Increase of FMRP expression, raised levels of *FMR1* mRNA, and clonal selection in proliferating cells with unmethylated fragile X repeat expansions: a clue to the sex bias in the transmission of full mutations?

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Abstract

Fragile X syndrome is a triplet repeat disorder caused by expansions of a CGG repeat in the fragile X mental retardation gene (*FMR1*) to more than 220 triplets (full mutation) that usually coincide with hypermethylation and transcriptional silencing. The disease phenotype results from deficiency or loss of *FMR1* protein (FMRP) and occurs in both sexes. The underlying full mutations arise exclusively on transmission from a mother who carries a premutation allele (60–200 CGGs). While the absolute requirement of female transmission could result from different mechanisms, current evidence favours selection or contraction processes acting at gametogenesis of pre- and full mutation males. To address these questions experimentally, we used a model system of cultured fibroblasts from a male who presented heterogeneous unmethylated expansions in the pre- and full mutation size range. On continual cell proliferation to 30 doublings we re-examined the behaviour of the expanded repeats on Southern blots and also determined the expression of the *FMR1* gene by FMRP immunocytochemistry, western analysis, and RT-PCR. With increasing population doublings, expansion patterns changed and showed accumulation of shorter alleles. The FMRP levels were below normal but increased continuously while the cells that were immunoreactive for FMRP accumulated. The level of *FMR1* mRNA was raised with even higher levels of mRNA measured at higher passages. Current results support the theory of a selection advantage of FMRP positive over FMRP deficient cells. During extensive proliferation of spermatogonia in fragile X males, this selection mechanism would eventually replace all full mutations by shorter alleles allowing more efficient FMRP translation. At the proliferation of oogonia of carrier females, the same mechanism would, in theory, favour transmission of any expanded *FMR1* allele on inactive X chromosomes.

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Fragile X syndrome is a triplet repeat expansion syndrome of mental retardation caused by mutational expansion of an untranslated CGG repeat located in the first exon of the fragile X mental retardation gene (*FMR1*).[1–3] Disease alleles, containing more than 220 CGG triplets and designated full mutations, only arise upon transmission from a mother who carries a premutation allele that is characterised by repeat sizes between 60 and 200 and is not associated with intellectual deficits.[4–7] Repeat expansion into the full mutation size range usually coincides with de novo methylation of the fragile X chromosome region.[3 8] De novo methylation is thought to be initiated at embryogenesis by attraction of DNA methyl transferase to hairpin-like unimolecular fold backs of CGG repeat sequences expanded beyond a threshold of repeat length.[9 10] By lateral spreading, each individual CpG dinucleotide may be involved[11–13] particularly in the CGG repeat and the *FMR1* promoter.[14–17] Hypermethylation of the fragile X chromosome region is associated with histone deacetylation and chromatin remodelling,[18] that is, processes that by themselves could cause transcriptional silencing of the *FMR1* gene,[19–21] followed by lack of *FMR1* protein (FMRP), which is required to allow normal brain development.[22–24] The phenotype of fragile X probands includes physical, behavioural, and cognitive features[25–28] and fragile X syndrome is the most frequent inherited cause of mental retardation.[29 30]

Transition from pre- to full mutation requires large gains in size, usually from about 100 to more than 1000 triplets, which cannot be created by a simple polymerase slippage mechanism.[31 32] Large scale expansion most probably results from DNA slippage mediated by hairpin-like single stranded DNA structures that can only be formed on sufficiently large repeats and could also be responsible for induction of abnormal de novo methylation at embryogenesis.[9 33 34] A long standing question is the timing of the transition from pre- to full mutation and its absolute requirement for female transmission.

Independent of their length, full mutation alleles exhibit considerable mitotic stability if they are methylated.[35] In early fetal life, somatic

mosaic patterns of different expansions exist in fragile X full mutation fetuses.[36 37] These mosaic patterns result from postzygotic mitotic repeat instability. When fully methylated, however, there is striking homogeneity of patterns among different tissues[36–38] and no significant changes of mutation patterns are observed upon continual mitotic activity of cultured fibroblasts.[39] On the other hand, direct and indirect evidence indicates that unmethylated full expansions,[40] large unmethylated premutations,[41] and extensively expanded CTG repeats in the *DMPK* gene of myotonic dystrophy[38] experience significant mitotic instability both in vitro and in vivo. These findings clearly place the time of unstable large scale repeat expansion into the full mutation size range at pre- and postzygotic developmental stages when expanded CGG repeats, independent of their sizes, remain unmethylated.[35 41]

The parent of origin effect in fragile X syndrome could result from different mechanisms depending on the timing of full expansion. Postzygotic transition from premutation to full mutation requires an (imprinting) mechanism to distinguish between paternally and maternally derived premutation.[42 43] Prezygotic transition requires some selection or contraction mechanism acting specifically at gametogenesis,[44–47] particularly in full mutation fragile X males who have only (unmethylated) premutations in their sperm.[44]

