Reduced FMRP and increased FMR1 transcription is proportionally associated with CGG repeat number in intermediate-length and premutation carriers

Aileen Kenneson, Fuping Zhang, Curt H. Hagedorn¹ and Stephen T. Warren*

Howard Hughes Medical Institute and Department of Biochemistry, Department of Genetics and Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322, USA and ¹Department of Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA

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The 5′ untranslated CGG repeat in the fragile X mental retardation-1 (FMR1) gene is expanded in families with fragile X syndrome, with more than 200 CGGs resulting in mental retardation due to the absence of the encoded fragile X mental retardation protein (FMRP). Intermediate and premutation alleles, containing between approximately 40 and 200 repeats, express grossly normal FMRP levels and such carriers are widely believed to be non-penetrant, despite continued reports of subtle cognitive/psychosocial impairment and other phenotypes. Using a highly sensitive quantification assay, we demonstrate significantly diminished FMRP levels in carriers, negatively correlated with repeat number. Despite reduced FMRP, these carrier alleles over-express FMR1, resulting in a positive correlation between repeat number and FMR1 message level. These biochemical deviations associated with intermediate and premutation FMR1 alleles, found in ~4% of the population, suggest that the phenotypic spectrum of fragile X syndrome may need to be revisited.

INTRODUCTION

The fragile X mental retardation protein (FMRP) is a selective RNA-binding protein (1) which is necessary for normal cognitive function, as the lack of FMRP results in mental retardation as a component of the fragile X syndrome. FMRP shuttles between the cytoplasm and the nucleus (2), although at steady-state it is predominantly located in the cytoplasm where it is bound to mRNAs (3) and associates with translating polyribosomes (3). The fragile X mental retardation gene (FMR1) contains a polymorphic trinucleotide CGG repeat tract in the 5′-untranslated region (5′-UTR) of the FMR1 message, with the most common normal allele containing 30 repeats. The vast majority of fragile X cases are the result of a large expansion in the CGG tract (more than 200 repeats), called full mutation alleles. Full mutations abolish FMR1 transcription (4), and the consequent lack of protein product, FMRP, results in fragile X syndrome. Carriers have alleles containing approximately 60–200 repeats, referred to as premutations. Premutation alleles are a functional category, defined by their propensity to change repeat number upon transmission to offspring. This change is particularly significant in the offspring of female premutation carriers, who are at risk of inheriting an expanded, full mutation-length allele. Because of the dynamic relationship between premutation and full mutation alleles, males may be obligate carriers based on their position in their family, but not affected with fragile X syndrome if they are carriers of premutation-length alleles. Recently, an additional category of high-end normal alleles of approximately 40–60 repeats, referred to as intermediate alleles, has been suggested based on evidence suggestive of a mild phenotype (5,6).

The mental retardation associated with fragile X syndrome ranges from mild to severe, and patients present with subtle but characteristic somatic signs, such as prominent ears, prominent mandible, long face and macro-orchidism (7). Although fragile X patients do not typically meet the diagnostic criteria for autism (8,9), many have autistic-like features such as repetitive motor mannerisms, impaired verbal communication and gaze aversion (8,10–12). Social anxiety and shyness are also prevalent (13,14).

While carriers of intermediate-length and premutation alleles are widely regarded as normal in terms of cognitive function (6,15–19), there have been persistent reports of subtle fragile X-like features in premutation carriers, including social anxiety (15,18,19–21), affective disorder (22), obsessive-compulsive disorder (23) and prominent ears (22,24–26). Furthermore, recent studies demonstrate an increased prevalence of intermediate-length (40–60) alleles in the special needs population, suggesting that these FMR1 alleles may also be associated with a mild fragile X-like phenotype (27,28). Ovarian dysfunction, including premature menopause, also occurs at a substantially increased frequency in premutation carrier females, although paradoxically does not appear in women carrying full mutations (29–32).

