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ARTICLE

Characterisation and expression of a large, 13.7 kb *FMR2* isoform.

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FMR2 **is the gene associated with** ***FRAXE*** **fragile site non-specific mental retardation (*****FRAXE*** **MRX). Previously a male patient was identified with developmental delay and speech problems who had a deletion within intron 3 of** ***FMR2.*** **No known** ***FMR2*** **exonic sequences were missing in this patient. Detailed northern blot analysis revealed existence of a new large isoform of** ***FRM2*** **in foetal brain. This isoform was characterised and found to be due entirely to an addition of an extra 4.9 kb of the 3' UTR to the previously characterised 8.755 kb** ***FMR2*** **transcript. This excluded involvement of the large** ***FMR2*** **isoform in the MRX phenotype of three individuals now known to have the same deletion of intron 3** ***FMR2*** **sequences. Expression studies on the new 13.7 kb** ***FMR2*** **isoform show that it is expressed predominantly in foetal brain and adult pituitary gland, whilst the expression of the shorter previously characterised 8.755 kb isoform is broader, including testis, thymus and placenta. Possible consequences of the alternative processing and expression of** ***FMR2*** **for the molecular pathology of** ***FRAXE*** **MRX are discussed.**

Keywords: FMR2; alternative splicing; non-specific X-linked mental retardation; MRX; expression

Introduction

FMR2 is the gene transcriptionally silenced in patients with *FRAXE* mental retardation (MR).[1,2] The 22 exons are spread across an approximate 650 kb genomic region in Xq28, encoding a transcript of 8.7 kb.[3] *FMR2* expression is high in brain and placenta and present in lung, fibroblasts and chorionic villi.[4] The gene is translated into a 1311 aminoacids long serine–threonine rich protein which is localised to the cell nucleus. It possesses at least one putative transcription activation domain.[3] Together with *AF4*,[5] *LAF4*[6] and an as yet uncharacterised gene from chromosome 5q31[3,7] it forms a new family of transcription regulators.

Several *FMR2* isoforms generated either as a consequence of alternative splicing of internal exons or different 3' end processing were detected and characterised.[3] One of them, *Ox*19[8] containing only the first 9 exons of *FMR2* would lack both the nuclear localisation addresses (NLS1 and NLS2) as well as the transcription activation domain.[3] More recently a foetal northern blot hybridisation revealed the presence of an additional, large, about 12 kb *FMR2* isoform expressed at a high level in foetal brain.[3]

Three cases of developmental delay and speech problems with submicroscopic deletions within the large (about 140 kb) intron 3 of *FMR2* are known,[9] (J Gecz, unpublished). We hypothesised that as yet

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uncharacterised sequences of the large, about 12 kb, foetal *FMR2* isoform might be involved in these deletions.

Materials and Methods

mRNA Isolation and RT PCR Experiments

Cytoplasmic mRNA was isolated using direct mRNA purification on magnetic beads (Dynal, Oslo, Norway). Approximately 10–200 ng of purified mRNA was random primed for first strand cDNA synthesis using SuperScript II RNase H-reverse transcriptase (Life Technologies, Gaithersburg, USA). Reverse transcription was carried out for 1 h at 45°C in a water bath. Negative controls where the reverse transcriptase was omitted were run at the same time, although the primers were chosen to amplify across large introns. PCR amplifications using either *FMR2* gene specific primers 90 (5'-GAG AGA AAC CAA CGC ACC AG-3') and 93 (5'-GTA GAG GAG CAG AAC CAT CAG-3') or control primers X5 (5'-CTG CCT TTG ACT TCT TTA TGC TC-3') and X6 (5'-GAT GAA GCC TTA TCT AAA TCA GTG-3') designed from the *XNP/ATRX* control gene[3,10] were performed on 1/20 of the original RT reaction. Altogether 35 cycles of PCR were carrying out (96°C for 30 s; 60°C for 30 s; and 70°C for 1 min) in a final 100 μl volume containing 100 mM Tris-HCl, pH = 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP; 50 pmol of each primer; and 2 U of Taq polymerase (Boehringer, Mannheim, Germany).