While postzygotic expansion and imprinting remained a formal theory, the prezygotic expansion model has previously received some support, as reviewed by Imbert *et al*.[48] Unmethylated full mutation alleles were detected in the oocytes of a 16 week old female fetus who showed methylated full expansions of similar size in other tissues.[47] In a 13 week old full mutation fetus with no detectable premutation in somatic tissues, no expression of FMRP could be detected in testicular tissues by monoclonal antibody testing, whereas FMRP expression was found in a few testicular cells of a 17 week old full mutation male fetus who also presented an additional faint premutation band on Southern analysis of testicular, but not of neuronal DNA.[47] Early FMRP expression in the testis could be associated with embryonal proliferation of spermatogonia.[45 46]

Selection or contraction mechanisms to eliminate full mutation alleles prezygotically from the gametocytes of affected fragile X males and to prevent expansion from premutation to full mutation at gametogenesis in normal premutation carriers should act during mitotic proliferation of spermatogonia, developmental processes that cannot be examined directly. Therefore, we studied a model system of cultured fibroblasts from a male carrier of heterogeneous expansions in the premutation and full mutation size range associated with an unmethylated promoter, allowing for *FMR1* gene expression on cell proliferation. As preliminary data obtained on such a model system were suggestive of clonal selection in favour of cells with premutation alleles,[40] we repeated the experiments on another cell population from the same donor and extended our investigation by studying *FMR1* gene expression by RT-PCR, immunocytochemistry, and western analysis.

Material and methods

Primary cultures of fibroblasts were established from skin biopsies as previously described.[41] Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, antibiotics, and fungizone. The number of population doublings at the nth passage, d(n), was calculated as $d(n) = \Sigma (2s_i)^{-1}$, $i = 1 .. n$, where s_i is the split ratio at the i^{th} passage. For example, 12 passages at a split ratio of 1/3 at each passage correspond to 18 population doublings.

One of the donors was a high functioning fragile X male showing a population of different length alleles distributed throughout the premutation and the full mutation size range, as reported previously.[41] Fibroblasts from a male with a normal *FMR1* gene and from an affected fragile X male with fully methylated full mutation alleles were used as positive and negative controls.

SOUTHERN ANALYSIS

Genomic DNA was isolated by salt extraction.[49] Aliquots (20 µg) were cleaved with restriction endonuclease *Eco*RI plus *Eag*I or with *Pst*I, size separated by electrophoresis through 0.8% agarose gels, blotted onto a positively charged nylon membrane, and hybridised to [α-^{32}P]dCTP oligolabelled probes Ox0.55 or Ox1.9, respectively, as described previously.[6] Expansion size was measured as CGG repeat index[50] given by the difference in size (base pairs) of normal and mutant bands, dividing by 3, and adding 30, the most common CGG repeat number of normal alleles in the German population.

WESTERN ANALYSIS

Total protein extracts from proliferating fibroblasts in semiconfluent cultures were prepared in $TGEK_{50}$ (Tris, Glycerol, EDTA, 50 mmol/l KCl) and 0.5 mmol/l PMSF, 1/1000 protease inhibitor cocktail, by freezing in liquid nitrogen and thawing three times. The supernatant containing the proteins was sampled after centrifugation at 16 000 g and stored at −80°C. Total protein in the samples was determined by the Bradford method at 595 nm wavelength. For blotting, protein extracts were adjusted to 1 × loading buffer (DTT, SDS Tris, glycerol), denatured at 95°C for five minutes, and the same amounts (10-30 µg) of protein were loaded onto a 10% SDS-polyacrylamide gel. Proteins were separated in 1 × Lämmli buffer and transferred to a 0.45 µm nitrocellulose membrane by tank blotting in Lämmli buffer with 20% methanol. Immunodetection was performed using monoclonal antibody mAb1C3 (Euromedex) in a dilution of 1:10 000 for FMRP detection, and monoclonal antibody mAbβ-actin (Sigma) in a dilution of 1:10 000 to control for loaded protein amounts. The second antibody was a goat-anti-mouse Ab conjugated with horseradish peroxidase in a dilution of 1:10 000, detected with

the ECL western blotting detection reagents (Amersham), and sequentially exposed (10 seconds to 30 minutes) to chemiluminescent film. Films were densitometrically evaluated by the video densitometry software, version 3.1 (Wojciech Warchol).

IMMUNOCYTOCHEMISTRY

Fibroblasts with unmethylated, expanded *FMR1* alleles of different sizes were grown on microscope slides, together with FMRP positive and negative control cells in separate areas on the same slide. Immunocytochemistry was performed essentially following the procedure described for blood smears by Willemsen *et al.*[51] The cells were fixed in 3% paraformaldehyde, followed by permeabilisation in 100% methanol. Immunodetection cascade was carried out with first antibody mAb1C3 (Euromedex), directed against FMRP (1:2000), second antibody biotinylated goat-anti-mouse (DAKO, 1:200), followed by streptavidin biotin complex conjugated with alkaline phosphatase (DAKO), and detected by new fuchsin substrate chromogen (DAKO). Endogenous alkaline phosphatase was inhibited by adding levamisole in a final concentration of 1 mmol/l to the substrate solution. The slides were counterstained with haematoxylin, mounted, and microscopically analysed. For quantitation, 100 cells per specimen were counted for FMRP positive staining or FMRP negative staining in relation to the positive and negative controls on the same slide.

