A majority of fragile X males with methylated, full mutation alleles have significant levels of FMR1 messenger RNA

F Tassone, R J Hagerman, A K Taylor, P J Hagerman

Abstract

FMR1 mRNA levels were determined in peripheral blood leucocytes for 48 fragile X males with methylated, full mutation alleles that are resistant to cleavage by methylation sensitive enzymes. Using quantitative (fluorescence) RT-PCR, we observed that more than half of these males produce FMR1 mRNA, with some mRNA levels approaching those found in normal subjects. In none of the samples analysed was there any evidence of premutation alleles. These results suggest that the assumed relationship between enzyme resistance and FMR1 gene silencing may not be generally valid. Despite the presence of FMR1 mRNA in some subjects, no FMRP production was detected by either immunocytochemistry or western blotting. The low/absent FMRP levels are probably a reflection of a post-transcriptional effect such as a defect in translation.

Keywords: trinucleotide repeat; gene silencing; quantitative RT-PCR; neurodevelopment

Fragile X syndrome nearly always arises as a consequence of a large expansion of a CGG trinucleotide repeat in the CG rich promoter region of the fragile X mental retardation 1 (FMR1) gene. Expansion of the CGG repeat into the full mutation range (>200 repeats) usually leads to hypermethylation of the CG rich region and transcriptional silencing.1 2 The consequent absence of FMR1 protein (FMRP) results in fragile X syndrome.

From the general relationship between hypermethylation and silencing, hypermethylation per se (or the absence of FMRP) is often taken as evidence that the FMR1 gene is transcriptionally silent. Thus, expression of FMRP in patients with hypermethylated, full mutation alleles is usually interpreted as resulting from either a small percentage of premutation alleles within the cell population being examined or to epigenetic (methylation) mosaicism. In this regard, hypermethylation is generally defined operationally in terms of resistance to cleavage of the CG rich promoter region by methylation sensitive restriction enzymes (for example, NruI, EagI, RsHIII).

In this report, we present evidence that a substantial fraction of males with full mutation alleles that are resistant to cleavage by methylation sensitive enzymes, and which produce little or no FMRP, nevertheless produce FMR1 mRNA, with some mRNA levels approaching those found in normal subjects. We show further that the mRNA is unlikely to be produced from low levels of premutation alleles. Thus, it is not generally valid to assume that enzyme resistance and/or absence of FMRP are indicative of a transcriptionally silent FMR1 gene.

Methods

Genomic DNA was isolated from peripheral blood leucocytes using Puregene kits (Gentra Inc). Allele sizes and methylation status were determined by standard Southern blot analysis, using NruI as the methyl sensitive restriction enzyme1 and the FMR1 specific probe StB12.3.1 For the detection of premutation alleles, CGG repeat lengths were determined by PCR analysis using primers 1 and 3.4 Total RNA was isolated from peripheral blood using Purescript kits (Gentra Inc). cDNA synthesis was as described in Tassone et al.5 Quantitative RT-PCR measurements of relative FMR1 mRNA levels were carried out using the 5' fluorogenic RT-PCR assay.6 7 Details and probe specific sequences for the FMR1 gene and for the reference gene (glucoronidase) were as described in Tassone et al.6

![Figure 1](http://jmg.bmj.com/)

**Figure 1** FMR1 mRNA levels in peripheral blood leucocytes for males with methylated, full mutation alleles, plotted as a function of the CGG repeat number (smallest allele when multiple bands are present). FMR1 mRNA levels are normalised to the mean level for normal controls.1 Grey triangles indicate positions of smallest CGG repeats for subjects with no detectable FMR1 mRNA after 30 cycles of fluorescent RT-PCR (<0.1%). Open/closed squares indicate repeat mRNA analyses from separate blood samples for two subjects.
were repeated in the six lanes to the left, with lane separation to minimise lane overlap detection at the 1% level for both premutation alleles. Lower mixing concentrations (allele sizes are indicated over the lanes. Repeat analyses (data not shown) always showed amounts of premutation alleles (81 or 126 CGG repeats) have been added. Percentages and

---

**Figure 2.** Southern blot analysis of the methylation status of the FMR1 CG rich region for four males with full mutation alleles. For each subject, 5 µg of genomic DNA isolated from peripheral blood leucocytes was digested with EcoRI and either NruI (N), EagI (E), or BssHII (B). The blot was probed with StB12.3 as previously described. A female (premutation) control (C) is also presented (cleavage with NruI). The positions of the normal unmethylated (2.8 kb) and normal methylated (5.2 kb) bands are indicated.

