Original articles

X inactivation of the FMR1 fragile X mental retardation gene

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Abstract
X chromosome inactivation has been hypothesised to play a role in the aetiology and clinical expression of the fragile X syndrome. The identification of the FMR1 gene involved in fragile X syndrome allows testing of the assumption that the fragile X locus is normally subject to X inactivation. We studied the expression of the FMR1 gene from inactive X chromosomes by reverse transcription of RNA followed by PCR (RT-PCR), both in somatic cell hybrids which retain an active or inactive human X chromosome and in a female patient with a large deletion surrounding the FMR1 gene. In both analyses, the data indicate that FMR1 is not normally expressed from the inactive X chromosome and is, therefore, subject to X chromosome inactivation. This finding is consistent with the results of previous studies of DNA methylation of FMR1 on active and inactive X chromosomes, verifies previous assumptions about the fragile X locus, and supports the involvement of X inactivation in the variable phenotype of females with full mutations of the FMR1 gene.

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Fragile X syndrome, characterised cytogenetically by a fragile site at Xq27.3, is the most common form of inherited mental retardation, with one in 1250 males and one in approximately 3000 females being affected.[1-3] The genetics of this syndrome have long been puzzling, as 20% of the males who carry the gene are normal while one-third of carrier females show some form of mental impairment.[4 5] In addition, the risk of mental retardation seems to depend on the position of a person in the pedigree; brothers and daughters of normal transmitting males are infrequently affected while their grandsons have an increased risk of being affected (the so called Sherman paradox).[4 5]

While many of the genetic features of this syndrome have been explained by the cloning of the fragile X mental retardation gene (FMR1) and by the study of sequence instability in this gene,[6-11] the mental impairment observed in some carrier females might be explained by X chromosome inactivation of the normal allele and expression of the mutant allele in a threshold proportion of cells.[12-15] Manifestations of the disease phenotype are directly correlated with the amplification of the CGG trinucleotide repeat within the FMR1 gene,[13 14] and affected females (like affected males) have an increase of their CGG repeat copy number above a critical length of approximately 230 repeats, which is associated with transcriptional suppression of FMR1 by DNA methylation. However, only about half of females with such full FMR1 expansions are penetrant and those who are penetrant are often less severely affected than males with a similar mutation.[15 16]

Laird[17] proposed that the fragile X syndrome is caused by abnormal chromosome imprinting in which the fragile X mutation interferes with the normal reactivation of an inactive X chromosome during oogenesis, resulting in an abnormally methylated and genetically inactive domain containing the fragile X syndrome gene on otherwise active X chromosomes in affected sons of carrier females. Laird's hypothesis was based on the assumption that the fragile X gene is subject to X chromosome inactivation. The inactivation of one of the two X chromosomes in females serves as a mechanism to achieve dosage compensation, and it has been generally assumed that most genes on the X chromosome are subject to inactivation.[18] Recent studies of the expression of genes from the inactive X chromosome have shown, however, that a growing number of genes in fact "escape" the process of inactivation.[18 19] Therefore, the question of whether X inactivation of the FMR1 gene plays a role in the fragile X syndrome requires direct examination.

In this study, we examined the expression of the FMR1 gene in somatic cell hybrids retaining one human X chromosome in its active or inactive state by RT-PCR analysis. This work is complemented by expression studies in a human cell line obtained from a female patient who shows non-random inactivation of her normal X chromosome and carries a 3–5 cM deletion of the fragile X region on her active X.[20-22] Thus, her two X chromosomes can be distinguished genetically, the inactive X with a normal copy of FMR1 and the active X

with the FMR1 gene deleted. In both assay systems, we show that the FMR1 gene is subject to X chromosome inactivation and is not expressed from the inactive X chromosome.

Materials and methods

CELL LINES

Human/mouse somatic cell hybrids retaining one human X chromosome in either its active or inactive state were used: t60-12 and AHA-11aB1 contain one active X chromosome each[23][24] and t11-4Aaz5, t48-1a-1Daz4A, and t75-2maz34-4a contain an inactive human X chromosome.[24] Fibroblast cell lines obtained from a patient with a deletion spanning the fragile X and IDS region[20-22] and her parents were provided by Dr J Clarke (Hospital for Sick Children, Toronto).

