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Short report

FMR3 is a novel gene associated with *FRAXE* CpG island and transcriptionally silent in *FRAXE* full mutations

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Abstract

We have identified a novel gene, *FMR3*, originating from the *FRAXE* CpG island. The *FMR3* gene is transcribed from the opposite strand to the *FMR2* gene. Analogous to the silencing of the *FMR1* and *FMR2* genes, *FMR3* transcription is extinguished by *FRAXE* full mutation. Although the role of *FMR3* in *FRAXE* associated mild to borderline mental retardation is not yet clear, lack of expression of *FMR3* in *FRAXE* full mutation males means that the *FMR3* gene is potentially involved.

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Keywords: *FMR2*; *FMR3*; *FRAXE*; mental retardation

FRAXE associated mental retardation (*FRAXE* MR[1]) is the most prevalent form of non-specific X linked mental retardation (MRX) affecting at least 1 in 23 423 males.[2–4] The molecular basis for this disorder has been identified as the absence[5 6] or truncation[5] of the FMR2 protein. This is a consequence of either methylation of the *FRAXE* CpG island in subjects with the full mutation (>200 CCG copies) and consequent transcription silencing of the *FMR2* gene,[7] or deletion within the *FMR2* gene.[5 8] No *FMR2* specific point mutations have been reported so far. The 5' end of the *FMR2* gene lies within the *FRAXE* CpG island. Although the transcription of the *FRAXE* CCG repeat as part of the *FMR2* gene has been suggested,[9] there are currently no experimental data supporting this.

In silico search for *FMR3* was prompted by identification of a mildly mentally retarded patient with a submicroscopic deletion of *FMR2* intron 3 sequences,[8] which has no apparent effect on expression of the *FMR2* gene (unpublished data). Database searches (EST division of GenBank) with genomic sequence around *FRAXE* identified a single EST, AI470948. The sequence of this EST starts from the *FRAXE* *Not*I site, only 3 bp from the putative *FMR2* transcription start site[9] and only 23 bp proximal to the *FRAXE* CCG repeat (fig 1A). Comparison of EST AI470948 and *FRAXE* genomic sequence (AC002368) identified two exons separated by a 2.1 kb intron (fig 1B). RT-PCR analysis across this intron showed that this EST originated from a new gene, *FMR3*, transcribed in the opposite orientation to *FMR2*. Transcription of *FMR3* from the opposite strand to that of the *FMR2* gene was also confirmed using gene specific primer synthesis of cDNA followed by PCR (results not shown).

A

```
accccacggc tgtttgtgct gtcttttcta gttcaaaact aattcccagt ggtgggggtg ggctgggctt   70
cagagagggg tcccattccc gaagcccgcg tcctggtccc gccagcccac ctcctgtatc cctgccacgg  140
cccctgcgtc ccgggtccgg gtccgggtcc tcagctctgt ggccgctccc actagctgat tttgacagtg  210
gggggtggga gagggttccc gaaaggcagg ggcgggcgcg gcgagcccct gccagatccg gggatgataa  280
                                                603 primer
cgtgcCTGCT CCATGTCGCG GCTTCTAGCT GTCCAGGCTC CGCCCCCTGT GAGTGTGTAA GTGTGTGATG  350
       FMR3
CTGCCGCGGC CGCcgcCGCC GCCTGTGCAG CCGCTGCCGC CGCCGCCGCC GCCGCCGCCG CCGCCGCCGC  420
                FMR2 putative
1. ..........................
    2. ..........................
CGCCGCCGCC GCTGCCGCCC CGGCTGCCGC GCCGCGCCGC TGCCTCTGCC CCGGCCGCCC CCGCCGCCGC  490
                        FMR2 exp
                3. ............................................
         5. ............................................
TGCCGCCGCC GGCCCGCAGC CAGCCAGGCG GGCGGCCCAG CCCGCCTGAG CCCGCAGCGG CTGCCGCCGC  560
..........
4. .............................................................
AGCGTCGGGT CGCTGGGTGC GCGGGCTACC GCGGACCGAG CGGACCCGAG TGGGCGACCA GGCGCTTGCC  630
                      598 primer
CGCCCAGTGC CACTGCCGCC GCTTCCTCGC CGGAGCACAG GACCAGACAC CTCCAGCGCC CGCTGCTGCT  700
GCCGATGCGG CCCGGACACT TTTAGCTGGG CGGGAGGGCT GGAGAGCCGG GGGCCGCCGA GAACCGCCAG  770
CGAGCTGTGC CGAGAGCCGC GCCGACCCGC TGCGATCAGG GACAGGCGCC CGCCCGCCGC CGCCGCCTGG  850
CCGCTATGGA TCTATTCGAC TTTTTCAGAG ACTGGGACTT GGAGCAGCAG TGgtaggtgt tgatttttgc  920
     M  D   L  F  D   F  F   R   D W  D  L  E  Q  Q   C                        16
```

B

FRAXE (CCG)$_n$
*Not*I
E2 E1 E1 ORF E2 tel
I 1, 2.1 kb
I 1, 151 kb
FMR3
FMR2

Figure 1 Diagram of FRAXE CpG island and FMR2 and FMR3 genes. (A) Sequence around FRAXE CCG repeat (boxed) indicating transcription start of FMR3 and FMR2 genes. Original primers 603 and 598 used for FRAXE CCG PCR[1] are shown by arrows. Dotted lines indicate deletions within the FRAXE CpG island found either in females with POF[11] (rows 1-4) or a male with mental retardation[10] (row 5). Upper case letters indicate exons, lower case letters indicate intronic sequences. (B) Position and orientation of transcription of the FMR3 gene in respect to the FRAXE CCG repeat and the FMR2 gene is shown (not drawn to scale).

