Genotype, Molecular Phenotype, and Cognitive Phenotype: Correlations in Fragile X Syndrome

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The study of the neurobehavioral consequences of mutations of FMR1, the gene responsible for fragile X syndrome (FraX), has been based largely on correlations between mutation patterns and cognitive profile. Following the characterization of FMRP, the FMR1 gene product, preliminary correlations between FMRP levels, and neurologic phenotype have been established. However, most of these investigations have focused on individuals at both ends of the genetic and cognitive spectra of FraX, subjects with normal or premutation (PM) alleles or males with the FMR1 full mutation (FM). The present study is designed to characterize FMRP expression and to correlate it with IQ, in a sample representing a wide spectrum of FMR1 mutations. For this purpose we developed a highly sensitive immunoblotting assay using peripheral leukocytes. Three distinct patterns of FMRP immunoreactivity (-ir) emerged. Individuals with normal (n = 28) and PM (n = 8) alleles as well as most females with the FM (n = 14) showed the highest levels with multiple ~70–80 kDa FMRP-ir bands. Males with the FM (n = 10) demonstrated only a 70 kDa FMRP-ir band, and had significantly lower levels when compared with any previous groups. Males with mosaicism and three of 14 females with FM displayed a doublet with equal amounts of the highest and lowest molecular weight FMRP-ir bands. Multiple regression models that adjust for the effect of parental IQ indicated that both activation ratio and FMRP-ir are significantly correlated to subject IQ. We conclude that FMRP-ir offers promise as an indicator of the impact of FMR1 mutations upon neurologic function. Furthermore, our unexpected finding of FMRP-ir in all males with FM suggests that most of them are not transcriptionally silent. Am. J. Med. Genet. 83:286–295, 1999. © 1999 Wiley-Liss, Inc.

KEY WORDS: fragile X; FMRP; immunoblotting; leukocytes; IQ; FMR1; activation ratio

INTRODUCTION

Fragile X syndrome (FraX) is the most prevalent form of inherited mental retardation [Rousseau et al., 1994, 1995; Warren and Nelson, 1994]. The mutation, in most of the cases, consists of an unstable expansion of a CGG trinucleotide repeat within the 5′ untranslated region of the FMR1 gene [Oberlé et al., 1991; Verkerk et al., 1991]. Based on the size of this expansion, individuals are classified as having normal (5–50 repeats), premutation (PM) (50–200 repeats), or full mutation (FM) (200–2,000 repeats) alleles [Fu et al., 1991; Oberlé et al., 1991; Snow et al., 1993; Rousseau et al., 1994, 1995]. A mixed pattern of FMR1 mutation in which PM and FM alleles are combined is termed mosaicism (typical) [Pieretti et al., 1991]. Prior to the characterization of the FMR1 gene product, the fragile X mental retardation protein, or FMRP, evaluations of the consequences of FMR1 mutations were based on correlations between genotype and physical and neurobehavioral phenotypes. Thus, the typical findings in FraX in males, which include mental retardation, testicular enlargement, and minor facial abnormalities, concur with FM-size repeat expansions and hypermethylation [Oostra and Halley, 1995]. By contrast,
individuals with PM alleles show minimal or no physical or neurologic abnormalities [Hansen et al., 1992; Mazzocco et al., 1993; Reiss et al., 1993; Oberlé et al., 1991; Yu et al., 1992]. However, the most complex relationships between FMR1 mutations and phenotype are present in those individuals who are mosaic, both with the classical genotype (PM and FM alleles) and with combinations of FM and normal alleles. The latter is the typical genotype of females with the FM, who show a more variable and milder phenotype with less severe cognitive and behavioral anomalies [Oostra and Halley, 1995; Reiss et al., 1995].

