Am. J. Hum. Genet. 66:6–15, 2000

Elevated Levels of *FMR1* mRNA in Carrier Males: A New Mechanism of Involvement in the Fragile-X Syndrome

Flora Tassone,[1] Randi J. Hagerman,[2,3] Annette K. Taylor,[4] Louise W. Gane,[3] Tony E. Godfrey,[5] and Paul J. Hagerman[1]

[1]Departments of Biochemistry and Molecular Genetics and [2]Pediatrics, University of Colorado School of Medicine, [3]Child Development Unit and Fragile X Treatment and Research Center, The Children's Hospital, and [4]Kimball Genetics, Inc., Denver; and [5]University of California San Francisco Cancer Center, San Francisco

Summary

Fragile-X syndrome is a trinucleotide-repeat–expansion disorder in which the clinical phenotype is believed to result from transcriptional silencing of the fragile-X mental retardation 1 (*FMR1*) gene as the number of CGG repeats exceeds ~200. For premutation alleles (~55–200 repeats), no abnormalities in *FMR1*-gene expression have been described, despite growing evidence of clinical involvement in premutation carriers. To address this (apparent) paradox, we have determined, for 16 carrier males (55–192 repeats), the relative levels of leukocyte *FMR1* mRNA, by use of automated fluorescence-detection reverse transcriptase–PCR, and the percent of lymphocytes that are immunoreactive for *FMR1* protein (FMRP). For some alleles with >100 repeats, there was a reduction in the number of FMRP-positive cells. Unexpectedly, *FMR1* mRNA levels were elevated at least fivefold within this same range. No significant increase in *FMR1* mRNA stability was observed in a lymphoblastoid cell line (160 repeats) derived from one of the carrier males, suggesting that the increased message levels are due to an increased rate of transcription. Current results support a mechanism of involvement in premutation carriers, in which reduced translational efficiency is at least partially compensated through increased transcriptional activity. Thus, diminished translational efficiency may be important throughout much of the premutation range, with a mechanistic switch occurring in the full-mutation range as the *FMR1* gene is silenced.

Received August 18, 1999; accepted for publication October 6, 1999; electronically published January 10, 2000.

Address for correspondence and reprints: Dr. Paul J. Hagerman, Department of Biochemistry and Molecular Genetics, University of Colorado School of Medicine, B-121, 4200 East Ninth Avenue, Denver, CO 80262. E-mail: paul.hagerman@uchsc.edu

0002-9297/2000/6601-0004$02.00

Introduction

In fragile-X syndrome (MIM 309550), clinical involvement is thought to be the result of reduced levels of the normal protein product of the fragile-X mental retardation 1 (*FMR1*) gene. In the standard model of *FMR1* expression, protein levels are reduced as a direct consequence of transcriptional silencing of the *FMR1* gene, which occurs when it becomes fully expanded (>~200 CGG repeats; full mutation) and methylated (Pieretti et al. 1991). In accord with this model, nearly all males with methylated, full-mutation alleles have mental retardation and have little (<10% of normal) or no *FMR1* protein (FMRP) in the peripheral lymphocytes (Willemsen et al. 1997; Tassone et al. 1999). Modest FMRP levels can occur in individuals who have fully expanded alleles with a partial or complete lack of methylation, or they can occur as a consequence of somatic mosaicism, in which some cells harbor alleles with <200 repeats. Individuals with higher FMRP levels usually present with a milder clinical phenotype, with learning disabilities occurring in the absence of mental retardation (Smeets et al. 1995; de Vries et al. 1996; Hagerman 1996; Tassone et al. 1999).

For those individuals with CGG-repeat numbers in the range of ~55–200, the term "premutation" has been coined, to reflect both the propensity for allele expansion in subsequent generations and the absence of direct clinical involvement (Fu et al. 1991; Oberlè et al. 1991). The findings of most clinical investigations of individuals—mainly females—with premutation alleles have demonstrated that the individuals have normal intellectual abilities (Mazzocco et al. 1993; Reiss et al. 1993; Rousseau et al. 1994). However, a limited number of females with the premutation do have mild physical and/or emotional problems (Franke et al. 1998; Riddle et al. 1998). Males with the premutation have also been characterized, and the results of several studies have demonstrated that at least some males have cognitive impairments (Loesch et al. 1987, 1994; Dorn et al. 1994; Rousseau et al. 1994; Smits et al. 1994; Hagerman et al. 1996; Steyaert et al. 1996). Findings of clinical in-

volvement in the premutation range suggest the need to reexamine the standard model—and the concept of the premutation—at the molecular level.

Expression of the *FMR1* gene has not been investigated, in a systematic fashion, for alleles in the premutation size range. *FMR1* mRNA and FMRP levels were reported to be normal in lymphocytes and in cultured lymphoblastoid and fibroblast cell lines from individual carriers of a premutation (Pieretti et al. 1991; Devys et al. 1993; Feng et al. 1995*a,* 1995*b;* Hmadcha et al. 1998). Barring the possibility of occult expansion in other tissues, these latter observations present an apparent paradox for the standard model, since clinical involvement in some males and females with premutation alleles leads to the expectation of reduced *FMR1* mRNA and/or FMRP levels. To examine this issue, we have employed a quantitative-fluorescence reverse transcriptase–PCR (RT-PCR) method (Livak et al. 1995; Heid et al. 1996), to obtain precise estimates of *FMR1* mRNA levels in peripheral blood leukocytes. This method employs a dual-labeled, fluorogenic hybridization probe, to provide accurate and reproducible quantification of mRNA levels. Quite unexpectedly, for premutation alleles in the 100–200-repeat range, *FMR1* message levels are approximately fivefold higher than the levels found in normal individuals. These elevated mRNA levels are present even in the face of lowered percentages of FMRP-positive (FMRP[+]) lymphocytes in this repeat range. Our observations suggest that mechanisms other than reduced transcription (e.g., blocks in nuclear export or translation) are responsible for the FMRP deficit and, ultimately, for clinical involvement in the premutation range.