RNA ISOLATION AND PCR ANALYSIS

Total RNA was extracted from confluent cells in culture with RNAzolB according to the manufacturer's instructions (BiotecX). cDNA was obtained from total RNA by reverse transcription as previously described.[23] cDNA aliquots in a series of 10-fold dilutions were amplified in 100 μl reactions with Promega *Taq* polymerase according to the manufacturer's instructions. Thirty cycles of amplification were performed in an Ericomp Twin Block System. Denaturation was for one minute at 94°C, annealing for one minute at 55°C, and primer extension for four minutes at 72°C. A final elongation step of 20 minutes at 72°C was also performed. Aliquots of the reaction product (20 μl) were electrophoresed on a 1·5% agarose gel in TAE (0·4 mol/l Tris/Cl, 0·013 mol/l sodium acetate, 0·002 mol/l EDTA, pH = 8·0), stained with ethidium bromide, and photographed under ultraviolet light.

OLIGONUCLEOTIDE PRIMERS

Primers for the amplification of a 428 bp product of FMR1 were designed spanning two introns, based on comparing sequences of the cloned human and murine genes[25]: 5'-GGCGCTAGCAGGGCTGAA-3' and 5'-CCGTAAGTCTTCTGGCACA-3'. For control reactions, products of the IDS, MIC2, and PDHA1 genes were amplified as described above, using primers described previously.[23][26] The primer concentrations were 20 ng per reaction for the FMR1 amplification, 40 ng per reaction for MIC2 and IDS, and 60 ng per reaction for PDHA1. In the duplex reactions of FMRI and PDHA1, the primer concentration for PDHA1 was lowered to 5 ng reaction.

Results

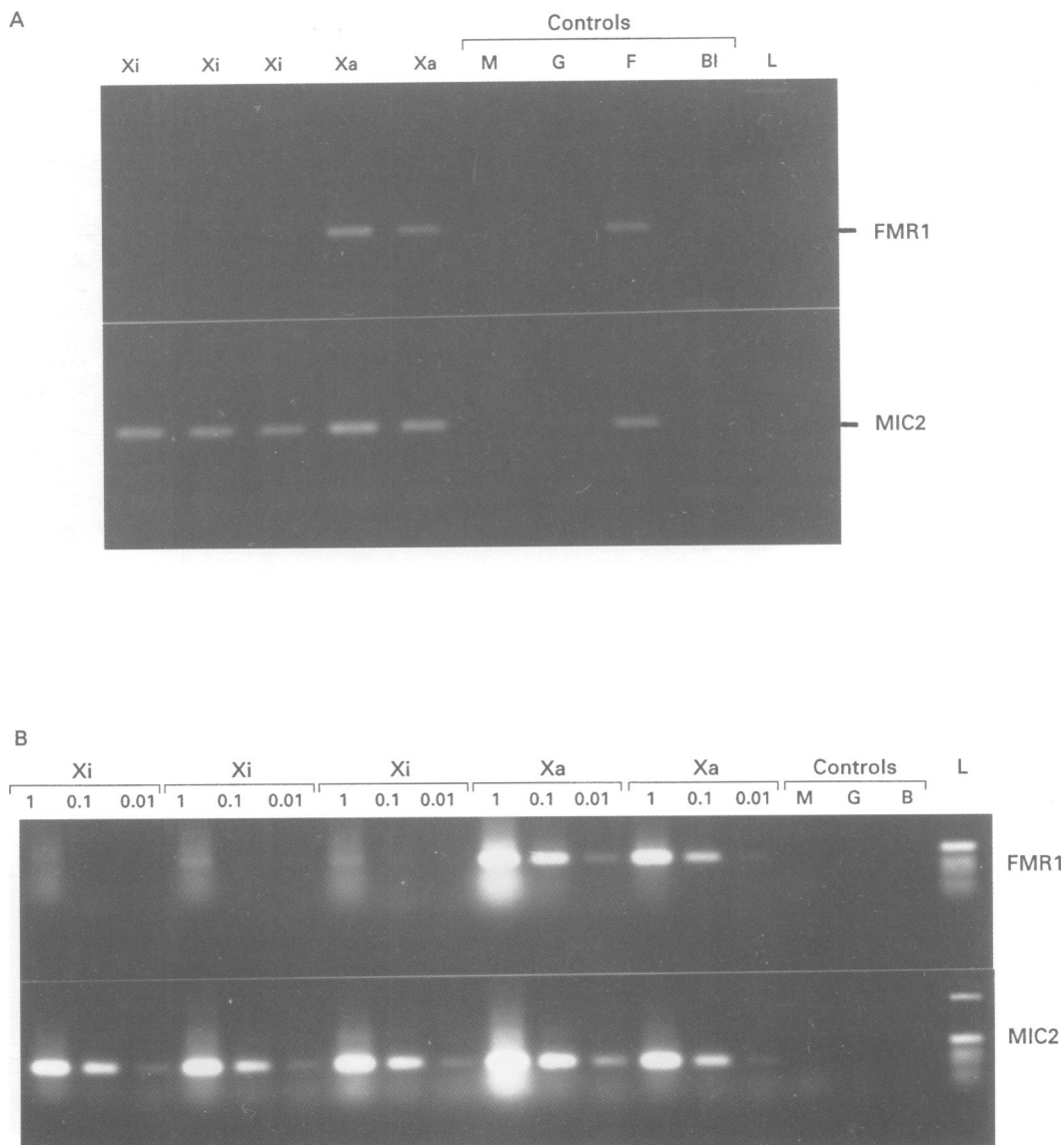### ANALYSIS OF THE INACTIVATION STATUS OF THE FMR1 GENE BY RT-PCR IN SOMATIC CELL HYBRIDS