RNA ANALYSIS

Total cellular RNA was isolated from cultured fibroblasts using the RNeasy™ Mini Kit (Quiagen) following the manufacturer's instructions. As we were unable to detect the heterogeneous population of *FMR1* mRNA in the fibroblasts of the high functioning fragile X donor male by northern analysis (not shown), RT-PCR was performed using the GeneAmp Kit (Perkin Elmer). Samples of RNA (1 μg) were reverse transcribed for 15 minutes at 42°C in 1 × reaction buffer containing 5 mmol/l $MgCl_2$, 2.5 U/l random hexamer primers, 1 U/μl RNAsin, 1 mmol/l dNTP, and 100 U Mu-MLV RT. Samples were then denatured for five minutes at 99°C and stored at 5°C. Using the total reverse transcribed sample, first strand cDNA was PCR amplified in a duplex reaction with the primer pairs 5'-CAC TTT CGG AGT CTG CGC AC-3' (FMR1E7) and 5'-TAG CTC CAA TCT GTC GCA ACT GC-3' (FMR1E14), 5'-AAT TAT GGA CAG GAC TGA ACG TC-3' (HPRTE2) and 5'-CGT GGG GTC CTT TTC ACC AGC AAG-3' (HPRTE7), designed to amplify the coding regions spanning exons 7-14 (772 bp) of the *FMR1* cDNA and exons 2-7 (350 bp) of the *HPRT* gene.[18] PCR was performed in 1 × Ampli*Taq* buffer (Perkin Elmer), 2 mmol/l $MgCl_2$, 2.5 U Ampli*Taq* polymerase, 30 pmol of each *FMR1* primer, and 20 pmol of each *HPRT* primer. After four minutes at 94°C, samples were subjected to 20, 30, or 40 cycles of 94°C for 30 seconds, 52°C for 60 seconds, and 72°C for two minutes.

PCR products were precipitated with ethanol, dissolved in distilled water, denatured for five minutes at 95°C in loading buffer containing formamide, electophoresed through 6% polyacrylamide/TBE gels, and vacuum blotted onto nylon membrane. PCR products were visualised by hybridisation to [α-^{32}P]dATP end labelled oligonucleotides FMR1E7 and HPRTE2. Alternatively, RT-PCR products were electrophoresed through 1.5% agarose gels and visualised by ethidium bromide staining.

DENSITOMETRY OF RT-PCR PRODUCTS

Hybridised filters were evaluated on a Phospho-Imager (Amersham Pharmacia Biotech). After three hours of exposure, signals were evaluated with the Imagequant software. Ethidium bromide stained agarose gels were photographed by a CCD camera. Prints were evaluated using the video densitometry software, version 3.1 (Wojciech Warchol).

SODIUM BISULFITE CONVERSION

The bisulfite conversion of genomic DNA was carried out following a minor modification of the protocol developed by Clark *et al.*[52] DNA (5 μg) was cleaved with 40 U *Hin*dIII restriction enzyme, precipitated, dissolved in 70 μl degassed aqua injectabilia (Braun), and denatured by adding 8 μl freshly prepared 3 mol/l NaOH for 15 minutes at 37°C plus two minutes at 95°C. The bisulfite solution was freshly prepared and contained 10 mg sodium bisulfite (Sigma) in 15 ml degassed aqua injectabilia plus 1 ml 40 mmol/l hydroquinone and adjusted to pH 5. Bisulfite solution (1.5 ml) was added gently to the denatured DNA. The reaction mixture was then overlaid with mineral oil and incubated in the dark for six hours at 55°C. After recovering the aqueous phase, the DNA was precipitated using 5 μl glass milk supplied with the GeneClean Kit (Bio 101 Inc), following the supplier's instructions, and the dried DNA pellets were resolved in 50 μl TE buffer. The purified DNA sample was subsequently mixed and incubated with 11 μl of freshly prepared 3 mol/l NaOH at 37°C for 15 minutes. Finally, the denatured, converted DNA was precipitated with ethanol, resolved in 10 μl aqua injectabilia, frozen, and stored until aliquots were used as PCR templates.

PCR AND SEQUENCING OF BISULFITE CONVERTED DNA

Nested PCR was carried out with primers designed to amplify bisulfite converted DNA (upper strand) of the *FMR1* promoter, previously identified by in vivo DNA footprinting analysis.[16] The primer sequences were 5'-TGA GTG TAT TTT TGT AGA AAT GGG C/T G-3' (1F), 5'-CTC AAA AAC A/G AC CCT CCA CC A/G-3' (1R), 5'-GGT AAC GCC AGG GGT TTC CGG TTT T C/T G C/T GA GGT AGT GTG ACT AAA ACC-3' (M13-2V), 5'-GAA ACA GCT ATG ACC ATG A/G AA ACT AAA C A/G C CTA ACT AAA ACC-3' (M13-2R). The fragment spanning the identified regulatory elements was amplified on