Material and Methods

Isolation and Analysis of Genomic DNA

Genomic DNA was isolated from peripheral blood leukocytes that were derived from 3–5 ml of blood obtained from carrier males or normal controls and from lymphoblastoid cell lines. This research was performed with protocols approved by the local institutional review board and with signed, informed consent. DNA isolation employed Puregene kits (Gentra). Southern blot analyses were performed for all carrier males, as described elsewhere (Taylor et al. 1994). For each analysis, 5 μg DNA were digested with the use of *Eco*RI and *Nru*I; this was followed by electrophoresis in 1% agarose/Tris-acetate gels and by transfer to nylon membranes. Blots were hybridized with the *FMR1*-specific probe StB12.3 (Oberlè et al. 1991). Standard PCR analysis was also used to determine the number of CGG repeats in carrier males, by use of primers "1" and "3," as described elsewhere (Brown et al. 1993). Of the 16 males with CGG-expansion (premutation) alleles represented in the present investigation, 9 possess alleles within the 55–100-repeat range (n = 55, 58, 60, 66, 73, 81, 84, 85, and 94), and 7 possess alleles within the 100–200-repeat range $(CGG)_n$, n = 113, 126, 130, 133, 180+, 183, and 190); the plus sign (+) designates a smear of unmethylated alleles extending from 180 to 280 repeats, on the basis of the results of a Southern blot. The aforementioned groups were compared with eight control individuals with alleles within the normal repeat range (n = 25, 28, 29, 30, 35, 39, 44, and 54).

Total-RNA Isolation and cDNA Synthesis

Total cellular RNA was prepared from 3–5 ml of blood, by use of standard methods (Purescript kits [Gentra]; Trizol [BRL]), and was quantified by total absorbance at 260 nm. RT reactions were performed in 100-μl aliquots containing 1 × PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl) (Gibco/BRL), 5.5 mM $MgCl_2$, 1 mM each dNTP, 5 μM random sequence deoxyoligonucleotide hexamers (Gibco/BRL), 0.4 U RNAse inhibitor (Gibco/BRL), and 2.5 U Moloney murine leukemia-virus RT (Gibco/BRL). At least three concentrations of total RNA (500 ng, 250 ng, and 125 ng, per 100-μl reaction) were used for each sample, to ensure linearity of the RT-PCR response. The RT temperature profile was as follows: 25°C for 10 min, 48°C for 40 min, 95°C for 5 min, and final cooling to 4°C. As a control for genomic contamination, 500 ng total RNA were treated as described above, with the exception that the RT was omitted.

Quantitative Fluorescence PCR for Determination of Relative FMR1 *mRNA Levels*

Fluorescence PCR reactions were performed with the use of the 7700 Sequence Detector (PE Biosystems) (Livak et al. 1995; Heid et al. 1996). The instrument determines relative abundances of mRNA, by use of real-time fluorescence detection of dual-labeled (TaqMan) probes, 5′-FAM (6-carboxyfluorescein)-deoxyoligonucleotide-TAMRA (6-carboxytetramethylrhodamine)-3′ (PE Biosystems), which are complementary to the PCR amplicon. A passive reference dye, ROX (PE Biosystems User Bulletin), is used to normalize all fluorescence signal intensities. During PCR amplification, the fluorescence intensity of the reporter dye (f_{FAM}) is normalized to the fluorescence intensity of the reference dye (f_{ROX}) ($R_n \equiv f_{FAM}/f_{ROX}$). The baseline value for R_n is determined during the first 10–15 cycles (Heid et al. 1996), as is the standard deviation (SD) of $R_n(\sigma_{Rn})$. The change in R_n, $\Delta R_n(\equiv R_n - R_{n,baseline})$, is directly proportional to the number of copies of the amplicon (Gibson et al. 1996). PCR primers were synthesized from β-cyanoethylphosphoramidites, by use of a Millipore Expedite

automated synthesizer, model 8909. TaqMan probes were obtained from Applied Biosystems/PE or Synthetic Genetics. Cross-comparisons of quantitative-PCR results were performed with the use of at least two of three 7700 Sequence Detectors, one of which was located at the University of California, San Francisco Cancer Center, and two of which were located at the University of Colorado School of Medicine. There was no instance of a significant difference in estimated mRNA level among the instruments.

In the present investigation, the *FMR1* amplicon is a 122-bp product that spans the junction between exons 3 and 4 of the *FMR1* gene (GenBank accession number L29074), thus eliminating false signals resulting from genomic contamination. The following *FMR1* primer/probe set was used: forward (F) primer (F-*FMR1*), 5′-GCA GAT TCC ATT TCA TGA TGT CA-3′; reverse (R) primer (R-*FMR1*), 5′-ACC ACC AAC AGC AAG GCT CT-3'; and TaqMan probe, 5′-(FAM)-TGA TGA AGT TGA GGT GTA TTC CAG AGC AAA TGA-(TAMRA)-3′. The relative abundance of *FMR1* mRNA, as well as variations encountered during sample preparation, was assessed by use of a reference (81-bp) amplicon derived from β-glucoronidase (*GUS*) mRNA (GenBank accession number NM000181). *GUS* mRNA represents a convenient reference, since we have observed that normal peripheral blood leukocytes maintain comparable levels of *FMR1* and *GUS* mRNAs. The *GUS* amplicon spans the junction between exons 11 and 12 of the *GUS* gene and utilizes the following primer/probe set: F-*GUS*, 5′-CTC ATT TGG AAT TTT GCC GAT T-3′; R-*GUS*, 5′-CCG AGT GAA GAT CCC CTT TTT A-3′; and *Taq*Man (sense) probe, 5′-(FAM)-TGA ACA GTC ACC GAC GAG AGT GCT GG-(TAMRA)-3′.

For each blood sample, quantitative-PCR reactions were performed in duplicate for each starting total RNA concentration, for both *FMR1* and *GUS* amplicons (a total of 12 reactions). Control reactions (*FMR1* and *GUS*) were run in parallel (an additional 12 reactions), with the use of a standard lymphoblastoid line known as "RP" (RPM 7666; normal lymphoblasts [ATCC CCL114]). The control reactions are necessary for correction of potential variations in buffer composition and/or pipetting errors and for correction of relative efficiencies of RT reactions. PCR amplifications were performed in 50-μl reaction volumes containing 1 × PCR buffer (Applied Biosystems/PE), 5.5 mM $MgCl_2$, 0.1 μM each primer and probe, 200 μM each dNTP, and 0.025 U *Taq* Gold (Applied Biosystems/PE)/μl. Cycle parameters were as follows: 95°C for 12 min, then (95°C for 15 s, 60°C for 1 min) × 50 cycles, and then a final temperature of 25°C.