The expression of the FMR1 gene was studied in somatic cell hybrids which retain only one human X chromosome in either its active or inactive state.[23][24] RNA was reverse transcribed and the resulting cDNA amplified by PCR (RT-PCR) with human specific primers for FMR1 and MIC2. MIC2 served as a positive control, as it has been shown to escape inactivation.[23][27] The FMR1 primers are species specific, such that only cDNA from the human X chromosome and not from the murine parental cell line was amplified. Further, the primers span two introns, so that any amplification results exclusively from cDNA and not from potentially contaminating genomic DNA. The results of the RT-PCR amplification in somatic cell hybrids is shown in fig 1. In initial experiments, 1 μl of the reverse transcription reaction, representing 0·25 μg total RNA, was used for amplification. In three independent inactive X hybrids examined, no expression of FMR1 was detected, although MIC2 expression could be readily detected, as expected (fig 1A). Two active X hybrids expressed both FMR1 and MIC2 with comparable signal strength. Mouse cDNA, prepared from the parental cell line of four of the hybrids used in this study, did not show expression of either FMR1 or MIC2, confirming the species specificity of this analysis.

In order to quantify the expression differences between FMR1 and MIC2, an experiment based on 10-fold serial dilutions was conducted. Dilutions of the reverse transcription reaction were again amplified with either the FMR1 or MIC2 primers (fig 1B). In two of the inactive X hybrids (t48-1a-1Daz4A and t75-2maz34-4a), a faint band can be seen only in the lanes with the highest concentration of inactive X cDNA as substrate. However, comparing the signal intensities between FMR1 and MIC2 or between active X and inactive X hybrids, it appears that FMR1 expression from the inactive X is at least 100-fold reduced compared to MIC2 or FMR1 expression from the active X.