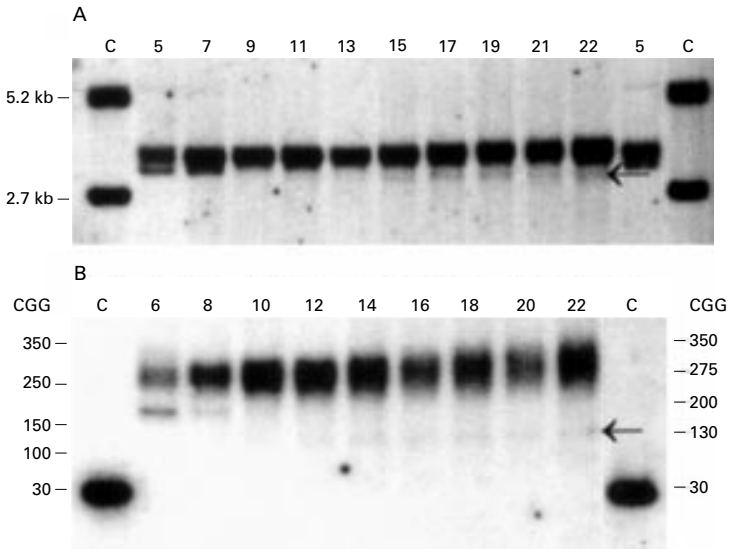

Figure 1 Changes in the expansion patterns upon proliferation of fibroblasts from the high functioning fragile X donor male. DNA was isolated from successive subcultures, digested with EcoRI plus EagI (A) and with PstI (B), and hybridised to probe Ox0.55 on Southern blots. The numbers above the lanes correspond to the passage numbers with one passage corresponding to 1.5 population doublings. C=control DNA from a female. CGG numbers are given as CGG repeat index (see Methods). Methylation of the restriction site of EagI results in fragments of 5.2 kb (normal allele on the inactive X) or larger (A). The arrows point to a faint signal of premutations (130-150 CGGs) that were first recognised at passage 10.

a robocycler (Stratagene) by nested PCR in 20 µl reaction mixtures containing 1 × Ampli*Taq* buffer (Perkin Elmer), 2 mmol/l $MgCl_2$, 0.25 mmol/l dNTPs, 10 pmol of each primer, and 5 U Ampli*Taq* polymerase. In the first PCR, 1.5 µl bisulfite treated genomic DNA was used as template and amplified with primers 1F and 1R. The second PCR mixture contained 1.5 µl reaction mixture from the first PCR and primers M13-2F and M13-2R. After four minutes at 95°C, samples were subjected to 30 cycles of 95°C for 20 seconds, 51-60°C gradient for 30 seconds, 72°C for 45 seconds, and one cycle at 72°C for five minutes.

Products of the second PCR were purified by use of MicroSpin S-400 HR columns (Pharmacia Biotech). Purified products were diluted (1:6) and sequenced according to Sanger using Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing kit (Amersham) with 7-deaza GTP, 2 pmol of each fluorescent labelled primer (M13 forward, M13 reverse), 4 µl of purified DNA samples per ddA, ddC, ddG, and ddT reaction. Sequence reactions were carried out on a robocycler (Stratagene). Reaction steps were 95°C for five minutes for one cycle; 95°C for 30 seconds, 60°C for 10 seconds, 72°C for 20 seconds for 30 cycles; 72°C for five minutes for one cycle. Samples were then denatured at 95°C for two minutes and electrophoresed through 3 mm ReproGel (Pharmacia Biotech) on an ALFexpress sequencer (Pharmacia Biotech).

Results

As illustrated in fig 1, the population of fibroblasts grown from the skin biopsy of the high functioning fragile X donor male was very heterogeneous with respect to *FMR1* CGG repeat size with most repeat indices ranging from 200 to 300. Methylation analysis of the *Eag*I restriction site in the *FMR1* promoter showed that the vast majority of alleles were unmethylated. Only faint signals of restriction fragments above 5.2 kb were detected on *Eco*RI plus *Eag*I blots. Such signals could result either from unmethylated alleles with more than 850 CGG repeats or from shorter methylated alleles (fig 1A). Analysis of bisulphite converted