Northern Blot Hybridisation

Human RNA Master Blot (Clontech, Palo Alto, USA) was hybridised at first with the large *FMR2* specific probe generated with the primers 90 and 93 (see above and Figure 1) and then with the probe from the 5' end of the *FMR2* gene (probe 2311).[4] Probes were hybridised to the filter overnight following the conditions recommended by the manufacturer and washed several times in 0.5 X SSC, 0.5% SDS at 65°C. Shorter (24 h) and longer (4 days) exposures were taken.

Results

Characterisation of the Large FMR2 *Isoform*

To characterise the large foetal brain *FMR2* isoform several approaches were used. Firstly, the whole open reading frame (ORF) was screened by RT PCR to search for additional internal, foetal brain specific exons which may not previously have been detected. No new exons were found, not even within intron 1 (about 150 kb) or intron 3 (about 140 kb). Secondly, different portions of the *FMR2* cDNA were hybridised to the foetal northern blot (Clontech, Palo Alto, USA), all detecting both *FMR2* isoforms of 8.7 kb and about 12 kb (results not shown). These results indicated that the large *FMR2* isoform may be due to an extra large 3' UTR.

Genomic sequence from the region generated at the Baylor College of Medicine, AF007262 was initially masked for repeat sequences using Repeat Masker 2 at http://ftp.genome.washington.edu/cgi-bin/RepeatMasker.

Subsequently, blastn searches were performed against dbEST database using BLAST 2.0 program[11] at http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/. Three new ESTs F03405, F08763 and Z41797 were identified and shown to belong to the *FMR2* gene (see below). The *FMR2* 3' end structure and processing is schematically shown in Figure 1c. The major 8.755 kb *FMR2* isoform uses the pA1 polyadenylation signal. This polyadenylation signal is only 200 bp downstream of intron 21 which contains the G-rich $(G_5T)_3(G_7T)(G_6T)$ sequence.[3] RT PCR using primers 73 (5'-TCTCAGACATTAATCCTACCATCTG-3') and 94 (5'-TTGAGCTTGCTACTGCAGCATC-3') (Figure 1c) shows that the alternative splicing over this intron is maintained also in the large isoform. The large *FMR2* transcript of 13.686 kb is an extension of the 8.755 kb isoform with the 3' end defined by the presence of the polyadenylation signal pA2 as well as the cytoplasmic polyadenylation element (CPE).[12] This adds 4.931 kb more of the 3' UTR to the 8.7 kb isoform, all in one exon 22A.

Expression of the 8.7 and 13.6 kb FMR2 *Isoforms*

RT PCR analysis using a variety of oligonucleotides designed from the ESTs F03405, F08763 and Z41797 showed this region to be transcribed in control foetal and infant brain and at a lower level in fibroblasts. To confirm that these ESTs belong to the *FMR2* transcript fibroblast cDNA from normal and *FRAXE* individuals was tested. Transcription was extinguished only in fibroblasts of individuals with *FRAXE* full mutations, as predicted only if these ESTs are part of a longer *FMR2* transcript (Figure 1a). Northern blot hybridisation with the 90/93 PCR product detected only the large *FMR2* isoform (results not shown) confirming the result of the RT PCR.

To assess the biological significance of the large *FMR2* isoform, expression of both isoforms was tested on poly A^+ mRNA from 50 different adult and foetal tissues (Figure 2, and Materials and Methods). The large 13.686 kb *FMR2* isoform (Figure 2a; probe 90/93 PCR product) showed highest expression in adult pituitary gland and total foetal brain with weak expression in most other tissues. The shorter 8.7 kb isoform was expressed in adult brain, cerebellum, testis,

