig 4), and the difference between the distribution of the NA/NA+NA ratio in the two age classes is significant ( $p=0.004$ ).

We also searched for a correlation between mental status and the inactivation pattern (fig 5). The proportion of normal active X was on average higher in mentally normal carriers (83%) than in those with mild or moderate mental retardation (61%). This difference was significant ( $p=0.04$ ) although our sample of female carriers with a large  $\Delta$  value is relatively small. It is interesting to note that the four females with less than 40% normal active X chromosomes are all mentally impaired and young (the oldest is 25 years old). We can assume that their inactivation pattern is less influenced by the age dependent selection process. Analysis of a larger number of cases will be needed to confirm if in

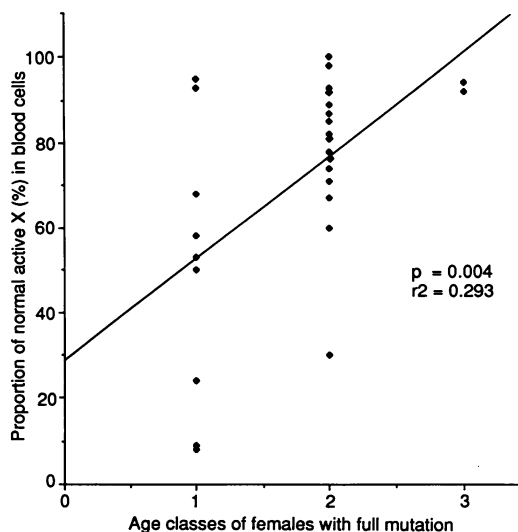


Figure 4 Correlation between age and proportion of normal active X chromosomes in female carriers with a large  $\Delta$ . Individual values of the NA/NA+NI ratio were plotted for each age class. Class 1: less than 20 years old, class 2: 21 to 40 years old, class 3: more than 40 years old (see Material and methods). The correlation coefficient ( $r^2$ ) is indicated, and significance of the influence of the age of carriers on the NA/NA+NI ratio in their blood cells was calculated by linear regression analysis ( $p=0.004$ ).

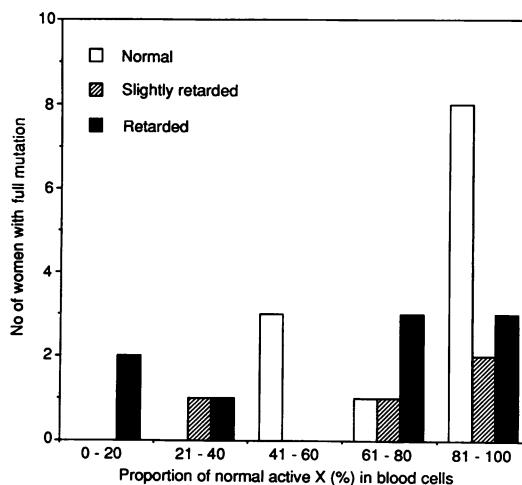


Figure 5 Correlation between mental status and proportion of normal active X chromosomes in female carriers with a large  $\Delta$ . Carrier women were divided into three classes according to the mental status reported to us. 'Slightly retarded' corresponds to borderline or mild impairment. For statistical evaluation, we merged the two retarded groups, and significance of the difference between normal and retarded was calculated by linear regression analysis, using the individual measured values of the NA:NA+NI ratio ( $p=0.04$ ).

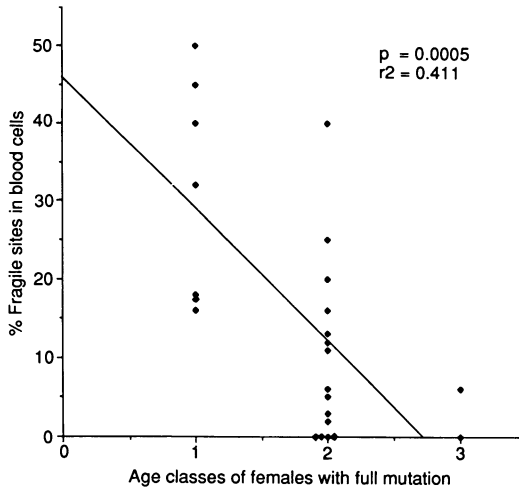


Figure 6 Decrease with age of the percentage of fragile site in female carriers with a large  $\Delta$ . Individual percentages of fragile X sites were plotted for each age class, and statistical significance of the influence of the age of carriers on their percentage of fragile X sites was evaluated by linear regression analysis.

