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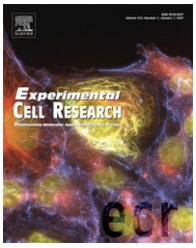

Research Article

Elevated *Fmr1* mRNA levels and reduced protein expression in a mouse model with an unmethylated Fragile X full mutation

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ABSTRACT

The human *FMR1* gene contains a CGG repeat in its 5′ untranslated region. The repeat length in the normal population is polymorphic (5–55 CGG repeats). Lengths beyond 200 CGGs (full mutation) result in the absence of the *FMR1* gene product, FMRP, through abnormal methylation and gene silencing. This causes Fragile X syndrome, the most common inherited form of mental retardation. Elderly carriers of the premutation, defined as a repeat length between 55 and 200 CGGs, can develop a progressive neurodegenerative syndrome: Fragile X-associated tremor/ataxia syndrome (FXTAS). In FXTAS, *FMR1* mRNA levels are elevated and it has been hypothesised that FXTAS is caused by a pathogenic RNA gain-of-function mechanism. We have developed a knock in mouse model carrying an expanded CGG repeat (98 repeats), which shows repeat instability and displays biochemical, phenotypic and neuropathological characteristics of FXTAS. Here, we report further repeat instability, up to 230 CGGs. An expansion bias was observed, with the largest expansion being 43 CGG units and the largest contraction 80 CGG repeats. In humans, this length would be considered a full mutation and would be expected to result in gene silencing. Mice carrying long repeats (~230 CGGs) display elevated mRNA levels and decreased FMRP levels, but absence of abnormal methylation, suggesting that modelling the Fragile X full mutation in mice requires additional repeats or other genetic manipulation.

Introduction

The Fragile X mental retardation gene 1 (*FMR1*), involved in Fragile X syndrome, contains a CGG repeat in its 5′-untranslated region. Depending on the length of this trinucleotide repeat, different clinical outcomes are possible. Repeats of normal individuals are within the range of 5 to 55 CGGs [1]. Repeat lengths greater than 200 CGGs (full mutation: FM) typically lead to methylation of both the CGG repeat and the *FMR1* promoter resulting in transcriptional silencing of the gene. The consequent absence of FMRP in neurons is the cause of the mental retardation in Fragile X patients. Fragile X syndrome is the most common genetic disorder associated with mental retardation [2].

The premutation (PM), comprising ~55 to ~200 unmethylated CGG repeats, was long thought to be associated only with a high risk of expansion to a full mutation upon maternal transmission. However, elevated *FMR1* mRNA levels and

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normal or slightly reduced *FMR1* protein (FMRP) were reported [3,4]. Additionally, 20% of female PM carriers are at risk of developing premature ovarian failure [5]. In 2001, the first evidence of a new neurological syndrome (Fragile X-associated tremor/ataxia syndrome: FXTAS) was published by Hagerman and colleagues, observed in five elderly male PM carriers. Patients presented with progressive intention tremor, leading to executive function deficits and generalised brain atrophy on MRI scans. Since this neurological syndrome is restricted to the PM range, reduced levels of FMRP are unlikely to be the underlying cause [6]. However, since elevation of *FMR1* mRNA levels seems to be correlated to the length of the CGG repeat [4,7,8], and cognitive and functional impairment increases with the number of CGG repeats [9], an RNA-gain-of-function effect has been proposed, in which elevated levels of *FMR1* mRNA containing an expanded CGG repeat lead to progressive neurodegeneration [6].

Little is known about when and how CGG repeat instability takes place. In order to be able to elucidate the timing and mechanism of CGG repeat instability and methylation of the *FMR1* gene, a mouse model was generated by Bontekoe and colleagues [10]. In this model, the endogenous mouse CGG repeat (8 trinucleotides) of a wild type mouse was exchanged with a human CGG repeat of 98 trinucleotide units, which is in the PM range in humans. This 'knock in' CGG triplet mouse shows moderate repeat instability upon both maternal and paternal transmission and displays biochemical, phenotypic and neuropathological characteristics of FXTAS [10,11].