FMR1 mutations in the FM range are associated with hypermethylation of a CpG island located proximal to the CGG polymorphism, which leads to FMR1 transcriptional silencing [Heitz et al., 1991; Pieretti et al., 1991; Wahrle et al., 1992]. Correspondingly, FMRP is not detected in males with FM alleles and full promoter region hypermethylation [Devys et al., 1993; Feng et al., 1995a; Verheij et al., 1993, 1995]. Conversely, normal and PM alleles are transcribed, their pre-mRNA alternatively spliced [Ashley et al., 1993], and translated into several FMRP isoforms in the 70–80 kDa range [Devys et al., 1993; Feng et al., 1995a]. Nevertheless, a particular combination of FMR1 alleles does not seem to predict FraX phenotype but rather the proportion of potentially transcribable DNA in the allelic mix. For instance, Hagerman et al. [1994] compared males with the FM who had different degrees of methylation. Subjects with incomplete or no methylation had a less severe physical and cognitive phenotype that was associated with lower but detectable FMRP expression [Hagerman et al., 1994]. Another attempt to address the relationship between FMR1 expression and FraX phenotype was made by Reiss et al. [1995], who demonstrated that activation ratio (AR) and not length of CGG expansion correlated with intellectual dysfunction in females with the FM. AR is a measure of the proportion of normal and PM alleles to total FMR1 as detected by Southern blotting of peripheral leukocytes [Abrams et al., 1994; Reiss et al., 1995].

The studies mentioned above suggest that the transcriptional/translational potential of the FMR1 mutation, and not the length of the CGG expansion, is the most important factor determining the FraX phenotype. Clarification of this issue is important not only for the understanding of the physical and neurobehavioral consequences of FMR1 mutations, but also for the development of appropriate therapeutic strategies. As most of the data on FMRP expression relates to males with the FM and individuals with the FM [Devys et al., 1993; Feng et al., 1995a; Verheij et al., 1993, 1995], the significance of intermediate FMRP levels is largely unknown.

The present study is designed to characterize the patterns of FMRP expression of a wide sample of FMR1 mutations, covering individuals throughout the FraX genetic and cognitive spectra. Subsequently, correlations between molecular phenotype (FMRP) and neurobehavioral phenotype (specifically IQ) have been carried out. For these purposes, we have developed an immunoblotting assay that measures FMRP levels in nontransformed peripheral leukocytes. This approach also allows direct correlations between genotype and molecular phenotype inFraX.

**METHODS**

**Research Subjects**

A total of 66 FMR1 DNA-typified individuals participated in the study. All subjects with FMR1 mutations have been followed at the Kennedy Krieger Institute as part of ongoing studies intending to characterize the neurobehavioral aspects of FraX, and the relationship between FMR1 mutation patterns and phenotype in FraX. Therefore, comprehensive cognitive and behavioral assessments of these individuals are available. Informed consent for the procedure was obtained directly from the subjects or from their legal guardians. Using standard Southern blotting techniques [Rousseau et al., 1991a], the individuals were classified as follows: 28 controls (14 males, 14 females), eight individuals with the PM (three males, five females), 24 subjects with the FM (10 males, 14 females), three males with typical mosaicism, and three males with atypical mosaicism and FMR1 deletions (<2.8 kb fragments) superimposed on PM and/or FM alleles. The median age and range (in years) for each major group were: control, 12.7 (3.0–26.8); premutation, 28.3 (4.1–46.2); full mutation, 13.0 (4.5–41.9) [males: 9.7 (4.5–25.9); females: 14.1 (7.7–41.9)]. Males with mosaicism or deletions had median ages of 9.4 and 17.2 years, respectively. The control group comprised both normal controls as well as subjects with mental retardation of unknown origin.

Two control monkeys, generously provided by Drs. S. Hendry and R. Lewis (Departments of Neuroscience and Neurology, Johns Hopkins Medical Institutions, respectively), were included for comparative evaluations of FMRP expression in primate brain and human peripheral leukocytes.

**Molecular Analyses**

Peripheral leukocyte samples were used for the FMR1 DNA and protein analyses in the human subjects. Fresh-frozen neocortical samples, specifically from primary and secondary visual areas, were obtained from two normal monkeys (Macaca mulatta) as described previously [Kaufmann et al., 1997a]. In the case of the FMRP analyses, peripheral leukocytes were separated using the CPT-Vacutainer system (Na Heparin/phosphate buffer, polyester gel, polysaccharide Na diatrizoate, without Ca or Mg) that enriches the sample to approximately 80% lymphocytes [Mole et al., 1994]. For DNA analyses, genomic DNA was isolated manually. For protein studies, both cell isolates and tissue samples were homogenized in a standard Tris buffer containing a cocktail of protease inhibitors [Kaufmann et al., 1997a] or in a Tris denaturing buffer containing sodium dodecyl sulfate (SDS) and urea (2% SDS, 10% β-mercaptoethanol, 10% glycerol, 8 M urea) according to Feng et al. [1995a].