Data Analysis

The number of cycles required for FAM fluorescence to reach a threshold level that is ~10 SDs (of fluctuations in background fluorescence, σR_n) above the mean background fluorescence ($\equiv C_t$ value) was determined for each PCR reaction (*FMR1*, *GUS*, and RNA concentration), by use of 7700 automated software. Plots of C_t versus total RNA were performed for each sample, to verify appropriate log-linear behavior ($2^{-C_t} \propto$ total RNA). For a perfect log-linear response, each cycle represents one doubling of the number of amplicon DNA molecules; thus, a ΔC_t of 1 reflects a twofold difference in RNA concentration. All relative *FMR1* mRNA levels were determined from the following relation: *FMR1* $mRNA_{rel} = 2^{-[\Delta C_t(\text{sample}) - \Delta C_t(\text{RP})]} = 2^{-\Delta\Delta C_t}$, where ΔC_t(sample) [$=C_t(FMR1) - C_t(GUS)$] is the difference, in threshold cycle number, between *FMR1* and *GUS,* for a given sample and total-RNA concentration; where ΔC_t(RP) is the same difference for the control cell line; and where $\Delta\Delta C_t$ is the cell-line–corrected difference. This approach will be discussed in more detail below. RT-PCR amplifications were also examined, by means of PAGE (12%), to verify the presence of the correct amplicon. After ethidium bromide staining, single bands were visible only at the expected positions for the *FMR1* and *GUS* amplicons. Furthermore, control experiments done with the use of an *FMR1* deletion (Gu et al. 1994) extending from ~80 kb upstream of the *FMR1* gene through exon 7 (thus deleting the entire 5′ regulatory region and the $[CGG]_n$ repeat as well as the targets of the PCR primers) did not produce detectable *FMR1* amplicon after 50 cycles of PCR. All analyses of C_t data, including nonpaired *t*-test and regression analyses, were performed on SigmaPlot (SPSS, Inc.).

Immunocytochemistry

The percentage of lymphocytes expressing detectable FMRP was determined by immunocytochemistry of blood smears (Willemsen et al. 1995, 1997; Tassone et al. 1999), with use of the anti-FMRP monoclonal antibody from hybridoma clone 1C3-a (Devys et al. 1993). The method is an indirect alkaline phosphatase–staining technique, in which cells fixed with 3% paraformaldehyde and permeabilized with methanol are incubated with FMRP-specific antibody, followed by incubation with goat anti-mouse immunoglobulin conjugated with biotin (DAKO) and a final incubation with streptavidin-biotinylated alkaline phosphatase (DAKO). Visualization of FMRP is accomplished with the use of a fuchsin-substrate chromagen system (DAKO). For each blood sample, 200 lymphocytes are counted, and FMRP(+) lymphocytes are scored for the presence of a red-staining cytoplasmic ring that indicates the presence of FMRP.

Cell Culture and Inhibition of Transcription

Lymphoblastoid cell lines were grown in RPMI 1640 supplemented with 12% FCS and glutamine. For deter-

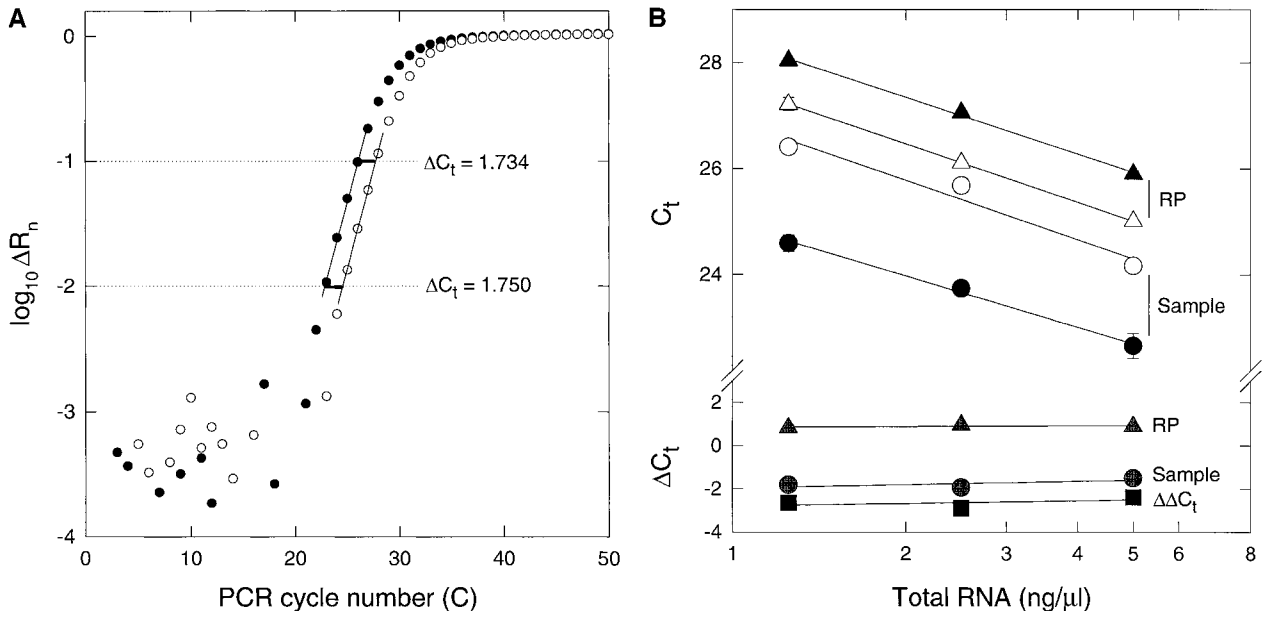

Figure 1 Outline of analysis of *FMR1* mRNA levels, by use of automated, fluorescence-detection RT-PCR, with peripheral blood leukocytes obtained from a male premutation carrier with 192 CGG repeats. *A,* Logarithm of the relative reporter (FAM) fluorescence intensities ΔR_n, for *FMR1* (*blackened circles*) and *GUS* (*unblackened circles*) probes, plotted as a function of the cycle number (C) in the PCR reaction (total cellular RNA 5 ng/µl). For a set of curves, C_t is the cycle number at a given threshold value of probe fluorescence (see the Material and Methods section). ΔC_t is the difference in cycle number for a given fluorescence threshold. *B,* Plots of C_t and ΔC_t, as a function of total cellular RNA. C_t plots: blackened triangles denote the *FMR1* probe, control (RP) cell line; unblackened triangles, *GUS* probe, RP cell line; unblackened circles, *GUS* probe, carrier male; blackened circles, *FMR1* probe, carrier male. ΔC_t plots: gray-shaded circles, C_t(*FMR*1) − C_t(*GUS*), carrier male; gray-shaded triangles, C_t(*FMR*1) − C_t(*GUS*), RP cell line. $\Delta\Delta C_t$ (*blackened squares*) represents the corrected value for the difference in cycle number for *FMR1* mRNA versus *GUS* mRNA and is used to determine relative mRNA levels (see the Material and Methods section).

mination of *FMR1* mRNA stabilities, actinomycin D (10 µg/ml) was used to block transcription (Baeyens and Cornett, 1993; Feng et al. 1995*a*). Lymphoblastoid cell lines that were established, by means of Epstein-Barr virus transformation, from normal males and carrier males were plated at a density of 1×10^6 cells/ml in 50 ml media. After 24 h, actinomycin D was added, and aliquots of suspended cells were removed at designated times. Cells were washed in PBS, and total RNA was isolated as described above. Samples were subjected to fluorescence RT-PCR, which was performed as described above.

