Frequency and replication status of the fragile X, fra(X)(q27–28), in a pair of monozygotic twins of markedly differing intelligence

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SUMMARY Chromosome analysis using conventional staining, G banding, and, after BUdR incorporation, two R banding methods, one using Hoechst and one acridine orange, were performed on lymphocytes from a pair of female monozygotic twins. The culture conditions were designed to show the presence of the fragile X (q27–28) which had previously been found to be segregating in the family. One twin was of higher than normal intelligence and the other had been diagnosed as mentally retarded. The frequency of the occurrence of the early/active fragile X compared to the overall total of informative fragile X was determined using both methods described above and was also compared with previous published data in the form of a graph showing percentage of early/active fragile X against intelligence.

Wide variation in levels of intelligence are found in females heterozygous for the fragile X syndrome. Sherman *et al.*,¹ Mikkelsen,² and Webb *et al.*³ suggest that approximately one-third of such females can be mentally retarded, causing difficulties in antenatal diagnosis. Mikkelsen suggests that most parents would choose termination of all positive fra(X) females.

Sherman *et al.*,¹ Schmidt,⁴ and Chudley *et al.*⁵ report a correlation between a high frequency of the fragile X in stressed lymphocyte cultures and mental retardation in female subjects. A familial level of fragile X in retarded males together with an increased level of fragile X in mentally retarded carrier females has been reported by Soudek *et al.*⁶

In 1980 Jacobs *et al.*⁷ suggested that the mental status of the female heterozygote could be explained by Lyonisation, and in 1982 this was supported by Froster-Iskenius *et al.*⁸ after demonstrating a preponderance of active fragile Xs in a mentally retarded boy with both Klinefelter's syndrome and the fragile X.

Recent results from Uchida and Joyce⁹ and Howell and McDermott¹⁰ support Froster-Iskenius *et al.*,⁸ although the correlation with Lyonisation has been recently disputed by Mikkelsen.¹¹ However, Mikkelsen's results were pooled, whereas Uchida

and Joyce and Howell and McDermott both calculated the percentages of active and inactive fragile X per individual subject.

When one of a pair of female monozygotic twins, the other of whom was mentally retarded, presented for genetic counselling, and both were found to have equal levels (7%) of the fragile X in their lymphocytes, this seemed an ideal opportunity to test the Lyon hypothesis as an explanation of their differing levels of intelligence.

Case reports

The family (fig 1) was ascertained through KS, a married woman teacher aged 27, who presented for genetic counselling. She has two older mentally retarded brothers (HF and IF) and a retarded twin sister (SF).

HF is shown in figs 2 and 3. There were no problems relating to his birth and, apart from some retardation of speech, no other abnormality was observed when he went to a normal infant school at the age of 5. However, by the age of 7 he was having learning difficulties and he was transferred to an ESN(M) school. Now, at the age of 40, he lives at home and attends a training centre daily. He cannot read and is unable to do shopping. He is short and stocky, with a height of 162 cm (below the 3rd centile) and a head circumference of 54 cm (on the

FIG 1 Pedigree of family F.

FIG 2 Full length view of HF aged 40 years.

FIG 3 Face of HF.

2nd centile for his age). He has a big nose, protruding ears, no abnormal neurological signs, and a testicular volume which is above the 95th centile.

IF (fig 4) also had a normal birth history and was noted to have indistinct speech as a toddler. He started at a normal infant school but was transferred to an ESN(M) school at the age of 7 and was later transferred to an ESN(S) school. Now, aged 30, he lives at home and works in a factory. He is retarded and stocky, like his brother, with a height of 173 cm.

KS (the proband) and SF were twins, born at 40 weeks' gestation. The second twin (SF) was reported by her father to have been blue at birth. However, she was not transferred to a Special Care Baby Unit; the Maternity Hospital records for their year of birth (1954) have been destroyed.

The twin girls were initially thought to be identical (fig 5) but later their parents considered them to be non-identical since SF had learning difficulties and was transferred to an ESN(M) school at the age of 10. Also, their facial appearances diverged as they grew older. KS has a long, thin, attractive face (fig 6), whereas SF has a broad lower jaw and greasy skin (fig 7). However, blood grouping and HLA typing have demonstrated that they have a 98 to 99% probability of being monozygous (GWG Bird, 1983, personal communication). Both women are A, CCDee, Le (a+b-), P₁+, MM, S-Fy^a-, Jk(a-b+), K- (DCT-), HLA-A3, A10, Bw35, B12 (44).

There were no other retarded relatives. The mother (II.4) had died in 1979 of leukaemia. The clinical diagnosis of the two affected males, HF and IF, was that of X linked mental retardation. The father of II.4 was in his late 30s or early 40s when II.4 was born.

FIG 4 Full length view of IF aged 30 years.

FIG 5 KS and SF at the age of one year. Their family cannot tell one from the other.

FIG 6 Face of KS aged 27 years.