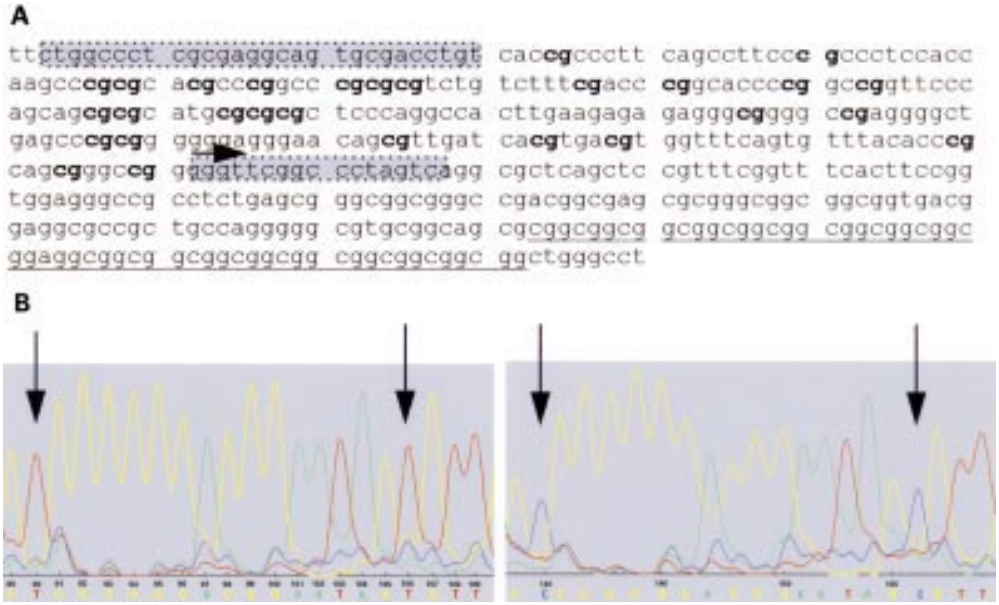

Figure 2 Methylation analysis of the FMR1 promoter previously defined by in vivo footprinting analysis.[16] (A) The promoter region of transcription factor binding includes 28 CpG dinucleotides which are printed in bold. The CGG repeat sequence is underlined. The arrow marks the transcription start site. Shading indicates the position of primers (2F, 2R) used to PCR amplify a promoter fragment from bisulfite converted genomic DNA. Sequencing of PCR fragments from successive subcultures of the high functioning fragile X donor male's fibroblasts gave no evidence of methylation at any CpG site. (B) Results of two CpGs are shown as examples. In the donor's fibroblasts (left) the CpGs (arrows) are unmethylated (C converted to T, red signal) whereas the same CpGs are methylated in the control cells from an affected fragile X full mutation male (right). C=resistant to conversion (blue signal).

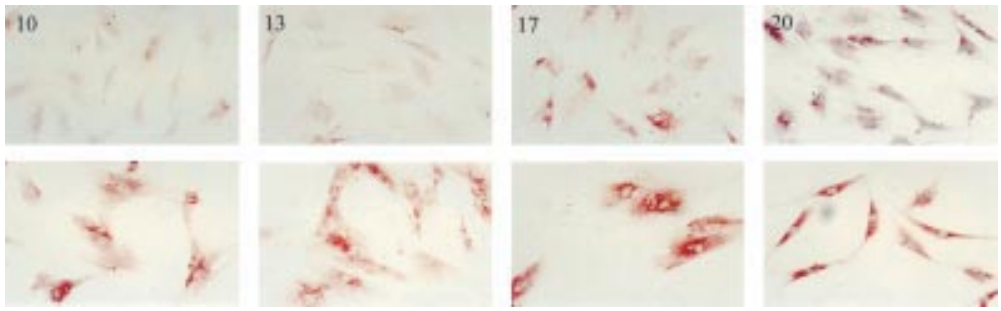

Figure 3 FMRP immunostaining of fibroblasts from the high functioning fragile X donor male at successive passages (upper row with passage numbers given in the figures), compared to control fibroblasts from a normal male (lower row). With increasing passage numbers of the donor's fibroblasts, increase of FMRP expression in subsequent subcultures of the donor's cells is indicated by an increase of immunoreactive cells.

genomic DNA was performed at each individual passage and did not identify any methylation in the *FMR1* promoter region spanning 28 CpG dinucleotides (fig 2). These data exclude significant methylation or accumulation of cells with methylated alleles during cell proliferation.

CHANGES IN THE EXPANSION PATTERNS

When the heterogeneous population of fibroblasts from the high functioning fragile X donor male was examined on successive passages during continual cell proliferation to 30 doublings (20 passages), significant changes in the overall pattern of expansions were noticed (fig 1). A faint signal of a potentially methylated allele, which was detected at passage 5 on a fragment larger than 5.2 kb on the *Eco*RI plus *Eag*I blot (fig 1A), disappeared with higher passage numbers. A blurred signal of a group of expansions between 230 and 275 CGGs in the patterns of early passages (lanes 5-7) appeared to have experienced both expansion and contraction, as this signal was recognised in subsequent subcultures as a broadening smear of expansions (fig 1B). At passage 22, the sizes of these alleles ranged from around 240 to 350 CGGs. Another fragment of approximately 175 CGGs, present in earlier passages (lanes 5-7), was not seen at higher passages.

When the proliferating cells had experienced at least 15 population doublings at passages 10-12, surprisingly a faint new signal of a premutation fragment with about 130 CGGs became detectable on both *Eco*RI plus *Eag*I (fig 1A) and *Pst*I digests (fig 1B). The density of this premutation signal slightly increased from passage 10 to 22 while the allele size expanded from 130 to 150 triplets.