With the notable exception of ovarian dysfunction, many of the phenotypes reported for premutation and intermediate allele carriers appear as more subtle versions of the fragile...
X phenotype caused by the total lack of FMRP. Thus, it is reasonable to predict that decreased levels of FMRP are responsible for these subtle features. However, most studies have found FMRP at grossly normal levels in carriers, although a few reports, using non-quantitative methods, have noted occasional FMRP reduction in premutation carriers, most often with large premutations, repeat-length mosaicism, and associated with cognitive impairment (33–36). To explore the relationship between FMR1 repeat number and phenotype, we developed a quantitative assay for the accurate measurement of FMRP levels in cells and found diminished FMRP levels over a very broad range of CGG repeat lengths.

RESULTS

Using purified FMRP (1) and purified eIF4E (37) as standards, we developed a slot-blot-based assay for the accurate measurement of FMRP levels in cell lysates (Fig. 1). eIF4E is the rate-limiting factor in eukaryotic translation initiation (38,39). In Epstein–Barr virus (EBV)-transformed human male B lymphoblastoid cell lines, we find with this assay $1.10 \times 10^6$ molecules of FMRP per cell ($n = 7$, SD = $0.19 \times 10^6$) and $5.35 \times 10^6$ molecules of eIF4E per cell ($n = 7$, SD = $0.81 \times 10^6$). As this assay was able to achieve a higher level of sensitivity for FMRP measurement than was previously possible, we re-analyzed the level of this protein relative to the FMR1 CGG repeat tract length in lymphoblastoid cells. The molar ratio of FMRP:eIF4E for male cells containing single FMR1 alleles (due to X chromosome linkage) with 19–35 CGG repeats was 0.218 (SE = 0.009, $t = 6.8254$, $P < 0.005$) 40% reduction of FMRP level compared to males with CGG repeat numbers in the normal range.

To examine the relationship between FMRP level and CGG repeat number in smaller premutation alleles, we determined FMRP levels in four premutation males with alleles containing 105–130 repeats. Compared to cells containing normal alleles, a 22% reduction in FMRP was observed relative to the control protein eIF4E (FMRP:eIF4E ratio = 0.171, SE = 0.004), a significant reduction ($t = 3.22$, $P < 0.01$). Furthermore, two males with alleles containing intermediate repeat numbers, 48 and 55 CGG repeats, below the length normally considered a premutation, showed a 17% reduction of FMRP when compared with males with normal alleles ($t = 2.12$, $P = 0.05$), suggesting that even within this intermediate range, FMRP levels may be affected. As illustrated in Figure 2, these data suggest that FMRP levels are negatively correlated with repeat numbers over a wide range of repeat tract lengths.

If the reduction in FMRP levels reported here over a wide range of CGG repeat lengths is biologically relevant, we should be able to detect proportionally increased FMR1 mRNA levels due to the transcriptional compensation mechanism proposed by Tassone et al. (40). We therefore examined FMR1 mRNA levels using reverse transcriptase PCR in the same cells in which we measured FMRP levels (Fig. 3). Normalizing to the amount of X chromosome-linked HPRT mRNA, normal cells had an FMR1 transcript level of 0.89 (SE = 0.098, $n = 7$). Cells carrying intermediate repeat-length alleles and premutation alleles displayed ratios increased 1.4- and 2.3-fold relative to normal cells ($t = 1.29$, SE = 0.040, $n = 2$ and 2.07, SE = 0.313, $n = 4$, respectively). The two cell lines carrying 140 and 190 repeats exhibited an FMR1 transcript level of 2.33 (SE = 0.785), although these two samples varied considerably (ratios of 1.54 and 3.11, respectively). However, taken together, these data demonstrate a positive correlation between FMR1 transcript levels and repeat number ($R = 0.87$) which

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**Figure 1.** Generation of standard curves using purified FMRP and eIF4E. (A) Representative slot-blot of purified FMRP (row 1), purified eIF4E (row 3) and samples (rows 2 and 4), probed with anti-FMRP (rows 1 and 2) and anti-eIF4E (rows 2 and 4) antibodies. Samples shown contain 29, 19, 105, 124, 130 and 140 repeats (left to right), and include one affected individual (140 repeats). (B) Standard curves of intensities for purified FMRP and eIF4E.