After culture of blood lymphocytes under conditions designed to demonstrate the presence of the fra(X)(q27-28), HF, IF, KS, and SF were all found to have the marker in a percentage of their cells (table 1), showing X linked mental retardation to be segregating within the family.

FIG 7 Face of SF aged 27 years.

Materials and methods

Short term (72 hour) lymphocyte cultures were established on the day of collection of the blood samples and repeated the following day with a further aliquot.

A total of 0.4 ml peripheral whole blood was added to 5.0 ml of the following media.

- (1) TC199 + 2% fetal calf serum (FCS).
- (2) TC199 + 2% FCS + methotrexate (10^{-7} mol/l).
- (3) TC199 + 2% FCS + FUdR (10^{-5} mol/l).
- (4) TC199 + 2% FCS + methionine (100 μ g/ml).
- (5) RPM1 1640 + methotrexate (10^{-7} mol/l) (10% FCS).
- (6) RPM1 1640 + FUdR (10^{-5} mol/l) (10% FCS).
- (7) RPM1 1640 + methionine (100 μ g/ml) (10% FCS).
- (8) TC199 + 2% FCS + BUdR (50 μ g/ml).

Altogether six cultures per female subject were set up in the presence of BUdR in order to distinguish between the active and the inactive X. BUdR was added six hours before harvesting and the cultures were kept in the dark thereafter. Colchicine was added to all cultures one hour before harvesting.

Cells from cultures without BUdR were stained with either orcein or Giemsa, in order to maximise scoring of fra(X). Banding studies were performed on cells grown in 199 or medium M (modified F10

TABLE 1 Expression of fragile X in family F.

	TC199 + 2% FCS				RPM1 1640		
		+MTX	+FUdR	+Methionine	+MTX	+FUdR	+Methionine
KS	4/60	7/25	4/30	1/7	4/9	16/164	
SF	3/40	5/15	11/23		9/35	21/50	
HF	4/50	NM	3/11		14/30	2/10	
IF	11/100	NM	NM		3/50	1/31	

NM=no mitoses.

without folate or thymidine) in order to confirm the fragility of the X chromosome.

Cells from BUdR cultures were initially stained using a modified R banding method for the light microscope. Slides were stained in Hoechst 0.5 ml stock solution (0.1 mg/ml distilled H₂O) in 50 ml Giemsa buffer, pH 6.8, for three hours, washed in buffer pH 6.8, mounted in the same buffer, well blotted, sealed, and exposed to ultraviolet light for three hours. Coverslips were then removed with distilled water and the slides stained in 50% Giemsa in phosphate buffer, pH 6.8, for 30 minutes. The slides were then viewed without coverslips and the position of the active and inactive Xs noted, usually with a small sketch, and fragile Xs, if visible, recorded. Slides were then destained in methanol overnight, restained with 50% Giemsa, and mounted in DPX. The chromosomes take up more stain the second time and, although some banding may be lost, it becomes more obvious whether an X is fragile. The slides were then viewed again and fragile sites recorded over the top of the original analyses.

Later slides were stained for ultraviolet microscopy using a modification of the method of Howell and McDermott.¹⁰ Slides were stained first in Leishman diluted 1:4 with phosphate buffer, pH 6.8. They were sealed in buffer under a coverslip, references taken, and a sketch made of fra(X) positive cells. The coverslip was washed off with tap water, the slide dried, and rapidly destained with two changes of methanol. After drying, the slide was immersed in normal saline, pH 6.0, and then restained in acridine orange (0.005% in saline, pH 6.0) for 30 minutes, washed twice in normal saline,

pH 6.0, mounted in saline, blotted well, and sealed. After exposure to ultraviolet light for 15 minutes, the fra(X) positive cells were re-examined under the ultraviolet microscope and the active and inactive Xs noted over the previous analysis.

After removal of the coverslip the slides were dried and reverse banded using 2% Leishman in buffer, pH 6.8, for 5 minutes, dried, and mounted in DPX in order to make a permanent preparation.

Results

Culture in TC199 with 2% FCS revealed 7% of the cells to show the fragile site Xq27-28 in both sisters (table 1). Incorporation of methotrexate (MTX) or FUdR also induced the expression of the fragile site but decreased the mitotic index. Incorporation of BUdR decreased the percentage of fra(X) to 4% in both sisters.

RESULTS OF INCORPORATION OF BUdR

Method 1 using Hoechst (fig 8)

(1) KS. Of 13 fra(X) positive cells the inactive X was fragile in nine and the active X fragile in four. One cell was fra(X) positive but uninformative.

(2) SF. Out of 14 fra(X) positive cells the inactive X was fragile in two and the active X fragile in 12.

Method 2 using acridine orange (fig 9)

(1) KS. A total of 50 informative fra(X) cells was obtained. There were 13 other cells with a fra(X) in which the active and inactive X chromosomes could not be distinguished, including one cell in which both Xs were fragile (fig 10). This is probably due to the effect of BUdR and has been previously

FIG 8 Arrows point to X chromosomes. (a) The fragile site in the late replicating X chromosome, (b) the fragile site in the early replicating X chromosome after BUdR incorporation and staining with Hoechst.