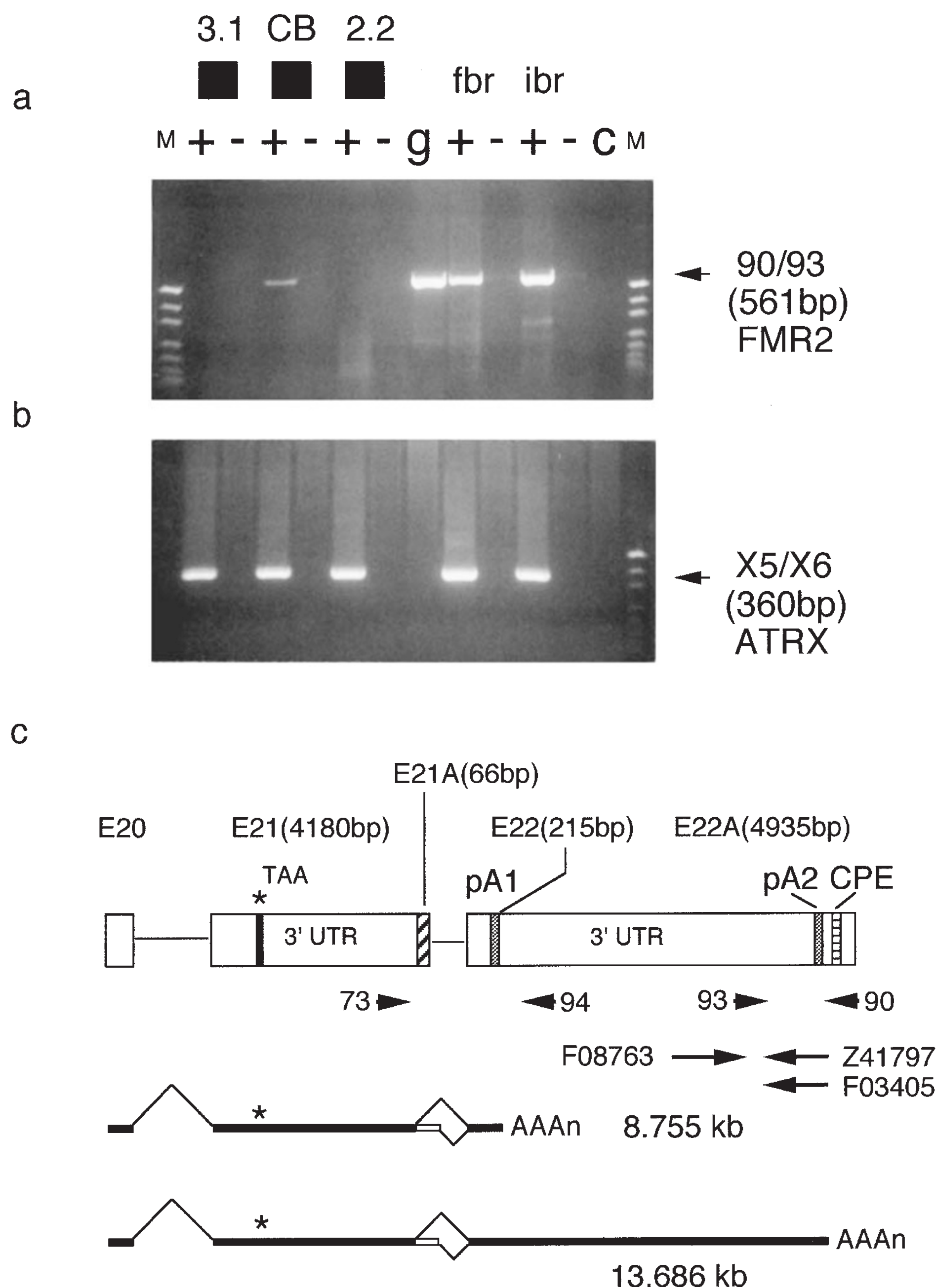
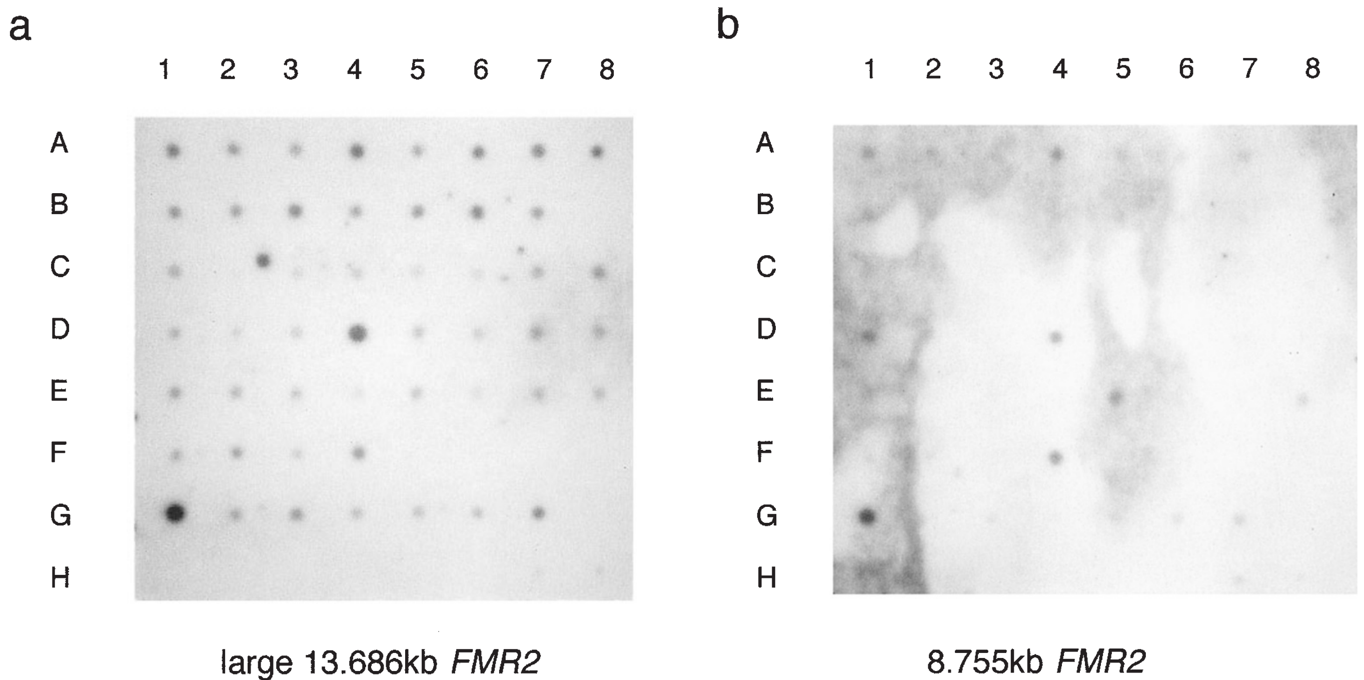