ANALYSIS OF THE INACTIVATION STATUS OF THE FMR1 GENE BY RT-PCR IN HUMAN CELLS

In order to complement the results obtained with somatic cell hybrids with results from analysis of a human cell line, FMR1 expression was studied in a mentally retarded female patient with a de novo deletion spanning the fragile X locus and iduronate sulphatase gene (IDS) region, who shows complete non-random inactivation of her normal X chromosome.[20-22] If these genes are subject to X inactivation, one would not expect to detect any expression of FMR1 or IDS, as these genes are present only on the inactive X chromosome of this patient. Indeed, as shown in fig 2A, no signal can be detected for the IDS and FMR1 RT-PCR amplification products in this female patient. As a positive control, FMR1 expression was studied in a duplex reaction with PDHA1, the X linked gene encoding a subunit of pyruvate dehydrogenase, which is known to be subject to X inactivation[28] (L Carrel, H F Willard, unpublished data). The expected 240 bp product of PDHA1 is detected in the patient's cells (presumably reflecting expression
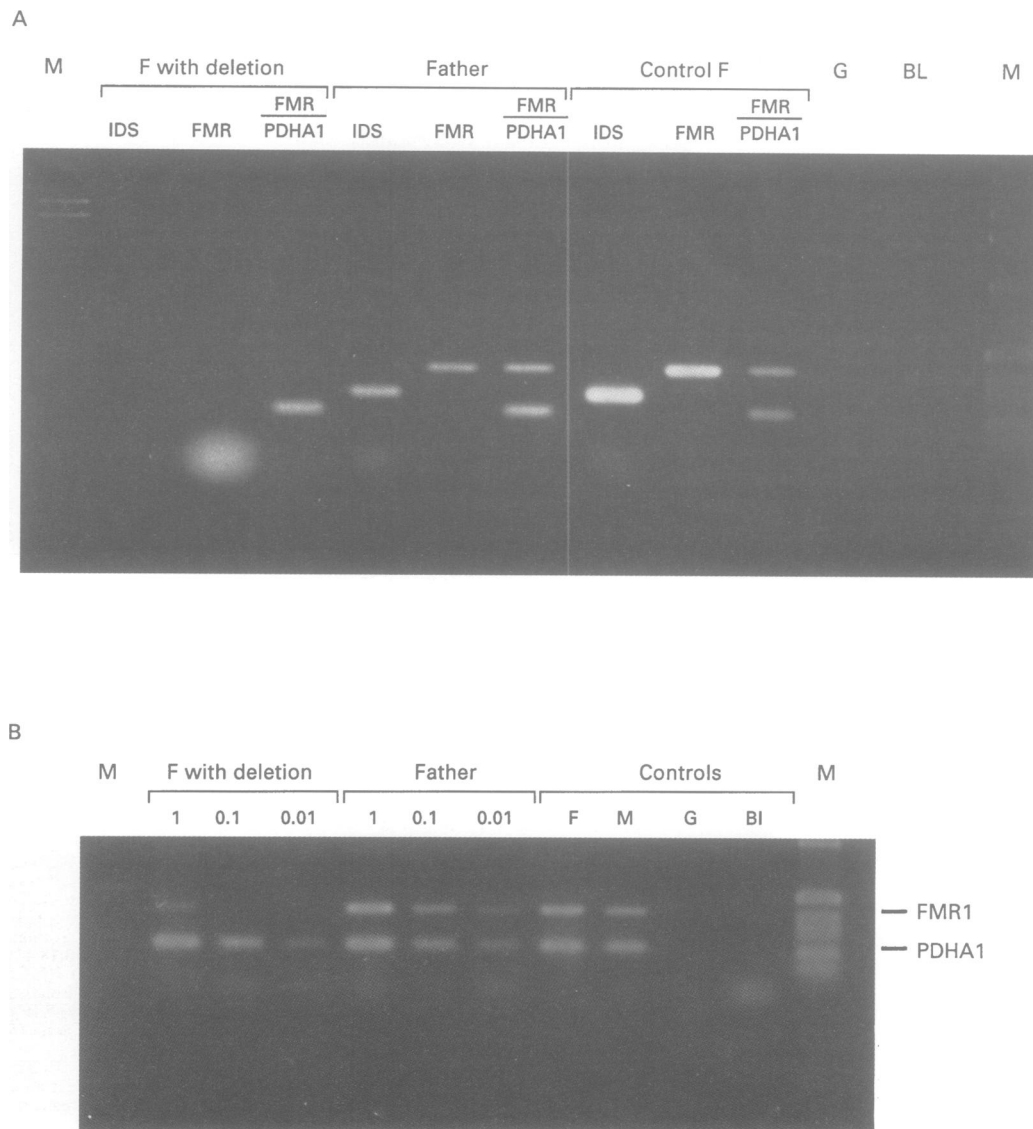
Figure 1 *Analysis of the inactivation status of the FMR1 gene by RT-PCR in somatic cell hybrids retaining either the inactive or active X chromosome. (A) Expression of a 428 bp product of the FMR1 gene (upper panel) and expression of a 400 bp product of MIC2 as a control (lower panel). Lanes 1–3: hybrids with inactive X chromosomes (Xi); lanes 4 and 5: hybrids with active X chromosomes (Xa); lanes 6–9 contain controls: mouse cDNA from the parental cell line tsA1S9(M), human genomic DNA (G), human female cDNA(F), and a no DNA blank reaction (Bl). Lane 10 contains a size standard ladder (L). (B) The upper and lower panels show the detection of FMR1 and MIC2 expression, as in (A). Lanes 1–9 contain sets of three 10-fold dilutions of cDNA from three independent inactive X hybrids (Xi); lanes 10–15 contain sets of three 10-fold dilutions of cDNA from active X hybrids (Xa); lanes 16–18 contain control reactions (0·1 concentration), as in (A).*

from the deleted active X), while the FMR1 product is absent. Fig 2A also depicts the positive amplification results for IDS, FMR1, and FMR1/PDHA1 duplexed in samples from the father of the patient (since his X chromosome is the inactive X in his daughter[21]) and from a female control.

In addition, a 10-fold dilution series experiment was carried out, as described in the section above. Only limited expression of the FMR1 gene in the female with a deletion could be observed, while strong signals were discerned for the PDHA1 control (fig 2B). Therefore, expression of the FMR1 gene from the inactive X in this human cell system is reduced about 100-fold compared with the expression of PDHA1 or compared with FMR1 expression from her father's X chromosome, confirming the results of the somatic cell hybrid analysis.