![Southern blot analysis](image1)

% added 81 % added 126

1 0.5 0.1 1 0.5 0.1 0.1 0.5 1 2 5 10 20 100

% added 126

1 0.5 0.1 1 0.5 0.1 0.1 0.5 1 2 5 10 20 100

---

**Figure 3.** PCR analysis of a full mutation allele (263 CGG repeats) to which defined amounts of premutation alleles (81 or 126 CGG repeats) have been added. Percentages and allele sizes are indicated over the lanes. Repeat analyses (data not shown) always showed detection at the 1% level for both premutation alleles. Lower mixing concentrations (≤1%) were repeated in the six lanes to the left, with lane separation to minimise lane overlap (20-24 hour exposure).

![PCR analysis](image2)

---

FMRF expression was determined as the percentage of FMRF positive lymphocytes from blood smears using the immunocytochemical approach and anti-FMRF monoclonal antibody from hybridoma clone 1C3-a. Details of the method are described elsewhere. Western blot analysis on total protein isolated from lymphoblastoid cell lines was performed as described in Tassone et al.

**Results**

To address the linkage between enzyme resistance and silencing, we have examined FMR1 mRNA and FMRF levels in peripheral blood leucocytes from 50 males. Two males possessed deletions that were transcriptionally silent. Forty eight subjects possessed full mutation alleles, ranging from 230 to >700 CGG repeats, which were resistant to cleavage by at least one methyl sensitive restriction enzyme, and which did not include premutation alleles (<1%) as determined by combined Southern and PCR analysis.

Results of quantitative RT-PCR measurements of relative FMR1 mRNA levels are shown in fig 1. Surprisingly, we found that leucocytes from the majority (60%) of subjects continue to express some FMR1 mRNA, with six males (12%) having FMR1 mRNA levels in the same range as observed in normal subjects despite essentially complete resistance to cleavage by at least one restriction enzyme. Two additional males have FMR1 mRNA levels between 40–70% of normal, eight have levels between 10–40%, and 13 possess detectable levels (~1-10%). Nineteen males (40%) with alleles of comparable size did not yield any detectable FMR1 mRNA after 50 cycles of PCR (<0.1%). Thus, more than half of the full mutation males in the current group continue to produce some FMR1 mRNA in leucocytes despite satisfying the enzymatic criteria for hypermethylated, full mutation alleles.

The methylation status of additional restriction sites (EagI and BssHII) was also examined for four subjects with the highest levels of FMR1 mRNA (fig 2). Densiometric analysis of the film presented in fig 2 generally showed the absence of unmethylated alleles (<1% of total density between 2.8 kb and 5.2 kb bands) with at least one enzyme. We did note the presence of some density (~15%) in the BssHII lane of sample 3, with faint densities in the EagI lane of sample 3, and in the NruI and EagI lanes of sample 2. This last observation could reflect either incomplete methylation at the corresponding cleavage sites (although no unmethylated alleles were detected in the PCR assay, see below) or background contamination in those lanes. Consistent with this observation, Stöger et al observed incomplete methylation at the BssHII site for alleles that were completely methylated at the NruI site (intraallelic mosaicism of the methylation epigenotype) in addition to their observations of all or none methylation (interallelic mosaicism).

Genomic DNA from all 50 subjects (including the two deletions) was also analysed by PCR as described in Brown et al. None of the samples yielded any evidence of premutation alleles after 30 cycles, and for the four subjects presented in fig 2, no bands were detected after 35 cycles of PCR. To check the sensitivity of our PCR assay for low levels of premutation alleles, we performed replicate, standard PCR reactions in which a full mutation DNA sample (263 CGG repeats) was mixed with varying percentages (0.1 to 20%) of premutation alleles having either 81 or 126 repeats (fig 3). The full mutation allele was chosen based on its relatively high FMR1 mRNA level (1.28 ± 0.18, fig 2). In all PCR reactions, the total initial DNA concentration was held constant at 200 ng. In all experiments, premutation alleles were detected at the 1% level. This level of sensitivity (~1%) using PCR has also been reported by de Graaff et al. Thus, our PCR protocol allows us to exclude the presence of premutation alleles at the 1% level in the full mutation samples.
FMR1 gene expression

FMR1 gene expression

ated alleles in the full mutation range, are no

mRNA, so their

FMR1

jects with hypermethylated full mutation are

full mutation range. More than half of the sub-

corresponding transformed lines. In this study,

chemistry and western blotting, the latter using

reduced, as determined by both immunocyto-

ation range,5 despite moderate reductions in

More recently, we have extended these obser-

vations into the full mutation range for

unmethylated and partially methylated alle-

le.13 Again, FMR1 mRNA levels were found to

be increased by as much as six-fold over

ormal, with FMRP levels that were invariably

reduced, as determined by both immunocyto-

chemistry and western blotting, the latter using

Corresponding transformed lines. In this study,

we have extended these observations into the

full mutation range. More than half of the sub-

jects with hypermethylated full mutation are

producing significant amounts of FMR1

mRNA, so their FMR1 genes are clearly not

silenced.