Results

Automated Fluorescence-Detection RT-PCR as a Quantitative, Reproducible Method for Measurement of FMR1 *mRNA Levels*

We have used an automated, fluorescence-detection RT-PCR assay to quantify the levels of *FMR1* mRNA in carrier males with CGG-expansion alleles lying within the range of ~55–200 repeats (premutation alleles). The basis of this method is the accurate determination of the increase in *FMR1*-specific amplicon DNA concentration during the early (log-phase) cycles of the PCR process. In the fluorescence-based assay, the increase in DNA concentration is monitored by the use of a complementary, dual-fluorophore–labeled oligonucleotide probe (*Taq*Man probe), with the extent of fluorescence being directly proportional to DNA copy number. An overview of the analysis, for a carrier male with 192 CGG repeats, is presented in figure 1.

In the present study, we used *FMR1*- and *GUS*-based probes that possess a FAM reporter at the 5′ ends and a TAMRA quencher at the 3′ ends. During the extension phase of each PCR cycle, the FAM reporter is cleaved from the probe, as a result of the 5′ exonuclease activity of the polymerase, thereby resulting in an incremental increase in fluorescence intensity. The increases in fluorescence ΔR_n (see the Material and Methods section, above) for each cycle are monitored in real time, by use of a 7700 Sequence Detector (PE Biosystems).

Relative mRNA levels are determined by a comparison of the cycle numbers for which the relative fluorescence signal, ΔR_n, exceeds the threshold value (threshold cycle number is designated "C_t" (see the Material and Methods section above). The precise value of the threshold fluorescence is not critical, provided that it lies in the log-phase region. For example, for the carrier male

Table 1

Relative *FMR1* mRNA Levels in Peripheral Blood Leukocytes of Males with Premutation Alleles

CGG Repeat (n)[a]	Sample Size[b]	*FMR1* mRNA levels (Mean ± SD) Relative to *GUS*[c]	Relative to nl Sample Mean
$100 < n < 200$ (hp)	7 (14)	7.21 ± .63 (7.15 ± .47)	5.1 ± .68
$55 < n < 100$ (lp)	9 (14)	2.44 ± .32 (2.75 ± .25)	1.7 ± .41
$n < 55$ (nl)[d]	8	1.42 ± .25	1.00

[a] Groupings represent all individuals with alleles $(CGG)_n$ in the designated size range.

[b] Sample sizes in parentheses include repeat analyses of RNA of some individuals within the group. Results of repeat analyses are averaged but are otherwise unweighted for the smaller numbers.

[c] All *FMR1* mRNA levels relative to *GUS* are normalized to the level of *FMR1* mRNA relative to *GUS* in a reference cell line (RPM 7666), as described in the Material and Methods section. Numbers in parentheses include repeat analyses, as specified above (see footnote b).

[d] Normal control individuals include females; there is no significant difference, in *FMR1* mRNA levels, between males and females in this range.

(192 CGG repeats) represented in figure 1*A*, the ΔC_t value of −1.750 [= $C_t(FMR1_{carrier}) - C_t(GUS_{carrier})$], determined at $\Delta R_n = 0.01$, differs, by only ~1%, from the value determined at a 10-fold-higher fluorescence threshold level ($\Delta C_t = -1.734$, at $\Delta R_n = 0.1$); note, however, that the threshold level must be held constant for any given comparison. For all of the samples analyzed in the current investigation, C_t values fall within the range of ~25–30. Within this range, all samples display the expected log-linear dependence of C_t on total cellular RNA concentration.

Although blood samples are routinely processed <24 h after the blood draw, delays are sometimes encountered during transportation of the sample. We have investigated the influence of delayed RNA extraction, by isolating RNA, at various times, from aliquots of blood (stored at 4°C) obtained from a carrier female. For extractions performed 0, 2, 5, and 7 d postdraw, no reduction in either *FMR1* or *GUS* mRNAs was observed. In addition, we generally see no significant variation in *FMR1* mRNA levels for either normal individuals or individuals with premutations who have had blood drawn on more than one occasion.

Sets of replicate RT-PCR experiments for each blood sample are always accompanied by equivalent sets of reference measurements for the lymphoblastoid cell line (RPM 7666 [RP]; see the Material and Methods section above). The ΔC_t values determined for each blood sample ($\Delta C_{t,sample}$) are normalized to the corresponding ΔC_t values for the cell line ($\Delta C_{t,RP}$); thus, $\Delta\Delta C_{t,sample} = \Delta C_{t,sample} - \Delta C_{t,RP}$. This latter correction takes into account batch-to-batch variations in *Taq* polymerase, probe and/or primer efficiency, and variations in RT efficiency. This approach thus allows one to directly compare data generated in different experiments and reduces experimental uncertainty in relative mRNA levels. Failure to correct for such variation in RT-PCR experiments would severely degrade the precision of experimental comparisons of mRNA concentration. A second procedure—namely, an analysis of the concentration dependence of the C_t and ΔC_t values (fig. 1*B*)—is also performed for each sample and cell line control. Strong variation in ΔC_t may indicate problems with pipetting; such experiments can be repeated.

Significant Elevation of FMR1 *mRNA Levels in Carrier Males*

We have determined the relative *FMR1* mRNA levels for 16 carrier males, by means of the fluorescence-based RT-PCR approach. Nine males possess alleles with a range of 55–100 repeats (designated as "low premutation" [lp]), and 7 possess alleles with a range of ~100–200 repeats (designated as "high premutation" [hp]) (for allele sizes, see the Material and Methods section above). Longer exposures (3–5 d) of Southern blots were performed, to eliminate the possible presence of methylated alleles. Note that one member of this latter group (180+; see the Material and Methods section above) possesses a smear of allele sizes that extends from 180 repeats into the low full-mutation range (≤280 repeats). Because all of his alleles were unmethylated, which is typical of a premutation, his mRNA data were included. Allele sizes were determined by conventional PCR analysis, which was augmented in some instances by Southern gel analysis. These groups were compared with a group of eight normal control individuals. The

Table 2

Analysis of Significance for the Differences in *FMR1* mRNA Levels among Three Allele Ranges (Nonpaired *t*-Test)

Comparison[a]	P[b]
hp 7 (14) vs. lp 9 (14)	4.3×10^{-6} (1.2×10^{-8})
hp 7 (14) vs. nl 8	6.5×10^{-7} (3.3×10^{-8})
lp 9 (14) vs. nl 8	2.5×10^{-2} (2.5×10^{-3})

[a] Sample groupings are as in table 1.