Discussion

The expression of the FMR1 gene from inactive X chromosomes was studied by reverse transcription and PCR in two different cell systems: in somatic cell hybrids which retain one human X chromosome in either its active or inactive state and in a female patient with a large deletion of the FMR1 and IDS genes and nonrandom inactivation of her normal X chromosome. In both systems, our data indicate that FMR1 is not expressed from the inactive X chromosome. The verification of our hybrid results in a diploid human cell line supports the validity of using somatic cell hybrids as a model system to study the expression of X linked genes.[24] The faint expression of FMR1 detected in two of the inactive X hybrids (fig 1B) does not alter this conclusion. At the molecular level, many X linked genes that are

Figure 2 Analysis of the inactivation status of the FMR1 gene by RT-PCR in a female with non-random X inactivation. (A) Lanes 1 and 13 contain a size marker (M); lanes 2–4 contain the amplification products of the female with a deletion; lanes 5–7 contain the amplification products of the father of the female patient; lanes 8–10 contain the amplification products of a female control: IDS amplification, FMR1 amplification, or FMR1 and PDHA1 duplexed amplification are indicated above each lane; lanes 11 and 12 are controls as in fig 1. (B) Duplexed RT-PCR reactions to assess relative expression of FMR1 and PDHA1 in female with a deletion, her father, and controls. Ten-fold dilution series are as described in the legend to fig 1B. Control lanes are as in fig 1 with cDNA from a normal female (F) or normal male (M). M lanes (lanes 1 and 12) contain size standards.

nonetheless subject to inactivation show some expression (albeit at very low levels) from the inactive X (C Brown, L Carrel, H Willard, in preparation). The low levels of FMR1 expression seen here (at most, ~1% of active X levels) are consistent both with our experience with other genes and with available biochemical data on other genes that are subject to X inactivation.[18]

The main focus of this study was to address the question of X inactivation of FMR1. While X inactivation has long been assumed for this gene and has been supported by extensive studies of FMR1 DNA methylation,[11 12 29–32] it has not, to our knowledge, been formally demonstrated. In addition, we have also shown that the IDS gene is subject to X inactivation. No transcription of IDS was detected in the female patient with a deletion of the FMR1 and IDS genes on her active X chromosome. This result is in agreement with previous biochemical studies carried out in cells from carriers of Hunter syndrome.[33–35]

The correlation between the clinical phenotype of fragile X syndrome and the extensive amplification of the CGG trinucleotide repeat within the FMR1 gene has been well documented.[10 11] The change from an unstable premutation, constituting only a modest size increase of the repeat, to a full mutation with a large copy number of the repeat requires the transmission through the female germline.

We have shown in this study that the FMR1 gene is subject to X inactivation, a requirement if inactivation plays any role in the manifestation of the disease. Affected males not only show elongation of the trinucleotide repeat, but also show methylation of the CpG island of the FMR1 gene and no transcription of the gene.[9 29] Hansen *et al*[30] and Hornstra *et al*[31] have

established the full methylation of sites at the 5′ end of FMR1 in an inactive X chromosome derived from a hybrid cell line. In their methylation analysis, comprising many sites in the CpG island of the FMR1 gene, including sites within the $(CGG)_n$ repeats, they observed extensive methylation in affected males similar to the methylation pattern derived from the inactive X in normal females, but with some increases in the methylation frequency at certain sites. Based on the data reported here, the methylation pattern in affected males, therefore, can be directly correlated with the inactivated FMR1 gene on normal inactive X chromosomes.[29-32]

In addition to establishing the X inactivation status of FMR1, our data would appear to be relevant to the expression of fragile X syndrome in some carriers of FMR1 full mutations.[15 16] Reduced penetrance in carrier females can be explained not only by premutation allele carriers, but also by variation in random X inactivation of the X chromosome carrying a normal or fully mutated FMR1 allele. As is also seen in other X linked disorders,[18] X inactivation of FMR1 nicely accounts for the observation that only about half of females with full mutations are penetrant and that those who are penetrant are often less severely affected than males.[3 15 16]

Lastly, the finding of X inactivation of FMR1 suggests that the FMR1 protein may be cell autonomous in the brain, not circulating or cross correcting adjacent cells. Thus, the degree of mental impairment in carrier females may represent the inactivation of the X chromosome carrying the normal allele in a critical proportion of neurones.

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