**Southern Blotting**

A standard DNA diagnostic protocol was carried out. Briefly, DNA was simultaneously digested with EcoRI.
and the methylation-sensitive restriction nuclease EagI, and electrophoresed in a 1% agarose gel. Blots were incubated with P32-labeled StB12.3 probe [Rousseau et al., 1991a]. This probe hybridizes to sequences located 3' to the CGG polymorphic region, which in normal controls are positioned between a 5' EagI and a 3' EcoRI restriction sites. DNA patterns were analyzed in terms of expansion size and methylation status as previously reported [Abrams et al., 1994]. Normal active (NA: unmethylated) alleles are characterized by a 2.8-kb band, whereas normal inactive (NI: methylated) alleles are recognized by a 5.2-kb band. PM alleles, that are unmethylated, are detected as 2.8–5.2 kb (typically 2.8–3.3 kb) bands whereas FM expansions are typically smear-like bands at >5.2 kb. Correspondingly, activation ratios (AR) were calculated by the following equation: AR = [NA/(NA+NI)] + [PM/(PM+FM)] as previously described [Abrams et al., 1994; Reiss et al., 1995]. The three male subjects with apparent deletion mutations were assigned ARs as follows. In two male cases (randomly selected) were processed by the enhanced chemiluminescence (ECL) technique according to manufacturer's instructions (Amersham), showed a highly significant correlation with the ECL measurements ($R^2 = .609, p = .0006$). Consequently, all subsequent analyses were carried out with data obtained by the ECL method.

**Western Blotting**

After protein assays, by either Lowry or modified-Bradford (for most analyses of leukocytes) techniques, 20–30 μg of each sample were electrophoresed in 7.5–12% SDS/polyacrylamide minigels. All blots were incubated with the 1a monoclonal antibody (Chemicon), which recognizes an epitope spanning FMRP residues 1–140 [Devys et al., 1993; Verheij et al., 1995], and developed with the enhanced chemiluminescence (ECL) technique as previously published [Kaufmann et al., 1996, 1997a]. In addition, approximately half of the cases (randomly selected) were processed by the enhanced chemifluorescence (ECF) technique according to manufacturer's instructions (Amersham). The latter allows quantitations of immunoreactivity (ir), with a phosphorimager, after direct scanning of blots. FMRP-ir quantitations in lymphocyte homogenates were standardized as follows. First, linearity of FMRP-ir measurements was established by protein loading-optical density (OD) correlation curves (not shown) as previously reported [Kaufmann et al., 1997a]. Protein loads in the 0–80 μg range were directly proportional to FMRP-ir levels measured in arbitrary OD units by the method (R^2 = .984; p < .0001). Secondly, samples from a typical (for FMRP) normal control subject, which were included in every assay, served as FMRP-ir standard. Consequently, all measurements were expressed as absolute value as well as ratio to the standard sample. A third level of standardization was obtained by measuring simultaneously both FMRP and a reference protein [Kaufmann et al., 1997b]. We chose β-tubulin (Sigma), as reference, on the bases of its apparent lack of involvement in FraX and its previous use in similar analyses [Feng et al., 1995a]. As FMRP and β-tubulin have markedly different molecular weight, a combined primary monoclonal antibody incubation protocol was used. All FMRP-ir quantitative assays were carried out with 30 μg of lysate per sample and, at least, in duplicate. Every immunoblotting trial also included duplicate lanes, for each sample, that were incubated with standard ascitis fluid (Sigma) instead of the 1a antibody (contained in ascitis fluid, Chemicon). These duplicate blots controlled for nonspecific immunoreactivity dependent on the secondary antibody.

**Densitometric Analyses**

Southern and Western blotting quantitations of leukocyte samples were done by using intermediate darkness films as published earlier [Abrams et al., 1994; Kaufmann et al., 1997a]. After autoradiographic and ECL development, respectively, films were scanned on an Epson ES-1200C scanner (Epson America, Torrance, CA) using Adobe Photoshop 3.0b for image acquisition. After identification of significant bands by comparison with standards, bands were quantified in OD units using a modified version of the NIH Image software as described before [Abrams et al., 1994; Kaufmann et al., 1997a]. ARs were calculated from these data as mentioned above. FMRP-ir was calculated by subtracting from the positive lane (incubated with the 1a antibody) the OD values corresponding to the negative control (lane incubated with standard ascisit fluid), after the general film background was removed with assistance of the NIH Image software. The parallel FMRP-ir assays of random samples, done with the ECF system (Molecular Dynamics, Amersham, Sunnyvale, CA), showed a highly significant correlation with the ECL measurements ($R^2 = .691, p < .0001$). Consequently, all subsequent analyses were carried out with data obtained by the ECL method.