young female carriers of a full mutation a high proportion of mutated active X is correlated with a higher likelihood of being mentally retarded. An inverse relationship between age and cytogenetic expression of the fragile X syndrome in female carriers has already been documented by several cytogenetic studies,<sup>13,14</sup> although it was proposed that this effect could be caused by ascertainment bias.<sup>14</sup> The populations previously studied included both manifesting carriers and obligate carriers likely to have only a premutation. The age dependent decrease of the percentage of fragile X sites was, however, confirmed in our more homogeneous set of carriers with a full mutation (fig 6). It has been suggested that when the mutation is on the inactive X chromosome it is less inducible to fra(X) expression,<sup>15</sup> but it seemed to us unlikely that this would fully explain the decrease in fra(X) expression.

We thus searched for another age dependent variable. We had observed rather anecdotally that the mutation was found as a smear most often in children. Further analysis in our set of carrier females with the full mutation showed that the mutation appears more heterogeneous in the younger age class than in the older one, although this did not reach statistical significance given the relatively small number of cases analysed (fig 7). It can be noted, for instance, that only one female under 20 showed a single large  $\Delta$  fragment (she is in fact a 'mosaic' with a large  $\Delta$  of 2.0 kb). These observations, if confirmed, would further support the existence of a selection process in blood cells, linked to the presence of a full fragile X mutation, and probably

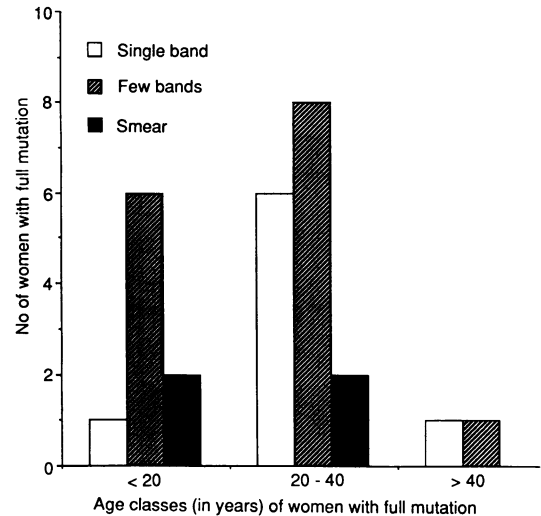


Figure 7 Relationship between the pattern of mutated fragments and age of female carriers with a large  $\Delta$ . The mutation pattern detected by probe StB12.3 in EcoRI + EagI digests was scored as corresponding to a single band (homogeneous pattern), a few discrete bands, or to a smear. The number of women in each age and mutation class is indicated by the height of the bars. For statistical evaluation, we merged the classes corresponding to a heterogeneous pattern (few bands or a smear), and used only two age classes (below and above 20). The trend towards a more homogeneous pattern in the older class did not reach significance ( $p = 0.30$  in a  $\chi^2$  test, with Yates's correction).

favouring the smaller mutations within the cell population.

## Discussion

We have observed two new age dependent features in females carrying a full fragile X mutation. The mutation is found preferentially on the inactive X chromosome in adult females, but not in younger ones. More preliminary data suggest that young females are more likely to show important somatic heterogeneity of the mutations than adults. We have also confirmed the decrease with age of fragile site expression in a population of females carrying a full mutation. Previous studies<sup>14</sup> on this latter effect could not discriminate between the two types of carrier, and might thus have been subject to ascertainment bias favouring, among older females, those carrying a premutation with a low percentage of fragile sites.