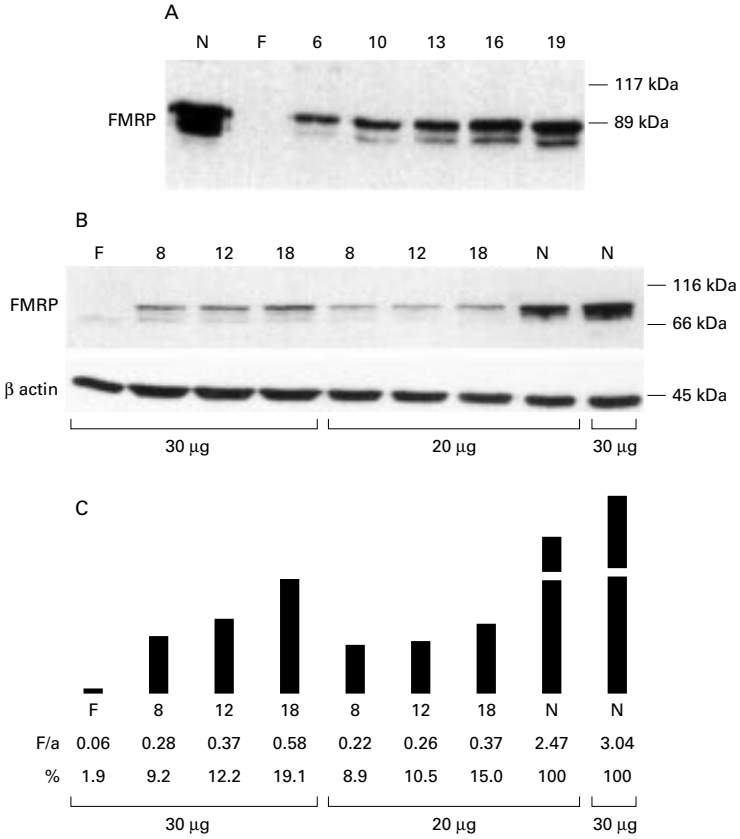

Figure 4 Increase of FMRP expression upon continual proliferation of fibroblasts from the high functioning fragile X donor male. (A) Western analysis of protein extracts from control fibroblasts of a normal male (N), an affected fragile X full mutation male (F), and of the donor's fibroblasts at passages 6, 10, 13, 16, and 19. (B) Western analysis and densitometry at passages 8, 12, and 18 with β-actin used as a reference. Relative amounts of FMRP are plotted as a quotient of signal densitities (F/a) and also given as percentages (%) of the FMRP/β-actin quotients of normal controls (N). In different experiments, 20 and 30 µg of total protein were loaded onto the gel. The faint band stained with FMRP antibody in the negative control (F) probably results from cross reaction of mAB1C3 with the FXR1P homologue.

CHANGES IN FMRP EXPRESSION

Immunocytochemical analysis showed a mosaic pattern of gene expression in the fibroblast population of the high functioning fragile X donor male (fig 3). In passage 10, about 5% of the cells showed a clear positive FMRP staining at levels corresponding to the positive control cells stained on the same slide, whereas 95% showed reduced or clearly negative staining, indicating partial or complete absence of FMRP. In passage 20, when the donor's fibroblasts had experienced 30 population doublings, the number of clearly FMRP positive cells was increased to 23%, whereas 77% of cells showed reduced staining or were negative.

Western analysis of total protein extracts isolated from the high functioning fragile X donor's fibroblasts was performed at each passage (fig 4). Amounts of FMRP were determined densitometrically using β-actin as a reference and were given as a percentage of normal FMRP levels in positive control cells. As illustrated in fig 4, and also clearly measured by densitometry, the fibroblasts of the high functioning donor male produced much lower amounts of FMRP compared to normal