In this paper, we report further repeat instability, up to lengths above 200 CGG units. In humans, this would implicate a full mutation, thus silencing of the gene. Mice carrying these long repeats (~230 CGGs) display elevated mRNA levels and decreased FMRP levels, but absence of CpG methylation, which would mean that in mice a full mutation has not occurred as yet. However, additional mice with long CGG repeats will be necessary to fully evaluate this issue.

Materials and methods

Mice

Both the knock in CGG triplet mice and wild type mice (parent of the knock in mouse, with a mouse endogenous $(CGG)_8$ repeat) were housed in standard conditions. All experiments were carried out with permission of the local ethical committee. Repeat lengths were determined for the whole mouse colony, but only male mice were used for the experiments.

Isolation of DNA from mouse tails

DNA was extracted from mouse tails by incubating with 0.2 mg/ml Proteinase K (Roche Diagnostics) in 335 μl lysis buffer (50 mM Tris–HCl pH 7.5, 10 mM EDTA, 150 mM NaCl, 1% SDS) overnight at 55°C. After adding 100 μl saturated NaCl solution the next day, the suspension was centrifuged. Two volumes of 100% ethanol were added and gently mixed. The appearing DNA cloud was fished out with a plastic pipet tip and subsequently washed and centrifuged in 500 μl 70% ethanol. The DNA was then dissolved in 100 μl milliQ-H_2O.

Repeat length determination

Following a method kindly provided by K. Usdin (personal communication), CGG repeat lengths were measured using the Expand High Fidelity Plus PCR System (Roche Diagnostics). Approximately 300 ng of tail DNA was added to a PCR mixture with a total volume of 50 μl, containing 0.2 μM of each primer, 200 μM of each dNTP (Invitrogen), 2% DMSO (Sigma), 2.5 M Betaine (Sigma), 5 U Expand High Fidelity Plus PCR System Enzyme and 10 μl of the 5× Expand HF buffer with Mg (7.5 μM). As forward primer 5′-CGGAGGCGCCGCTGCCAGG-3′ was used and 5′-TGCGGGCGCTCGAGGCCCAG-3′ as reverse. PCR conditions were 10 min initial denaturation at 95°C followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 65°C and elongation for 5 min at 75°C, with a final elongation step of 10 min at 75°C. Twenty five microliters of PCR product was mixed with an equal volume of loading dye (95% formamide, 0.1% bromephenol blue, 0.1% xylene cyanol) and loaded onto a denaturing 6% polyacrylamide (PA) (19:1) gel, which was run in 0.6× TBE at 18 W. Separated DNA was visualised on a UV lamp after soaking the gel in 0.6× TBE with ethidium bromide. Approximate repeat lengths were determined using a standard curve, based on DNA samples of which repeat lengths were determined with an ABI-based Fragile X size polymorphism assay in the past [10].

As these primers are specific to the knock in allele and do not detect wild type alleles, a separate PCR is necessary to distinguish females heterozygous or homozygous for the repeat. TaKaRa LA Taq was used with GC buffer II according to manufacturer's instructions (Takara Bio Inc), with 5′-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3′ as forward primer and 5′-AGCCCCGCACTTCCACCACCAGCTCCTCCA-3′ as reverse primer.

Transmission pattern of repeat alleles

The mode of transmission of the repeat allele was investigated, by comparison of the repeat lengths present in the tails of the breeding couple with those of their offspring. Since the ability of the PA gels to resolve lengths of PCR products in this range is limited, relatively large bin sizes were chosen to describe repeat length. Comparisons between parents and offspring were made using PCR products run on the same gel. Frequencies of contractions, stable transmissions and expansions were tested with a one-sample *t*-test. Magnitude and direction (contraction, stable transmission or expansion) of the repeat instability, gender of the offspring and parental origin of the repeat allele were taken into account. Transmission groups were tested for differences in magnitude of contractions and expansion separately, using one-way ANOVA. Effect of gender of offspring, as well as effect of parental allele of origin, was investigated with independent samples *t*-tests for both contractions and expansions. Because of small expected frequencies, Fisher's Exact Test was performed to test for possible differences in frequencies of contractions, stable transmissions or expansions, amongst the different transmission groups. The same test was used to determine the significance of differences in frequencies of an allele fitting into the categories distinguishing magnitude of instability (categories shown in Fig. 2). A difference in absolute