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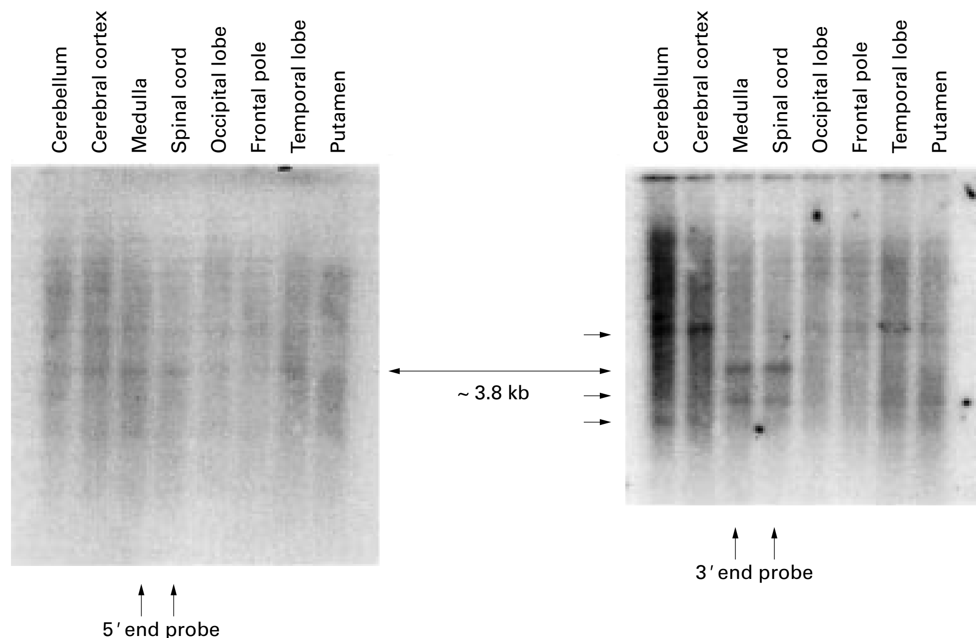

Figure 2 Northern blot analysis of FMR3. FMR3 probes (5' and 3') were hybridised to human brain II Northern blot (Clontech). FMR3 transcript of ~3.8 kb was detected in medulla and spinal cord. Additionally, two smaller and one larger transcript (of slightly lower abundance) were detected with the 3' end FMR3 probe (right panel, indicated by arrows). A 5' end probe 1.2 kb insert of EST AI470948 was used. The 3' end probe was derived by RT PCR based on the sequence of EST N66183 (see text).

Northern blot analysis with the 1.2 kb insert of the EST AI470948 identified ~3.8 kb low abundance transcript expressed in adult brain (medulla and spinal cord) (fig 2, left panel). In the search for the 3' end of *FMR3*, two ESTs derived from the same cDNA clone (N66183 and N99175) were identified more proximal (~6 kb) to the *FRAXE* CpG island. A 541 bp PCR probe just distal to the polyadenylation signal observed in the EST N66183 was designed and generated by RT-PCR on human adult brain mRNA using standard techniques.[9] The following oligonucleotide primers were used: F10 5'-CTA TGC TGC TAT GCA ACG ACG-3' and R5 5'-ACA CTT AGC ACT GCT GAT GTC ACC-3'. Northern blot hybridisation showed the same size transcript (~ 3.8 kb) as detected with the EST AI470948 clone insert (fig 2, right panel). However, in addition to the ~3.8 kb transcript one larger and two smaller sized transcripts were detected (arrows, fig 2, right panel). It is not yet clear whether these transcripts are the result of an alternative splicing of the *FMR3* gene or alternative transcription start sites.

In searching for additional introns and exons within the *FMR3* gene, EST AI470948 was fully sequenced and the region between ESTs AI470948 and N66183 was subdivided into 500-900 bp regions and amplified from oligo dT primed human adult brain cDNA. No additional exons were detected, which would indicate that the *FMR3* gene is composed of only two exons. Careful analysis of the region of the *FMR3* transcript did not show any protein coding open reading frame (ORF) within the *FMR3* transcript, although smaller ORFs (up to 51 amino acids) were present. Thus, based on currently available information, we can only speculate that the *FMR3* is a protein coding gene.