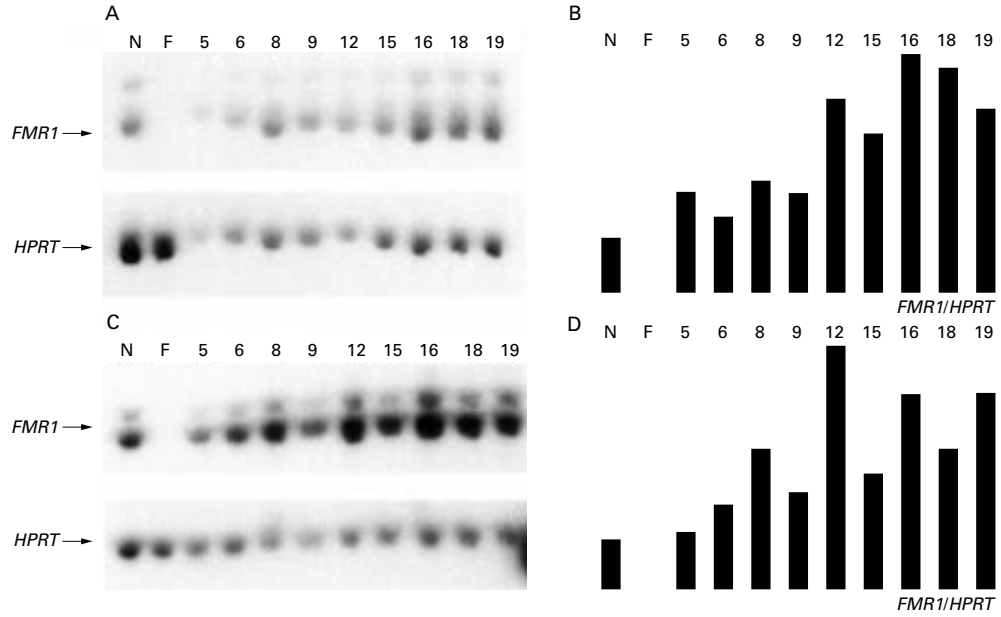

Figure 5 Increased FMR1 mRNA in the fibroblasts from the high functioning fragile X donor male. Results of two experiments of duplex RT-PCR with 20 (A, B) and 25 amplification cycles (C, D) are shown. (A, C) Signals of labelled FMR1 and HPRT oligonucleotides hybridised to PCR products blotted onto nylon membrane (see Methods). (B, D) Plots of the FMRP/HPRT quotients of signal densities. Aliquots of total RNA (500 ng) were extracted from control fibroblasts of a normal (N) and a fragile X full mutation male (F), and also from fibroblasts of the high functioning fragile X donor male at successive subcultures with passage numbers (5-19) given above the lanes. One passage corresponds to 1.5 doublings. Note the tendency of the raised FMR1 mRNA levels in the donor's fibroblasts to increase with increasing population doublings.

control cells. However, in successive subcultures, surprisingly, this low FMRP level increased continuously. On 15 population doublings, the FMRP levels increased from 9% to more than 15% of the normal level.

Semiquantitative duplex RT-PCR showed increased *FMR1* mRNA in the fibroblasts of the high functioning fragile X donor male. In these experiments, *FMR1* expression was determined in relation to *HPRT*, and given as quotient of signal densities (fig 5). At each passage analysed, the *FMR1* mRNA levels of the donor's fibroblasts were between 114 and 505% above the levels of normal fibroblasts that were used as positive controls. As these results were surprising, they were confirmed on repeat RT-PCR experiments when cycle numbers and amounts of total RNA template in the reaction mixtures varied. In contrast to FMRP, the amounts of *FMR1* mRNA did not increase continuously with each individual population doubling, as they showed considerable variation on repeated experiments. Densitometry nevertheless showed a tendency of the increased mRNA levels to increase further on continual cell proliferation. The increases were seen on densitometry of both blotted RT-PCR fragments (fig 5) and agarose gel photographs (not shown).

Discussion

The aim of our project was to study mechanisms that could eliminate full mutation alleles from the gametocytes of full mutation fragile X males and prevent expansion from premutation to full mutation by a contraction or selection process acting during mitotic proliferation of spermatogonia. In this project, we evaluated the mitotic behaviour of unmethylated *FMR1* alleles with CGG repeats expanded to pre- and full mutation sizes. These alleles were previously identified in cultured fibroblasts from a high functioning fragile X donor male.[41] Two populations of cultured fibroblasts, established from different skin biopsy materials from the same donor male, were studied as model systems for the extensive mitotic proliferation of spermatogonia occurring throughout the effective fertile life span with a number of premeiotic cell divisions for gamete production ranging from 50 to several hundred.[53] The first population was studied previously.[40] The results of the present study confirmed our previous findings of mitotic instability of expanded unmethylated alleles. Significant changes in the pattern of heterogeneous and unmethylated expansions were again found to occur during continual cell proliferation.

Mitotic instability is indicated on Southern analysis by the blurred and smeared appearance of expansion signals resembling those usually seen in adult myotonic dystrophy patients[38 41] and by a gain in size of fragments that could at times be followed individually through successive subcultures. Changes of repeat sizes were also detected when somatic cell hybrid clones were established to study individual expansions separately.[40] As reported recently,[54] another feature of mitotic instablity of full mutation alleles in somatic cell hybrids is the occurrence of contraction products that may be more or less stably maintained on further cell proliferation owing to their reduced size.[54] Such products were also detected in our previous somatic cell hybridisation experiments[40] with fibroblasts from our proband (unpublished results) but were not investigated further with respect to their transcriptional

activity and to a possible influence of the mitotic behaviour of the larger alleles in the same hybrid clone. The subpopulation of fibroblasts with 130 CGG repeats, detected only after 20 population doublings, probably also originated from unstable contraction of larger alleles.

The expanded *FMR1* alleles in the donor's fibroblasts were shown to have a completely unmethylated promoter with no detectable change of methylation upon cell proliferation to 30 population doublings. Hypermethylation of full mutation alleles is generally thought to begin by methylase binding to abnormal structures of single stranded DNA molecules formed within the expanded CGG repeat, followed by lateral spreading of methylation over the fragile X chromosome region.[9] Complete absence of methylation from each individual CpG dinucleotide in the *FMR1* promoter does, therefore, indicate that the expanded repeat in the same allele is also unmethylated. It is worth noting that de novo methylation of alleles with sufficiently large CGG repeats could not be detected during proliferation of the proband's fibroblasts. The origin of the unmethylated fully expanded alleles is still unknown. A global defect of DNA methylation can be ruled out by the good health of many of these males and also by the finding of normal methylation of other loci containing repetitive elements.[55] Interestingly, largely expanded unmethylated CGG repeats remained unmethylated when transferred into the background of apparently de novo methylation competent embryo carcinoma cells.[55]