FIG 9 Arrows point to the X chromosomes. (a) The fragile site in an X chromosome (Leishman stain), (b) the same cell to demonstrate that the fragile site is on the early replicating X chromosome (acridine-orange), (c) the same cell (RBA banding).

FIG 10 Cell showing an early and a late replicating fragile X.

reported by Nielsen *et al.*¹² Out of the 50 informative cells, the fragile X was inactive in 35 and active in 15.

(2) SF. As above, a total of 50 informative cells was obtained. In nine cells the active and inactive X chromosomes could not be distinguished and these were excluded. Out of the 50 informative cells the fragile X was inactive in seven cells and active in 43 cells.

Discussion

In an attempt to interpret these results, all available information was abstracted from published reports (table 2) and a graph was drawn using a rough division of IQ into severe retardation, dull, and normal along the ordinate and percentage of active fragile X, that is

$$\left(\frac{\text{Active fra(X)}}{\text{Total active + inactive fra(X)}} \times 100 \right),$$

TABLE 2 Replication status of the fragile X: previously published data and KS and SF.

Authors	Method	IQ	Age (y)	Sample size	% active fra(X)	% fra(X)	% fra(X) + BUdR	Culture
Lubs ¹³	H ₃ phase-contrast orcein	Normal	24	20	70	28		Micro 199
Jacobs <i>et al</i> ⁷	BUdR	Normal	67	4	25	0.0	7.0	(1) F10 + 15% FCS 72 h
	Hoechst	Normal	54	5	20	4.2	2.6	(2) 199 or 'M' + 5% FCS
		Normal	23	8	25	13.5	5.8	92 h
		Slow	29	9	56	12.7	8.4	
Nielsen <i>et al</i> ¹²	BUdR	Normal	29	26	54	7		199
	Acridine orange Giemsa					24] 32]		199 + MTX
Uchida and Joyce ⁹	BUdR	Slow	26	129	78	7.7	6.1	Macroculture 10 ml
	Giemsa	Slow	21	120	71	9.2	3.8	199 + 2.0 ml plasma
	Acridine orange	Normal	50	78	51	3.8	2.8	(± AB serum
	Giemsa	Normal	48	32	31	2.7	2.7	2.0 ml)
	Reverse banding							
Howell and McDermott ¹⁰	BUdR	Severe retardation	48	28	82			Micro TC 199 + methionine
	Leishman	Normal	33	23]	22			199 + 2.0 ml plasma
	Acridine orange	Normal	33	33]	54			(± AB serum
	Leishman Reverse banding			2]				2.0 ml)
KS	Hoechst	Normal	27	13	30	7		McCoys 5a+ 10% FBS+FUdR
SF	Acridine orange			50	30	7	4	199 + 2% FCS
	Hoechst	Dull	27	14	85	7		199 + 2% FCS
	Acridine orange			50	86	7	4	199 + 2% FCS

along the abscissa (fig 11). Age was not taken into account on the graph but is included in table 2.

From table 2 it can be seen that all the mentally retarded females with one exception have 70% or more of their fragile X chromosomes active. In the one exception, only nine cells were analysed.⁷ This may correlate with the observation that about one-third of fra(X) positive females are mentally retarded.

FIG 11 Approximate relation of IQ to percentage of early fragile X.

Four fragile X females with normal intelligence have a percentage of active fragile X of over 50. These are two possible explanations. (1) That the normal active X compensates for the fragile active X up to a point, perhaps by diffusion of gene product. (2) That the inactive fragile X is being selected against or lost. This could account for the reduction of fra(X) expression in the female heterozygote of normal IQ compared to the mentally retarded heterozygotes and hemizygotes. If the fragile site is the result of a condensation abnormality this may be masked in a chromosome which is in a condensed state much of the time.

In mammals X inactivation is believed to take place randomly around the time of implantation.¹⁴ At this stage gastrulation has not yet taken place and therefore both potential neural and mesodermal tissue could be expected to show a similar pattern of inactivation, although this will also depend on the number of stem cells that go to form each tissue and their relative mitotic activity. It is possible therefore that the inactivation pattern in lymphocytes could be similar to that in the central nervous system and could be used for antenatal diagnosis in a fra(X) positive female. Splitting of the zygote in this pair of monozygotic twins must also have taken place before X inactivation occurred.

Of the two methods, acridine orange is the more objective, as the fragile Xs are picked out without bias. Hoechst also has the disadvantage, as Jacobs *et al*⁷ have stated, that the banding pattern of the early X can resemble a fragile site and the late X can be so pale it can hardly be seen at all.

There has been some discussion as to whether BUdR may preferentially affect the expression of fragile sites on the active or inactive X.¹⁵ As the percentage of fragile X was reduced by the same amount in both sisters (from 7% to 4%) upon addition of BUdR this does not seem likely in our case.

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