**Figure 2.** FMRP levels as a function of CGG repeat tract length. Molar ratio (mean and SE) of FMRP:eIF4E versus number of repeats for 15 cell lines. Linear regression results: $y = -0.0006x + 0.229$; $r(13) = 0.785$; $P < 0.0001$; correlation coefficient = $-0.84$. The means and standard errors of each of the four groups are depicted on the left: normal (red); intermediate (blue); premutation (purple); and penetrant premutation (green) alleles.
parallels the inverse correlation between repeat length and FMRP levels ($R = -0.84$).

It is unclear if the cell senses diminished FMRP in order to signal the transcriptional enhancement of FMR1 or if the changes in transcription and translation are uncoupled events linked independently to repeat number. To address whether or not the cell senses FMRP function, we quantified the FMR1 mRNA levels in cells derived from a mentally retarded male with a normal CGG repeat length but harboring an I304N point mutation in FMR1 (41). This single amino acid change does not change the abundance of the mutant FMRP in the cells (3). However, unlike normal FMRP, which is largely associated with translating ribosomes at steady state, the I304N protein does not detectably associate with ribosomes and remains in the cytoplasm as an mRNP particle (3). The later observation has been taken as evidence for the critical role of the FMRP:ribosome association as this patient has a particularly severe form of fragile X syndrome. Therefore, if the cells sensed FMRP function, these cells would be expected to have altered FMR1 message levels. Analysis of I304N cells indicated that the FMR1 transcript level falls well within the normal range defined above with an FMR1 mRNA level of 1.01. Thus, assuming function is abrogated in these mutant cells, the transcriptional changes reported above do not seem to arise by a graded loss of FMRP function.

**DISCUSSION**

The important role of FMRP in cognitive function is manifest by the mental retardation arising in its absence. As a corollary, it is logical that the subtle fragile X-like cognitive, psychosocial and physical phenotypes associated with some premutation-length alleles may be due to decreased levels of FMRP. While most studies have failed to detect differential FMRP levels associated with premutation-length CGG tracts, reductions in FMRP levels have been observed previously in some mildly impaired premutation carriers. However, the available non-quantitative methods lacked the sensitivity to detect more subtle reductions that might be associated with smaller alleles.
**FMRI** mRNA with CGG repeat number result in tight regulation of FMRI transcription and FMRP abundance, where the latter parameter, based on Feng et al. (43), is influenced by the translational impediment posed by the 5′ untranslated trinucleotide repeat of the FMRI message. The mechanism at work here is unknown, although the normal FMRI mRNA level of the mutant I304N cell line, reported here and by others (44), appears to rule out the function of FMRP as a factor, as the I304N FMRP protein is present in normal levels while its lack of function does not result in increased FMRI transcription. We cannot rule out that the cell directly senses the FMR protein. However, there are data indicating that the I304N protein assumes a less folded conformation (45) and may not interact with its normal protein partners (46). Thus, it is also possible that non-FMRP mechanisms are responsible for the increased FMRI mRNA levels associated with increased CGG tract lengths, such as the expanded CGG tract leading to proportionally more open promoter conformation and proportionally enhanced transcription, as has been suggested by Raca et al. (47). Independently, the lengthened CGG tract in the 5′-UTR of the message could slow translation, leading to diminished FMRP levels and an apparent coupling of transcription/translation with repeat length. Additional studies of FMRI mRNA levels in conjunction with methylation and histone acetylation analyses in the repeat range between hyper-transcribed premutations and hypo-transcribed full mutations may be useful in elucidation of these molecular mechanisms. Regardless of the precise mechanism, the concomitant increase in FMRI transcript levels over a range of increasing repeat tract sizes, including both intermediate and premutation alleles, indicates the existence of a coupled or uncoupled compensatory response to decreased FMRP levels. The compensation is only partial, however, as normal FMRP levels are not achieved in cells with repeat numbers out of the normal range.