[b] Derived from nonpaired *t*-tests. Numbers outside the parentheses are based on unweighted averages of repeat measurements for individuals within each group (see table 1).

relative *FMR1* mRNA levels for these three groups are presented in table 1; an analysis of the significance of the differences reported for these three groups is presented in table 2. The relative *FMR1* mRNA levels for each individual are presented in figure 2.

It is evident, from the data presented in table 2, that *FMR1* mRNA levels are elevated in both subgroups of carrier males, relative to those in the normal control population. Moreover, there is a substantial (fivefold) elevation in the upper portion (hp) of the premutation range relative to the lower portion (lp). We recognize that the boundary between hp and lp (100 CGG repeats) is somewhat arbitrary in the absence of an underlying mechanistic basis; however, it does serve to underscore the growth in relative mRNA levels as one approaches the upper portion of the premutation range. Moreover, the current division yields a more conservative estimate for the increases in *FMR1* mRNA than do regression analyses (see the Discussion section, below). Also significant is the fact that, in our limited group, we have not observed any hp males with *FMR1* mRNA levels in the normal range. No *FMR1* mRNA was detected, after 50 PCR cycles, for a male with an *FMR1* deletion (Gu et al. 1994) that encompasses the 5′ portion of the *FMR1* gene. Finally, the absence of any reduction in *GUS* mRNA levels in hp samples relative to the levels in nl samples (data not shown) rules out general suppression of *GUS* in carrier leukocytes.

mRNA Decay Experiments as Evidence That the Elevated FMR1 *mRNA Levels Are Not a Result of Increased mRNA Stability*

To determine whether the increased *FMR1* mRNA levels are due to increased mRNA stability in carrier males, we have estimated the half-lives ($t_{\frac{1}{2}}$ values) for the decay of both *FMR1* and *GUS* mRNAs from an EBV-transformed lymphoblastoid line MM, derived from the carrier male with 183 CGG repeats. DNA analysis of the transformed cell line indicated a small reduction in repeat number (to 160 repeats). A second line (AG09391, "AG"; NIA Cell Repository) was used as a normal control. After treatment of cells in culture with the RNA polymerase II inhibitor actinomycin D (10 μg/ml), mRNA levels were measured, over time, by use of the fluorescence-based RT-PCR method. The results of this analysis are presented in figure 3 and in table 3. The absence of any significant increase in *FMR1* half-life in the premutation cell line, coupled with the approximately eightfold increase in mRNA level, indicates that increased stability cannot account for the higher mRNA level. This observation implies that the increase in *FMR1* mRNA levels in the carrier males is the result of an increased rate of mRNA production.

Immunocytochemical Staining as Revealing a Reduced Percentage of FMRP(+) Lymphocytes in Carrier Males with hp Alleles (100–200 CGG Repeats)

Production of FMRP is generally thought to be normal in the premutation range (Devys et al. 1993; Verheij et al. 1993; Feng et al. 1995*a;* Kaufmann et al. 1999; Tassone et al. 1999), although we have recently observed reduced fractions of FMRP(+) cells in three males with premutations with hp alleles (Tassone et al., in press). In two of the males, reduced FMRP production was also observed by western blot analysis (Hagerman et al. 1996; D. Nelson, personal communication). We have used the immunocytochemical staining method (Willemsen et al. 1995, 1997) to determine the percentage of

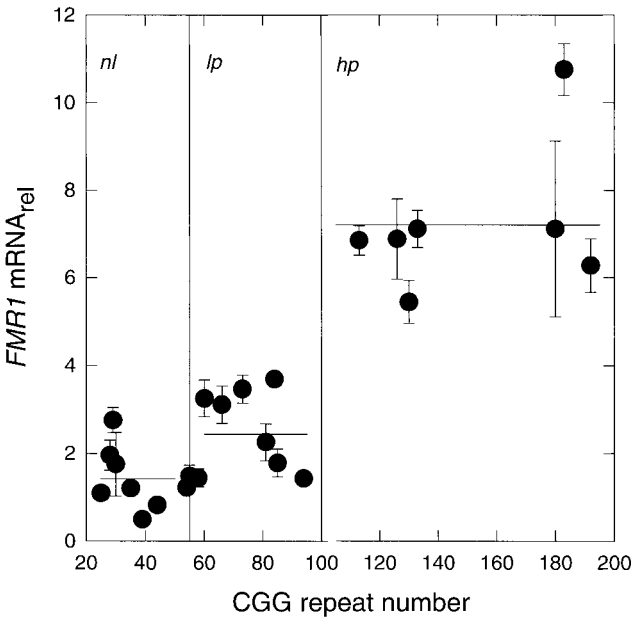

Figure 2 Plot of *FMR1* mRNA levels (relative to *GUS* mRNA [rel]), as a function of the CGG repeat number (n). nl, $n < 55$, lp = $55 \leqslant n < 100$, and hp = $100 \leqslant n \leqslant 200$. Horizontal lines represent the group (unweighted) means from table 1.

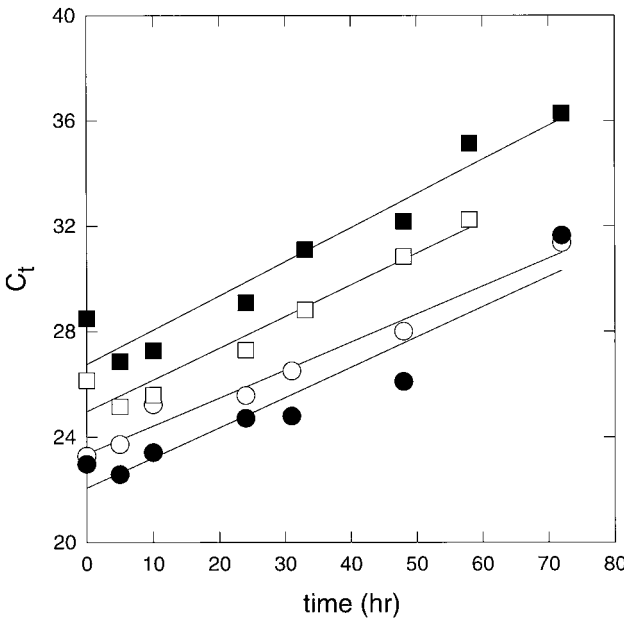

Figure 3 Plots of the increase in C_t values for *FMR1* and *GUS* mRNAs, as a function of time after treatment of cell lines with 10 μg actinomycin D/ml. *FMR1* (*blackened symbols*) and *GUS* (*unblackened symbols*) probes indicate results for both a normal control cell line, AG (*squares*), and a premutation cell line, MM (160 CGG repeats) (*circles*). Increasing C_t reflects decreasing mRNA level, with an increase of one unit representing a twofold decrease in probe-specific message.