The non-random inactivation pattern strongly suggests that blood cells with a full fragile X mutation on the active X chromosome have some selective disadvantage. Such mutations might confer on the cells a slower replication or a higher rate of cell

death, resulting in a progressive enrichment of white blood cells with their mutated X inactive. This may indicate that the gene(s) whose expression is impaired by the mutation in leucocytes, such as the recently cloned FMR-1 gene,<sup>16</sup> may have a cell autonomous function. The selection process appears only slowly progressive, indicating that the impairment does not have a dramatic impact on the blood cell physiology. It should also be pointed out that affected males do not show obvious impairment of white blood cell function.

The decrease with age of the heterogeneity of mutation patterns could stem from a gradual selection for the smallest mutations in leucocytes. Inactivation of the FMR-1 gene probably results from methylation of the CpG island rather than from the actual  $\Delta$  value of the full mutation, as no mRNA was found in leucocytes of 16 patients who had fully methylated mutations.<sup>16</sup> Thus it seems unlikely that this gene (or any other gene under the control of the same CpG island) would be involved in the selection process towards a homogeneous pattern of mutation. We assume that the fragile site induction *in vivo* or in cell culture is easier as  $\Delta$  is larger, owing to a greater difficulty in local replication. It could thus be suggested that the decrease in fragile site expression with age results from an age dependent selection favouring the smaller mutations in the cell population. *In vivo* expression of the fragile site could cause a deleterious effect by long range perturbation of chromatin structure or even by leading to deletion of the distal Xq28 segment in some cells. Such deletions have been observed *in vitro* after fragile site induction in 2 to 4% of cells<sup>17</sup> and were proposed to play a role in phenotypic expression of the syndrome if they occur in brain cells.<sup>18</sup> It has also been reported that iduronate sulphatase activity may be decreased in fragile X patients, although the corresponding gene is localised more than 1500 kb distal to the fragile X locus.<sup>19</sup> While the fragile site is expressed on both the active and inactive X chromosomes, it may be less inducible when it is on the inactive X chromosome<sup>15</sup> and this may also contribute to the age dependent reduction in the level of the fragile site.

In daughters of NTMs, we found a slight but significant deviation from the expected random inactivation pattern, as the proportion of normal active X chromosomes was only 40%. It is difficult to envisage that this could be because of selection in favour of the premutation being on the active X chromosome. One possible explanation would be that an imprint on the paternal X chromosome results in a slight skewing of the inactivation process. Preferential inactivation of the paternal X chromosome occurs in extraembryonic membranes of mice and rats and in somatic tissues in marsupials.<sup>20</sup> On the other hand, inactivation is believed

to be completely random in humans. Quantitative analysis at other X chromosome loci should indicate whether our observation reflects a general phenomenon or some particular feature owing to the presence of a fragile X premutation.

One important issue in prenatal diagnosis for a female fetus would be the ability to predict whether or not the future child will be mentally retarded. We have previously shown that the overall risk is about 50% in carriers of a full mutation.<sup>7</sup> Our present study indicates that the establishment of a correlation between the X inactivation pattern of a full mutation and mental status in females may be blurred by the progressive selection process taking place in leucocytes. More extended studies should thus focus on very young girls. It is unlikely, however, that even if some correlation was found, it would be of sufficient predictive value to be of real use in genetic counselling. Indeed, the inactivation pattern in leucocytes may well be different from that in cells whose malfunction is responsible for mental retardation. Furthermore, the pattern of methylation characteristic of the inactive X or of fragile X mutations is not established in chorionic villi<sup>47</sup> (unpublished observations), which would preclude analysis of the inactivation pattern in early prenatal diagnosis.

We wish to thank Ms C Kretz, V Biancalana, and S Blumenfeld for technical assistance in the analysis of fragile X families, and our clinical colleagues for providing us with blood samples and information on these families. This work was supported by grants from the Ministère de la Recherche et de la Technologie, and INSERM/CNAMTS to JLM, and by a fellowship of the Association Française contre les Myopathies to FR.

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