Figure 1 *RT PCR analysis and a schematic of the 3' end of the* FMR2 *gene.* **a**, *shows RT PCR result using primers 90 and 93 from the very 3' end UTR of the* FRM2 *gene (see Materials and Methods). Patients 3.1 and 2.2 have the* FRAXE *$(CCG)_n$ full mutation and complete methylation of the CpG island as reported previously;[4] CB is the patient with a deletion of exons 2 and 3 of* FMR2 *from which deletion interval the* FMR2 *gene was originally isolated.[1] Fibroblast mRNA was reverse transcribed using oligo(dT) with (+) or without (–) reverse transcriptase (SuperscriptII). As controls, foetal brain (fbr) and infant brain (ibr) mRNA as well as total genomic DNA (g) and no DNA (C) were used. M indicates the pUC19/*HpaII *molecular weight marker.* **b**, *shows the result on the same material as in* **a**, *using primers X5 and X6 designed from the* XNP/ATRX *control gene.[10]* **c**, *schematically shows the* FMR2 *3' end structure and processing. Two canonical AAUAAA polyadenylation signals (pA1 and pA2) are used for* FMR2 *transcript cleavage and polyadenylation giving rise to two major isoforms of 8755 and 13 686 bp respectively. CPE indicates the presence of the cytoplasmic polyadenylation element (UUUUUAU).[12] The stop codon in the* FMR2 *sequence is indicated with an asterisk. Position of the oligonucleotides is indicated by an arrowhead and a number and the three ESTs (F08763, F03405 and Z41797) identified are shown as arrows. The DNA sequence of the newly identified* FMR2 *isoform has been deposited into the GenBank no: U48436.*