FMRP(+) lymphocytes from several additional hp carrier males. On the basis of previous estimates of the fraction of FMRP(+) lymphocytes from 33 males with nonexpanded alleles (89% ± 9%) (Willemsen et al. 1997; also see Tassone et al. 1999), males with allele sizes that have a range of 100–200 repeats (fig. 4) appear to have reduced FMRP-protein expression. It should be noted that positive staining for FMRP does not directly report cellular levels of FMRP; rather, it reflects the presence of a threshold level of FMRP in each lymphocyte. Therefore, the results presented in figure 4 are interpreted simply as a reflection of reduced FMRP production in hp-carrier males.

Discussion

In the present study, we have demonstrated that males who are fragile-X carriers maintain substantially higher levels of *FMR1* message in peripheral blood leukocytes than do normal control individuals. Moreover, the magnitude of the increase appears to be approximately fivefold greater for larger alleles (~100–200 CGG repeats). Although our sample size is relatively limited, the increase is highly significant (table 2); we have not observed any males with alleles in that size range whose *FMR1* mRNA levels are not increased. Therefore, the current data establish the presence of an *FMR1*-specific, (molecular) phenotypic abnormality in males with alleles in the premutation size range.

There have been several previous reports of *FMR1* mRNA levels in the premutation range (Pieretti et al. 1991; Devys et al. 1993; Feng et al. 1995*a,* 1995*b;* Hmadcha et al. 1998). Pieretti et al. (1991) reported normal RNA levels in 10 females with premutations; however, no allele sizes were reported. Devys et al. (1993) reported normal mRNA levels in three lymphoblastoid lines with CGG repeats in the 90–100 range. Similar results were reported, by Feng et al. (1995*a*), for several lymphoblastoid lines with CGG repeats in the 84–104 range and, by Hmadcha et al. (1998), for a premutation male (leukocytes) with 101 repeats. These latter results are qualitatively consistent with the current results for the lp group, which show only a modest increase in mRNA level. Feng et al. (1995*b*) presented RNAse protection results for subcloned fibroblast cell lines from a single male with mosaicism. The relative *FMR1* mRNA levels were not reported for individual alleles within the premutation range, although the average mRNA level for all expanded alleles was stated to be only slightly (albeit not significantly) elevated, compared with the levels seen for normal alleles. It should be noted, however, that the average was taken over both premutation (lp and hp) and full-mutation alleles. Thus,

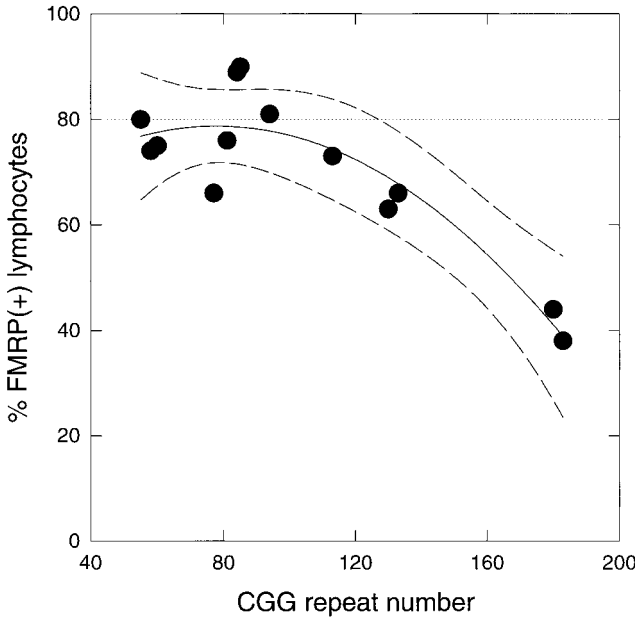

Figure 4 Plot of %FMRP(+) lymphocytes, as a function of CGG-repeat number. The standard error associated with each point is ~4%. The horizontal dotted line represents 1 SD below the mean value of the %FMRP(+) lymphocytes for males with nonexpanded alleles (Willemsen et al. 1997); dashed lines, 99% confidence limits for a second-order regression of the current data.

Table 3

Linear-Regression Analysis of Decay of *FMR1* and *GUS* mRNAs in Lymphoblastoid Cell Lines after Treatment with Actinomycin D

Cell Line[a]	$\Delta C_t(0)$[b]	$t_{\frac{1}{2}}(FMR1)$[c]	$t_{\frac{1}{2}}(GUS)$	$t_{\frac{1}{2}}(FMR1)/t_{\frac{1}{2}}(GUS)$
MM (CGG_{160})	−1.3	8.8 h	9.4 h	.94
AG09391	+1.8	7.7	8.3	.93

NOTE.—First-order regression analyses were performed on each of the decay profiles in figure 3, by use of the following functional form: $C_t = C_t(0) + (1/t_{\frac{1}{2}}) \times t(h)$.

[a] As described in the text.

[b] Values for each cell line were determined, from the *Y*-intercepts of first-order regression analyses of the *FMR1* and *GUS* mRNA decay curves, as the difference of the intercepts; thus, $\Delta C_t(0) = C_t(0)_{FMR1} - C_t(0)_{GUS}$. $\Delta\Delta C_t = 3.1$, $mRNA_{rel}(FMR1, MM) = 8.6$, where $\Delta\Delta C_t(0) = \Delta C_t(0)_{MM} - \Delta C_t(0)_{AG}$ and relative *FMR1* mRNA = $2^{-\Delta\Delta C_t(0)}$.

[c] Decay half-lives ($t_{\frac{1}{2}}$) are given by the reciprocal of the slopes of $C_t(t)$ for each curve.

it is difficult to make an allele-specific comparison of the results of the present study and those of the study by Feng et al. (1995*b*). Finally, it should be noted that the results of previous studies did not report controls for concentration dependence; such experiments are difficult to perform with the use of RNAse protection or gel-based RT-PCR methods. The ability to perform multiple control experiments represents a singular advantage of the automated method—one that we have found to be necessary for the accurate quantification of relative *FMR1* mRNA levels.

The current observations are particularly germane to the phenotypic status of males with premutations, since recognition of clinical involvement in this range is inconstant. Variation in reported prevalences of clinical involvement is due, in part, to the definition of involvement. In some studies, the definition is limited to cognitive impairment, whereas, in others, both cognitive and behavioral abnormalities are recognized (Dorn et al. 1994; Loesch et al. 1994; Hagerman et al. 1996; Franke et al. 1998; reviewed in Hagerman 1999). Furthermore, for at least some apparent carriers, cognitive and/or behavioral involvement may be the result of conditions unrelated to fragile-X syndrome (Hagerman 1999; Tassone et al., in press). Therefore, the observation of increased *FMR1* mRNA levels in males with premutations, apparent even for alleles with <100 repeats, establishes the presence of an abnormal, *FMR1*-specific molecular phenotype in the premutation range. The present observations provide a possible molecular foundation for the clinical observations in the premutation range, although the mechanistic linkage is not yet understood.