**Cognitive Assessment**

The cognitive component of our study included 43 of the 66 subjects using the following criteria. This subset of subjects had one or both parents with either normal or PM FMR1 alleles, who also received standardized IQ testing. Additionally, one control subject with a full-scale IQ (FSIQ) = 41 was excluded as he was specifically ascertained because of his severe learning problems. The mothers of 41 subjects, and 26 of their fathers, were assessed using an abbreviated (four subtest) WAIS-R IQ test [Silverstein, 1982]; therefore, mean parental IQ (MPIQ) for each subject was calculated as the average IQ of both parents ($n = 26$) or using the maternal IQ or paternal IQ alone ($n = 17$). Each subject's IQ assessment was carried out with age-appropriate scales (e.g., Bayley-II [Bayley, 1993], Stanford-Binet fourth edition [Thorndike et al., 1986], WISC-R [Wechsler, 1974], or WISC-III [Wechsler, 1991]), and the final FSIQ scores were adjusted to be consistent with the range and standard deviation of the WISC scores. The 43 assessed subjects had the following FMR1 patterns: 13 controls (seven males, six fe-
males), four PM (three males, one female), 20 FM (eight males, 12 females), three typical mosaic (all male), and three atypical mosaic/deletion (all male). The median age (years) and ranges for these groups were: control, 7.5 (3.0–13.6); PM, 11.3 (4.1–16.9); males with FM, 10.4 (4.5–25.6); females with FM 13.9 (7.7–19.2); typical mosaic, 9.4 (6.5–9.7); atypical mosaic/deletion, 17.2 (4.9–25.6). The median FSIQ and ranges were: control, 111 (76–135); PM, 107 (95–121); males with FM, 47 (39–60); females with FM, 88.8 (60–118); typical mosaic, 49 (43–52); and atypical mosaic/deletion, 69 (40–91).

**Statistical Analyses**

Statistical analyses were carried out with the program Statview 4.5. In addition to descriptive statistics, analyses of variance (ANOVA) of the following variables were performed: FMRP absolute OD values, FMRP subject/FMRP standard subject ratios, FMRP subject/β-tubulin subject ratios, and FMRP subject/β-tubulin subject/same measure from standard subject. Genotype to molecular phenotype correlations were carried out by simple regression analyses, with AR as independent variable and FMRP sample/FMRP standard ratio or FMRP/β-tubulin/standard ratio as dependent variables.

Genotype or molecular phenotype to cognitive phenotype correlations were assessed using previously described methods [Reiss et al., 1995]. Specifically, the independent contributions of MPIQ, FMR1 AR or FMRP-ir, and age, to intellectual measures were determined with hierarchical/stepwise regression analyses using a Model II error term. In order to elucidate the association between molecular and cognitive variables in subjects with presumed normal versus abnormal FMR1 gene function, the models were applied to the following subgroups from the complete sample of 43 subjects described in the section above: (a) subjects with normal FMR1 expression (control and PM alleles), as AR = 1 for each of these subjects; (b) FraX subjects with potential of FMR1 expression and, therefore, AR greater than 0 (to exclude males with the FM), and less than 1 (to exclude controls and PM); and (c) all subjects with reduced FMR1 expression and AR <1 (FM, typical mosaicism, atypical mosaicism/deletion). In the first step of each hierarchical/stepwise regression analysis, MPIQ was forced into the model as an initial independent variable to account for, measure, and remove the variance in subject FSIQ that is predicted by MPIQ. The standardized regression coefficient (β) for MPIQ was determined from this initial step. As a second step, the molecular variable, AR or FMRP-ir, was entered as an independent predictor variable in each regression analysis. If the F-value corresponding to the individual contribution of either independent variable in the model was ≥4.00 (approximating a P-value of <.05 for the degrees of freedom utilized in this design), the variable was entered into the regression model and the standardized regression coefficient and P-value determined.