Whether or not these drops in FMRP level influence the phenotype of carriers of intermediate and premutation alleles remains to be established and is not addressed here. However, while the number of samples presented here is small, the data reported above provide preliminary evidence for a molecular basis for the cognitive, psychological and somatic features that are sometimes observed in premutation carriers. Current techniques such as western blot analysis and assessments of immuno-positive cell frequencies in blood and hair roots are rapid and useful for biologically-relevant samples. However, the low sensitivity and indirect nature of these techniques, respectively, can be augmented by the quantitative and sensitive immuno-assay presented here. Moreover, this assay may prove valuable in the study of females carrying either premutation or full mutation alleles. Further adaptation of the technique to whole blood, particularly the extension to a sandwich ELISA assay for blood, will be required for large-scale use. However, the data reported now allow an approach to allow investigation of the entire spectrum of phenotypes ranging from fragile X syndrome as the most severe and to more subtle phenotypes, such as learning disabilities, which have recently been suggested to be associated with intermediate-length alleles, which may comprise as many as 4% of FMRI alleles in the population.

**MATERIALS AND METHODS**

**FMRI mRNA quantification**

EBV-transformed human B lymphoblastoid cells were lysed in cold RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.5, 2 mM EDTA, 0.5% Igepal, 0.1% SDS, 0.5% Triton X-100, 0.012 mM deoxycholic acid), boiled for 10 min, cooled on ice and sonicated. Protein concentrations were determined by Bradford assay. Cell extracts, purified FMRP (1) and purified eIF4E (37) were diluted to working concentrations in PBS, or PBS plus 300 mM NaCl for FMRP, and blotted onto nitrocellulose membrane using a Bio-Rad slot-blot apparatus. Proteins were detected using standard immunodetection technology with the anti-FMRP monoclonal antibody IC3 (48), anti-eIF4E monoclonal antibody (Transduction Laboratories) and HRP-conjugated goat anti-mouse antibody (Kirkegaard and Perry Laboratories) at saturating concentrations. Signals were generated by Enhanced Chemi Luminescence (Amersham) and detected by exposure to Hyperfilm (Amersham). Signal intensities were quantified by analysis of digital scans using the NIH Image 1.62b7f software to plot signal profiles, and areas under the plot profile were used as signal intensities. Molarity of FMRP or eIF4E in the cell lysates was determined using standard curves generated from the purified proteins, after subtracting out signals from the background and secondary antibody controls as appropriate. Calculations of cellular content of FMRP and eIF4E were based on a cell content of 106 picograms of total protein per cell as determined in our laboratory for these cell lines. Each sample was assayed a minimum of three times.

**FMRI mRNA quantification**

RNA was extracted from cells with the High Pure RNA Isolation Kit (Roche). RT–PCR of FMRI and HPRT mRNA was performed on a Roche Light Cycler using the SYBR Green I RNA Amplification Kit (Roche). Primers 5′-gatgaagatactgccattc-3′ and 5′-tagctccaatctgtcgcaactgc-3′ were used for FMRI, and primers 5′-ctggggtccttttcaccagcaag-3′ and 5′-aattatggacag-gacgagtc-3′ were used for HPRT. Melting temperatures for the RT–PCR reactions were 60°C and 62°C, respectively. Melting curve analysis was used to determine the amount of RT–PCR product [change in fluorescence (fluorimeter gain = 5) per change in temperature at 82°C for FMRI and 85°C for HPRT (DF/DT°C)]. Standard curves were generated from the DF/DT°C for 10–40 ng of RNA template from the normal cell line J1 (containing 29 repeats). Twenty to twenty-five nanograms of template from the other cell lines were amplified, and product level determined relative to that of J1.

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REFERENCES