It is also interesting to speculate as to whether the elevated mRNA levels may give rise to a clinical phenotype that is unique to the premutation range. Although the current work has focused on carrier males, there is evidence, in a subgroup of carrier females, of a unique phenotype that includes premature menopause and a higher rate of twinning, which are features unobserved in females with the full mutation (Turner et al. 1994; Murray et al. 1998; Allingham-Hawkins et al. 1999). This issue is currently under investigation; however, the levels of *FMR1* mRNA in premutation females are likely to be influenced by the activation ratio.

An immunocytochemical approach has been used to identify the percentage of cell populations in which reduced levels of FMRP are expressed (Willemsen et al. 1995, 1997). Although this approach is not particularly sensitive to mild reductions in the FMRP level, we do observe, for the larger alleles within the premutation range, significant reductions in the percentages of FMRP(+) lymphocytes (fig. 4). In view of the elevated mRNA levels in this range, reduced FMRP expression is likely to reflect a translation defect that is beginning even in the premutation range. A translation defect has been reported in fibroblast cell lines (derived from one male with mosaicism) in the full-mutation range (Feng et al. 1995*b*).

The mechanistic basis for the elevated mRNA levels in carrier males is not understood at present. At the most basic level, such elevations could be the result of either increased transcriptional activity of the *FMR1* gene or greater stability of the message itself (reduced rate of loss) or both. Elevated levels of mRNA have been reported for the myotonic dystrophy protein kinase gene (*DMPK*) of myotonic dystrophy, another trinucleotide-repeat–expansion disease that involves a CTG expansion in the 3′ UTR region (Sabourin et al. 1993), although this issue is controversial (Waring and Korneluk 1998, pp. 131–146). We have examined the question of mRNA stability, by measurement of the rates of decay of *FMR1* mRNA in a lymphoblastoid cell line (160 CGG repeats) derived from a carrier male (183 CGG repeats) and from a normal control. That the decay half-lives for the normal and premutation lines are nearly equal (table 3) indicates that altered (increased) stability cannot account for the increased steady-state level of the *FMR1* mRNA.

The absence of a significant increase in stability of the *FMR1* mRNA suggests that transcriptional activity of the *FMR1* gene is increased in carrier males. This possibility is particularly intriguing in light of the reduced number of FMRP(+) lymphocytes in at least some carrier males with large premutation alleles (see fig. 4). If defective translation of the FMR1 mRNA is occurring for premutation alleles, then an early manifestation of reduced translational efficiency may be feedback induction of the *FMR1* gene, in an attempt to overcome a mild protein deficit. Thus, normal or near-normal protein levels in the high-premutation range may only be maintained through abnormally high levels of message,

although no direct evidence for such a model currently exists.

In the presentation of our results, we have introduced a somewhat arbitrary division—at 100 CGG repeats—between two groups of carrier males. This distinction was introduced simply to illustrate the trend in message level. We have also performed first-order through third-order regression analyses of all data in figure 2. These three analyses yielded similar outcomes—namely, (*a*) relative mRNA levels that, for alleles of 200 repeats, are eightfold higher than the levels for alleles of 30 repeats and (*b*) coefficients of determination that are comparable ($r^2_{1st} = .76$; $r^2_{2d} = .76$; $r^2_{3d} = .78$). Thus, the current division yields a more conservative, model-free estimate for the degree of elevation, and it is, in any case, more appropriate in the absence of any underlying mechanism.

Finally, we wish to emphasize the utility of the fluorescence-based RT-PCR approach. Real-time measurements obtained throughout the PCR process enable one to define a range of cycle numbers in which the response of the fluorescent signal is linearly proportional to the original concentration of RNA. This feature greatly improves both precision and accuracy of the determination of relative mRNA levels. In addition, the current investigation involved >1,000 individual PCR reactions for full quantification of relative mRNA levels (concentration-dependence, secondary corrections, repetitions, etc.); such numbers of PCR reactions are facilitated by the relative ease of the automated measurements.

Acknowledgments

This work was supported by funding (to P.J.H. and R.J.H.) from the Boory Family Fund and the Mulvey Family Fund, a FRAXA Research Foundation Fellowship (to F.T.), National Institutes of Health grants GM35305 (to P.J.H.) and HD36071 (to R.J.H.), and Maternal Child Health Bureau grant MCJ-089413 (to R.J.H.). The authors wish to thank Janine Mills for her help with oligonucleotide preparation.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html (for *FMR1* gene [accession number L29074] and reference gene derived from *GUS* mRNA [accession number NM000181]

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for fragile-X syndrome [MIM 309550])

References

Allingham-Hawkins DJ, Babul-Hirji R, Chitayat D, Holden JJ, Yang KT, Lee C, Hudson R, et al (1999) Fragile X premutation is a significant risk factor for premature ovarian failure: the International Collaborative POF in Fragile X study—preliminary data. Am J Med Genet 83:322–325

Baeyens DA, Cornett LE (1993) Transcriptional and posttranscriptional regulation of hepatic beta 2-adrenergic receptor gene expression during development. J Cell Physiol 157:70–76

Brown WT, Houck GE Jr, Jeziorowska A, Levinson FN, Ding X, Dobkin C, Zhong N, et al (1993) Rapid fragile X carrier screening and prenatal diagnosis using a nonradioactive PCR test. JAMA 270:1569–1575

de Vries BBA, Jansen CCAM, Duits AA, Verheij C, Willemsen R, van Hemel JO, van den Ouweland AMW, et al (1996) Variable *FMR1* gene methylation of large expansions leads to variable phenotype in three males from one fragile X family. J Med Genet 33:1007–1010

Devys D, Lutz Y, Rouyer N, Bellocq J-P, Mandel J-L (1993) The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. Nat Genet 4:335–340

Dorn MB, Mazzocco MMM, Hagerman RJ (1994) Behavioral and psychiatric disorders in adult male carriers of fragile X. J Am Acad Child Adolesc Psychiatry 33:256–264

Feng Y, Lakkis L, Devys D, Warren ST (1995*a*) Quantitative comparison of *FMR1* gene expression in normal and premutation alleles. Am J Hum Genet 56:106–113