As shown in this report for the first time, the unstable behaviour of expanded unmethylated *FMR1* alleles in proliferating fibroblasts from the male donor was associated with FMRP expression increasing continuously with increasing numbers of population doublings. As shown by immunocytochemistry, only a proportion of fibroblasts contributed to the cell population's total FMRP level, and this proportion also increased upon continual cell proliferation. These data clearly indicate clonal selection in favour of cells with higher FMRP translation efficiencies, associated with changes of the *FMR1* repeat expansion patterns in the heterogeneous cell population. We did not directly compare the expansion sizes among FMRP positive and negative cells. As, however, Feng *et al*[56] and Sandberg *et al*[57] reported that premutations do not significantly reduce FMRP production in lymphocytes whereas expansions to larger repeat sizes do inhibit their own translation, the FMRP positive fibroblasts should carry the smaller expansions. In particular, the accumulating subpopulation, which was first detected at 20 doublings and carried expansions of about 130 CGGs, should make a major contribution to the increase of the total FMRP.

Although increasing in successive subcultures, FMRP expression of the proband's fibroblasts remained below normal. In contrast, the levels of transcripts measured by semiquantitative RT-PCR were higher than normal. Since our RT-PCR did not amplify any significant levels of transcripts in cells from full mutation males with fully methylated *FMR1* genes, the detected transcripts originated from the expanded *FMR1* alleles and not from the autosomal *FMR1* homologues *FXR1* and *FXR2*. Our results clearly confirm the findings of increased *FMR1* mRNA in males carrying fragile X premutations, reported only recently by Tassone *et al*.[58] By mRNA decay experiments, these authors did not observe a significant increase of *FMR1* mRNA stability in a lymphoblastoid cell line from a male with premutation alleles including 160 CGG triplets. Reduced translational efficiency of expanded *FMR1* mRNA molecules is suggested to be compensated for, at least partially, by upregulation of transcription.

If such a compensatory mechanism also results in increased transcription of unmethylated full mutation alleles, transcription factor binding to the *FMR1* promoter should be normal in all cells of males with unmethylated expansions regardless of allele size, but this is obviously not the case. In vivo footprinting analysis of the fibroblasts of our high functioning fragile X donor male showed absence of protein binding to the *FMR1* promoter in a large proportion of cells, indicating transcriptional inactivity of their *FMR1* alleles[59]. This proportion does not reflect the number of cells with methylated *FMR1* genes as previously thought. Whereas methylated alleles may exist, methylation could not be detected by sequencing of bisulfite converted genomic DNA.

In conclusion, the increased overall level of *FMR1* messages in the fibroblast population of our high functioning fragile X donor male probably results from a subpopulation with alleles in the premutation or in the lower full mutation size range, indicating an enormously increased transcriptional level in these particular cells. The increase of *FMR1* mRNA in subsequent subcultures may reflect the accumulation of such cells, which are able to compensate for reduced FMRP translation.

Accepting the heterogeneous population of cultured fibroblasts from our high functioning fragile X donor male as a model system for mitotically proliferating spermatogonia, current data are very much in favour of a replacement mechanism driven by FMRP dependent cellular selection resulting in the absence of full mutation alleles from mature sperm, regardless of the presence of pre- or full mutations in somatic tissue. In the germ cells, alleles of premutation size may exist initially in mosaic cases or develop by contraction of previously expanded and mitotically unstable alleles. FMRP dependent selection would require these expansions to be unmethylated at spermatogenesis, according to present knowledge on the methylation status of unstable repeats. When compared to somatic cells, sperm cells of premutation carriers have a broader range of CGG repeat size indicating higher repeat instability of the germ cells.[60]

FMRP itself is not necessary for normal male fertility.[61] As shown with our model system, however, during continuous mitotic activity of the heterogeneous population, the cells producing higher FMRP levels, such as

cells with premutations, have a selective advantage, leading to their accumulation in the population and eventually to complete replacement of cells with larger expansions hardly producing any FMRP. FMRP plays a role in translation as a shuttle of mRNA between the nucleus and the active translating cytoplasmic ribosomes and as a translation factor.[62–65] A specific role of FMRP in mitotic cell proliferation of spermatogonia, oogonia, and other cells is indicated by the finding that expression of this protein is upregulated during mitosis.[21 45 46 66] Cells that cannot upregulate their FMRP expression may well experience proliferative disadvantages compared to those that can.

At oogenesis, the same selection mechanism would favour transmission of any fragile X mutation carried on the inactive X chromosome. By random X inactivation, about 50% of oogonia will exhibit normal FMRP levels originating from the normal active X chromosome, whereas the FMRP level would be zero or reduced in cells carrying an expanded *FMR1* allele on the active X chromosome.

An unanswered question is why these expanded unmethylated *FMR1* alleles, whose origin remains to be elucidated, can persist in blood, skin, and other somatic tissues of high functioning fragile X males. The theory of allele replacement driven by FMRP dependent cell selection implies that a specific function of FMRP during cell proliferation might be taken over in particular cell types or developmental stages by another RNA binding protein.

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