**RESULTS**

**FMR1 Patterns and AR**

As mentioned in the preceding section, our sample included four main FMR1 mutation categories: 28 controls, eight individuals with the PM, 10 males with the FM, 14 females with the FM, and six males with either PM/FM mosaicism or deletions superimposed on PM or FM alleles. For most of our analyses, we separated the subjects with the FM according to sex because they represent two distinctive groups in terms of FMR1 potential for expression. There is substantial evidence supporting variable X chromosome inactivation in females with the FM [Reiss et al., 1995] that contributes to the heterogeneity of this group. Our findings on AR are in agreement with this concept. While all individuals with normal or PM alleles had an AR of 1, every male with the FM had an AR of 0. By contrast, females with the FM had a median AR of 0.639 and a 0.47–0.88 range. The group of males labeled as mosaic showed a predominant FM fully methylated component in combination with unmethylated expansions in the PM range. Accordingly, subjects with mosaicism had a median AR of 0.347 that ranged from 0.22 to 0.43. The group labeled as “deletion” was more heterogeneous and had AR that ranged from slightly above 0 to 0.88.

Considering the reported skewing toward the X chromosome carrying the normal FMR1 allele in older females with the FM [Rousseau et al., 1991b], age-AR regression analyses were carried out in this group. There was a moderately significant positive correlation between age and AR in females with the FM, with AR of −0.8 in adult subjects (R² = .334, P = .0304).

**FMRP Immunoblotting Patterns**

The immunoblotting pattern of FMRP immunoreactivity (ir) in leukocytes from control subjects was consistent with previous reports [Feng et al., 1995a; Khandjian et al., 1995; Verheij et al., 1993, 1995]. It was characterized by a major band at ~80 kDa and additional bands in the 70–78 kDa MW range (Fig. 1). However, only the leukocyte lysate obtained under denaturing conditions showed this pattern of FMRP-ir. When leukocytes were homogenized in standard Tris buffer, FMRP-ir appeared as four bands: a faint one at ~70 kDa, two closely migrating in the 50–65 kDa range, and the major one at ~45 kDa. The latter band resembled FMRP-ir in immunoprecipitates from kidney and other visceral organs from several species, including humans [Verheij et al., 1995]. Monkey neocortical homogenates, processed in parallel to the leukocyte lysates, displayed only the characteristic 70–80 kDa bands under both standard and denaturing conditions (Fig. 1). Based on these findings, all samples were subsequently processed in the SDS/urea buffer as reported by Feng et al. [1995a].

Qualitative analyses of leukocyte homogenates demonstrated that all subjects expressed FMRP-ir, although the levels in males with the FM were markedly low. Three different FMRP immunoblotting patterns were recognized. The first pattern was the typical one with multiple bands in the 70–80 kDa range, and it was detected in all controls (normal and mentally re-
tarded), all subjects with PM, and in 11 of the 14 females with the FM (Fig. 2). A second pattern of FMRP-ir was recognized in the three of the 14 females with the FM, two of the males with typical mosaicism, and in two of the males with shorter (deleted) unmethylated fragments associated with FM and PM alleles. This pattern consisted of only two weak bands of FMRP-ir of relatively equal intensity (Fig. 2). The heavier band seemed to correspond to the major ~80 kDa band, mentioned above, while the lighter one coincided with 70 kDa band present in controls. Finally, a third pattern of FMRP-ir that was present in all males with FM, and the remaining subjects with “deletions,” was characterized by a weak 70 kDa band that seemed to correspond to the lightest FMRP isoform normally detected (Fig. 2). There was complete agreement between two raters, blind to the DNA status, in assignment of all the 66 blots to one of the three categories mentioned before. As noted above, three males that were originally labeled by Southern blotting as having typical FM alleles did exhibit the second pattern with two FMRP-ir bands. A repeat DNA analysis showed that these subjects were, indeed, mosaics with predominance of FM bands.