The highest FMR1 mRNA levels found to
date, for either premutation or for unmethyl-

ated alleles in the full mutation range, are no

more than roughly ten-fold increased over nor-

nal levels.8 Therefore, premutation alleles, if

present at or below the 1% level, could not

account for FMR1 mRNA levels that are above

10% of normal levels, roughly one third of the

males in the current study. For males with rela-
tive mRNA levels in the 1-10% range (fig 1), we

cannot rule out the possibility that an unde-
tectably small fraction of unmethylated alleles
(<1%) gives rise to part or all of the residual

mRNA. These results, which agree with previ-

ous findings that fully methylated, full muta-
tion alleles are mitotically stable,16 17 establish

that the observed mRNA levels do not result

from an undetectably small fraction of cells

with premutation alleles. In addition, in a pre-

vious study, de Graaff et al noted that in a lung
tumour sample from a fragile X patient with a

fully methylated premutation allele (∼160

repeats), 30-40% of cells were expressing

FMRP by immunocytochemistry. These au-

thors argued that such a high percentage of

FMRP positive cells could not be the result of

smaller, unmethylated alleles, since those

alleles would not have gone undetected in their

DNA assays. Although the observations of de

Graaff et al4 were based on expression patterns

in tumour tissue, which may be substantially
dysregulated, their results, along with those of

the current work, raise an important caveat

regarding the assumed relationship between

resistance to enzyme cleavage and transcrip-
tional silencing.

Thus, for many full mutation alleles that are

resistant to cleavage with methylation sensitive

restriction enzymes, the FMR1 gene

nevertheless remains active. In fact, for six sub-

jects, FMR1 mRNA levels are approximately
equal to the mean level for normal subjects.1

However, it should be noted that in the context

of full mutation alleles, “normal” levels prob-

ably represent a significant transcriptional
deficit, since leucocytes with unmethylated

alleles of the same length have mRNA levels

that are four to six-fold increased.13

We do not yet know why some subjects in the
current group produce FMR1 mRNA while

others do not, although we favour the proposal

that methylation of a specific subset of CpG
elements is critical for silencing.13 14 18 Given

the broad range of mRNA levels in fig 1 (for

example, greater than 100-fold range of mRNA

levels among males with methylated alleles of

∼330 repeats), it is likely that expression levels

are influenced by methylation at several CpG

sites. We wish to stress that we cannot

distinguish between intra-allelic and interallelic

mosaicism for many of the subjects represented

in fig 1, particularly for those with relative

mRNA levels that are below 10-20% of

normal. However, for the alleles with the high-
est expression levels, interallelic mosaicism is

unlikely, since we would have been able to
detect 10-20% of completely unmethylated

alleles.

In the current study, none of the subjects

examined (including two in the highest mRNA

group) expressed more than 10% FMRP posi-
tive lymphocytes (range 0-10%, mean 4.6%,

99% CI 2.7-6.5%), measured by immuno-

cytochemical staining.16 17 The small percentages

of FMRP(+) cells may represent, in part,

intrinsic errors in the staining method, since

western analysis of protein levels for lympho-

blastoid lines derived from six members of the

current group, two with mRNA levels in the

6-8% range (263, 363 repeats, fig 1) and four

with no detectable mRNA, showed no FMRP

expression.

In summary, full mutation alleles that appear

to be predominantly methylated may not be

silent despite low/absent FMRP expression.

Thus, it is not always correct to conclude that

the FMR1 gene is “silent” on the basis of either

enzymatic criteria or low/absent protein levels.

The low protein levels probably reflect a post-

transcriptional effect such as a defect in transla-
tion.13 15 Any significant deficit in translation of

the FMR1 mRNA is of central importance

for therapeutic approaches aimed at recovering

function from the endogenous gene, since it

is not sufficient simply to reactivate the gene itself

without also addressing the subsequent prob-

lem of translation. Clearly, there may be differ-

ences between leucocytes and neurones in the

level of expression of the FMR1 gene, with the

possibility that hypermethylated alleles are not

transcribed in neuronal cells; a better under-

standing of this issue must await more detailed

analysis of neural tissues. Furthermore, be-

cause methyl sensitive restriction enzymes test

for methylation at only a few CpG positions, it

is possible that silencing of the FMR1 gene

is the result of focal CpG methylation events at

other positions that are not probed by the

www.jmedgenet.com
enzymes. Finally, it is possible that the range of mRNA levels reflects differences in the degree of histone acetylation for equivalent epigenotypes. These possibilities are currently being investigated.

This work was supported by the Cooper/Kraft/Fishman Family Fund and Booby Family Fund (to PJH and RJH), a FRAXA Research Foundation Fellowship (to FT), National Institutes of Health Grants GM35305 (to PJH) and HD36071 (to RJH), and a Maternal Child Health Bureau grant MCH-089413 (to RJH).