Feng Y, Zhang F, Lokey LK, Chastain JL, Lakkis L, Eberhart D, Warren ST (1995*b*) Translational suppression by trinucleotide repeat expansion at *FMR1*. Science 268:731–734

Franke P, Leboyer M, Gansicke M, Weiffenbach O, Biancalana V, Cornillet-Lefebre P, Croquette MF, et al (1998) Genotype-phenotype relationship in female carriers of the premutation and full mutation of *FMR-1*. Psychiatry Res 80:113–127

Fu YH, Kuhl DP, Pizzuti A, Pieretti M, Sutcliffe JS, Richards S, Verkerk AJ, et al (1991) Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell 67:1047–1058

Gibson UE, Heid CA, Williams PM (1996) A novel method for real time quantitative RT-PCR. Genome Res 6:995–1001

Gu Yanghong, Lugenbeel KA, Vockley JG, Grody WW, Nelson DL (1994) A de novo deletion in *FMR1* in a patient with developmental delay. Hum Mol Genet 3:1705–1706

Hagerman RJ (1996) Physical and behavioral phenotype. In: Hagerman RJ, Cronister AC (eds) Fragile X syndrome: diagnosis, treatment, and research, 2d ed. Johns Hopkins University Press, Baltimore, pp 3–87

——— (1999) Fragile X syndrome. In: Neurodevelopmental disorders: diagnosis and treatment. Oxford University Press, Oxford, pp 61–132

Hagerman RJ, Staley LW, O'Conner R, Lugenbeel K, Nelson D, McLean SD, Taylor AK (1996) Learning-disabled males with a fragile X CGG expansion in the upper premutation size range. Pediatrics 97:122–126

Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome Res 6:986–994

Hmadcha A, De Diego Y, Pintado E (1998) Assessment of

FMR1 expression by reverse transcriptase-polymerase chain reaction of KH domains. J Lab Clin Med 131:170–173

Kaufmann WE, Abrams MT, Chen W, Reiss AL (1999) Genotype, molecular phenotype, and cognitive phenotype: correlations in fragile X syndrome. Am J Med Genet 83:286–295

Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl 4: 357–362

Loesch DZ, Hay DA, Mulley J (1994) Transmitting males and carrier females in fragile X—revisited. Am J Med Genet 51: 392–399

Loesch DZ, Hay DA, Sutherland GR, Halliday J, Judge C, Webb GC (1987) Phenotypic variation in male-transmitted fragile X: genetic inferences. Am J Med Genet 27:401–417

Mazzocco MM, Pennington BF, Hagerman RJ (1993) The neurocognitive phenotype of female carriers of fragile X: additional evidence for specificity. J Dev Behav Pediatr 14:328–335

Murray A, Webb J, Grimley S, Conway G, Jacobs P (1998) Studies of FRAXA and FRAXE in women with premature ovarian failure. J Med Genet 35:637–640

Oberlè I, Rousseau F, Heitz D, Kretz C, Devys D, Hanauer A, Bouè J, et al (1991) Instability of a 550–base pair DNA segment and abnormal methylation in fragile X syndrome. Science 252:1097–1102

Pieretti M, Zhang FP, Fu YH, Warren ST, Oostra BA, Caskey CT, Nelson DL (1991) Absence of expression of the *FMR-1* gene in fragile X syndrome. Cell 66:817–822

Reiss AL, Freund L, Abrams MT, Boehm C, Kazazian H (1993) Neurobehavioral effects of the fragile X premutation in adult women: a controlled study. Am J Hum Genet 52:884–894

Riddle JE, Cheema A, Sobesky WE, Gardner SC, Taylor AK, Pennington BF, Hagerman RJ (1998) Phenotypic involvement in females with the *FMR1* gene mutation. Am J Ment Retard 102:590–601

Rousseau F, Heitz D, Tarleton J, MacPherson J, Malmgren H, Dahl N, Barnicoat A, et al (1994) A multicenter study on genotype-phenotype correlations in the fragile X syndrome, using direct diagnosis with probe StB12.3: the first 2,253 cases. Am J Hum Genet 55:225–237

Sabourin LA, Mahadevan MS, Narang M, Lee DSC, Surh LC, Korneluk RG (1993) Effect of the myotonic dystrophy (DM) mutation on mRNA levels of the DM gene. Nat Genet 4: 233–238

Smeets HJM, Smits APT, Verheij CE, Theelen JPG, Willemsen R, van de Burgt I, Hoogeveen AT, et al (1995) Normal phenotype in two brothers with a full *FMR1* mutation. Hum Mol Genet 4:2103–2108

Smits A, Smeets D, Hamel B, Dreesen J, de Haan A, van Oost B (1994) Prediction of mental status in carriers of the fragile X mutation using CGG repeat length. Am J Med Genet 51: 497–500

Steyaert J, Borghgraef M, Legius E, Fryns J-P (1996) Molecular-intelligence correlations in young fragile X males with a mild CGG repeat expansion in the *FMR1* gene. Am J Med Genet 64:274–277

Tassone F, Hagerman RJ, Ilke DN, Dyer PN, Lampe M, Willemsen R, Oostra BA, et al (1999) FMRP expression as a potential prognostic indicator in fragile X syndrome. Am J Med Genet 84:250–261

Tassone F, Hagerman RJ, Taylor AK, Mills JB, Wood S, Gane LW, Hagerman PJ. Clinical involvement and protein expression in individuals with the *FMR1* premutation. Am J Med Genet (in press)

Taylor AK, Safanda JF, Fall MZ, Quince C, Lang KA, Hull CE, Carpenter I, et al (1994) Molecular predictors of cognitive involvement in female carriers of fragile X syndrome. JAMA 271:507–514

Turner G, Robinson H, Wake S, Martin N (1994) Dizygous twinning and premature menopause in fragile X syndrome. Lancet 344:1500

Verheij C, Bakker CE, de Graaff E, Keulemans J, Willemsen R, Verkerk AJ, Galjaard H, et al (1993) Characterization and localization of the *FMR-1* gene product associated with fragile X syndrome. Nature 363:722–724

Waring JD, Korneluk RG (1998) Genetic studies of the myotonic dystrophy CTG repeat. In: Wells RD, Warren ST (eds) Genetic instabilities and hereditary neurological diseases. Academic Press, San Diego

Willemsen R, Mohkamsing S, de Vries BBA, Devys D, van den Ouweland A, Mandel J-L, Galjaard H, et al (1995) Rapid antibody test for fragile X syndrome. Lancet 345:1147–1148

Willemsen R, Smits A, Mohkamsing S, van Beerendonk H, de Haan A, de Vries B, van den Ouweland A, et al (1997) Rapid antibody test for diagnosing fragile X syndrome: a validation of the technique. Hum Genet 99:308–311