To investigate whether this gene is subject to transcriptional repression in subjects with *FRAXE* CCG full mutations, fibroblast RNA from seven *FRAXE* full mutations, one *FRAXE* premutation, two known *FMR2* deletion patients, and controls was analysed by RT-PCR.[7] The result of this experiment is shown in fig 3. As in the *FMR2* gene (fig 3, middle panel) the newly identified gene *FMR3* is transcriptionally silenced in *FRAXE* full mutations (fig 3, upper panel). *FMR3* transcripts were detected in a *FRAXE* premutation and in controls. *FMR3* transcription was also detected in two *FMR2* deletion patients. These results show that absence of *FMR3* expression might also contribute to the *FRAXE* MR phenotype.

The mental retardation in *FRAXE* is mild to borderline (50<IQ<85) without any dysmorphic features. There are at present six documented cases of *FRAXE* full mutations with a clinically normal phenotype,[4] although the level of *FMR2* expression was tested in only two of them.[7] Identification of yet another gene, *FMR3*, associated with the *FRAXE* fragile site is intriguing, especially the extent of any contribution this gene might have to *FRAXE* MR. Currently, there is only one *FMR2* mutation (deletion of exons 2 and 3) which affects only the *FMR2* gene (where a truncated protein would be produced) and not *FMR3*. All other *FRAXE* full mutations so far tested have had both *FMR2* and *FMR3* transcripts

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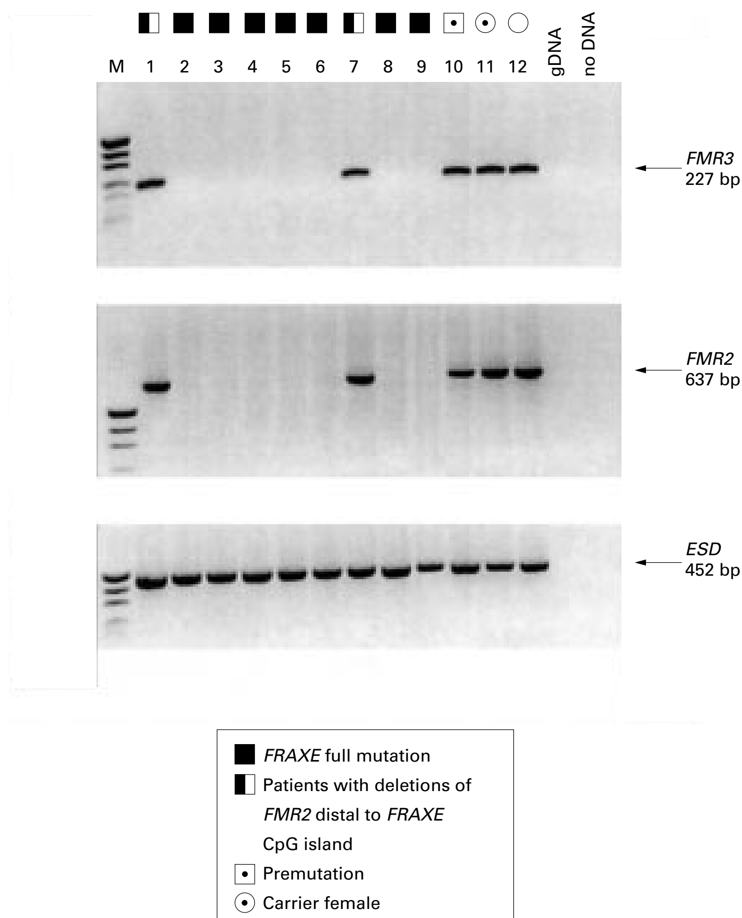

Figure 3 RT-PCR analysis of FMR3 and FMR2 genes in fibroblasts of FRAXE full mutations. FMR3 expression was not detected in subjects with a full mutation at FRAXE. For FMR3, initially 20 cycles were performed using primers F1, 5'-CGG AGC CTG GAC AGC TAG AAG C-3' and R1, 5'-AGT AGC TGA TGC TCA GTG AGC-3' followed by 30 cycles with primers F2, 5' - GCT AGA AGC CGC GAC ATG GAG C -3' and R2, 5' -TGT TTG AGG AGA GTG GCT AGG AAG -3'. For FMR2, primers 14 and 62 were used.[9] Esterase D (ESD) was used as a control.[9] gDNA denotes genomic DNA and M stands for pUC19/HpaII marker.

absent. Thus, it is difficult to estimate the extent of the contribution of *FMR3* to the *FRAXE* MR clinical phenotype and to establish whether the affected phenotype is the result of more than one gene.

Several deletions of the *FMR3/FMR2* 5' end region have been described. These were identified either from a *FRAXA* full mutation male[10] or females with premature ovarian failure (POF,[11] fig 1A). While the contribution of the *FMR2/FMR3* genes to the *FRAXA* phenotype of the deletion patient of Brown *et al*[10] is difficult to assess (because of the severity of the *FRAXA* phenotype), the four deletions reported by Murray *et al*[11] may help to determine the relative contributions of the *FMR2/FMR3* genes to POF, as these affect transcription starts of either the *FMR3* or the *FMR2* gene.

The identification of a second gene associated with the *FRAXE* $(CCG)_n$ repeat is novel and may help to resolve unanswered questions about *FRAXE* MR and the aetiology of POF.

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