c

	1	2	3	4	5	6	7	8
A	whole brain	amygdala	caudate nucleus	cerebellum	cerebral cortex	frontal lobe	hippo- campus	medulla oblongata
B	occipital lobe	putamen	substantia nigra	temporal lobe	thallamus	sub- thallamic nucleus	spinal cord	
C	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
D	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
E	kidney	liver	small intestine	spleen	thymus	peripheral leukocytes	lymph node	bone marrow
F	appendix	lung	trachea	placenta				
G	fetal brain	fetal heart	fetal kidney	fetal liver	fetal spleen	fetal thymus	fetal lung	
H	yeast total RNA 100 ng	yeast tRNA 100 ng	*E. coli* rRNA 100 ng	*E. coli* DNA 100 ng	Poly r(A) 100 ng	human Cot1 DNA 100 ng	human DNA 100 ng	human DNA 500 ng

Figure 2 FMR2 *expression on 50 adult and foetal tissue mRNAs.* **a**, *shows distribution and approximate expression levels of the large 13.7 kb* FMR2 *isoform. Highest expression was detected in adult pituitary gland (D4) and foetal brain (G1).* **b**, *shows expression of the 8.7 kb* FMR2 *isoform in addition to foetal brain (G1) and pituitary gland (D4). Relatively high expression was detected in cerebellum (A4) testis (D1), thymus (E5), and placenta (F4).* **c**, *loading pattern of the human RNA master blot.*

thymus and placenta, in addition to foetal brain and adult pituitary gland (Figure 2b).

Discussion

Three new *MRX* genes have recently been discovered *GDI1*,[13] oligophrenin[14] and *PAK3*.[15] All three seem to belong to signal transduction pathways through GTP-binding proteins.[16] *FMR2*, the first characterised gene responsible for non-specific X-linked mental retardation, however, does not appear to belong to the same pathway. Its function is not known, but it is speculated that it might act as a (co)activator of transcription.[3] Two major isoforms of *FMR2* were described[1,2,8] and characterised.[4] We have identified[3] and characterised (present study) an additional large *FMR2* isoform of 13.7 kb. The existence of this *FMR2* isoform with predominant expression in foetal brain and adult pituitary gland is puzzling as no additional protein sequence is encoded. Perhaps the unusually large foetal *FMR2* 3' UTR of 9177 bp contains some yet uncharacterised transcription/translation control elements. A link between transcription initiation, elongation, RNA polymerase II and processing of the 3' end of mRNA has been shown to be mediated by the cleavage-polyadenylation specificity factor (CPSF).[17] From the experiments on *Xenopus* oocytes it has been suggested that polyadenylation can induce 5' cap methylation which then can stimulate the translation of CPE-containing mRNAs. Alternatively, the translation dynamics can be repressed at a specific time of development. These alternatives have been observed from *Drosophila* and *Cenorhabditis elegans*.[18] Whether there are any elements contributing to *FMR2* transcription/translation control in the 3' UTR of either isoform can now be tested.

Recently, a mouse *fmr2* has been cloned showing 88% identity to the human *FMR2* at the protein level.[19] Expression studies showed the *fmr2* gene to be expressed in early embryos (maximum around day 11) and specific areas of brain thought to be involved in learning and cognitive processes (hippocampus, Purkinje cells). High expression of *FMR2* was found also in human hippocampus.[8] Increased expression of the large *FMR2* isoform in pituitary gland (present study) is interesting as there is an intimate connection between hippocampus and pituitary gland known as hippocampus/hypothalamus-pituitary-adrenocortical axis (HPA). HPA axis is speculated to have a role in learning and memory.[20] No alternative *fmr2* isoforms similar to those found in human *FMR2* were reported.[19]

Three apparently unrelated boys with developmental delay and deletion of intron 3 *FMR2* sequences have been identified. Fine mapping of the deletion revealed it to be identical in all three boys removing only intronic sequences of intron 3 *FMR2*[9] (J Gecz, unpublished). The hypothesis of involvement of the *FMR2* in the phenotype of these individuals with deletions of intron 3 *FMR2* sequences was not substantiated as no *FMR2* coding sequences were deleted. Whether there is another as yet unidentified *MRX* gene in intron 3 of *FMR2*, or the intronic deletions have an affect on *FMR2* processing, remains to be investigated.

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