An interesting observation was that three male subjects of our mosaic group (two of them corresponding to typical mosaicism and one to FM alleles associated with shorter unmethylated fragments), who displayed the distinctive two-band pattern of FMRP-ir, were ini-
FMRI Levels

Several quantitative estimates of FMRP levels were carried out, as mentioned in the Methods section. They included FMRP absolute values (in OD units), FMRP subject/FMRP standard ratios, FMRP subject/β-tubulin subject ratios, and FMRP subject/β-tubulin subject ratio/same measure from standard subject. In general, there was consistency in measurements of FMRP absolute levels across trials. Absolute FMRP levels, expressed as ratios to the standard control subject included in every assay, were highly correlated to FMRP/β-tubulin ratios ($R^2 = .723, P < .0001$). On the basis of its higher informative value, the ratio of FMRP/β-tubulin from each subject to the standard control was used as the main variable for all analyses. The highest levels of FMRP-ir corresponded to subjects showing the standard pattern of FMRP-ir, with particularly high values for those with normal and PM alleles that had FMRP-ir mean and standard error of 0.814 +/-0.041 and 0.913 +/-0.183, respectively. Females with the FM who, in most instances, showed the same pattern of FMRP-ir of controls, had slightly lower levels (0.698 +/-0.077). By contrast, males with the FM represented the lower end of the FMRP-ir spectrum with values of 0.103 +/-0.017. No differences in FMRP-ir were found between males and females in the control and PM groups. Finally, males with either typical or atypical mosaicism/deletion had FMRP-ir levels that were slightly higher than males with the FM: 0.132 +/-0.041 and 0.241 +/-0.112, respectively. Figure 3 displays these data in graphic form. ANOVA showed significant differences only when males with the FM were compared with controls, subjects with the PM, and females with the FM; the $P$-values for these comparisons (Scheffe’s post hoc analysis) were <.0001, .0003, and .0007, respectively. Based on the already mentioned bias toward FM allele inactivation in females with the FM [Rousseau et al., 1991b; Willemsen et al., 1995], age-FMRP-ir correlational analyses were done. The latter did not show a significant correlation between these variables ($R^2 = .10, P = .7493$).

AR—FMRP Correlations

AR is a measure of the potential for transcription of FMR1 [Reiss et al., 1995]. AR to FMRP-ir correlations, carried out by simple regression analysis, were highly significant for the entire sample ($R^2 = .527, P < .0001$). Due to the concern that individuals at both ends of the FMR1 mutation spectrum (subjects with normal and PM alleles and AR of 1, and males with the FM and AR of 0) would have an excessive influence on this correlation, an additional analysis was performed with only subjects with AR in the range 0<AR<1. The latter analysis also demonstrated a significant correlation but somewhat less strong than when the entire group was included: $R^2 = .245, P = .0311$. Both analyses are displayed in Figure 4.

Molecular-IQ Correlations

β-coefficients and $P$-values from the hierarchical/stepwise regression analyses appear in Table I. In both subgroups of subjects with FraX, both AR and FMRP-ir have a comparably strong effect on subject FSIQ level. In the control subjects alone, however, FMRP-ir is not predictive of FSIQ level. Conversely, MPIQ is a significant predictor of subject FSIQ in the control group, but not in FraX subjects.

To determine whether age-FSIQ correlations influenced the outcome of these analyses, a separate set of regression analyses were performed in which age was entered as an independent variable in the second step (with either activation ratio of FMRP-ir). These results

Fig. 4. Correlations between activation ratio (AR) and FMRP-ir levels. Regression analyses including every subject under study (A) demonstrated a more direct relationship between both variables ($P < .0001$) than when the calculations were made only with the group of subjects with intermediate AR (B). In the latter case, the $P$-value decreased to .0311.
(not shown) demonstrated that age was not a significant predictor of subject FSIQ. Plots of the each molecular variable against the residuals from the regression of MPIQ and subject FSIQ (see Fig. 5) demonstrate the molecular to IQ correlations. The two outlying points with AR>0 and AR<1 in the AR versus residual FSIQ plots are a typical mosaic male (residual IQ = −53, AR = 0.43), and a female with a full mutation (residual IQ = 37, AR = 0.56).

**DISCUSSION**

Our data demonstrate the feasibility of detecting and quantifying FMRP in peripheral leukocytes, without the need of establishing lymphoblastoid cell lines. Among the advantages of blotting techniques over immunocytochemical approaches, as those recently reported for FMRP-ir in blood smears [Willemsen et al. 1995, 1997], is that in addition to facile quantification they provide information regarding the molecular features of the protein under study. Changes in length, charge, or conformation are directly evaluated by immunoblotting [Kaufmann et al., 1997a]. Our initial assays demonstrated the instability of FMRP under standard conditions. Only when leukocytes were homogenized in a denaturing buffer [Feng et al., 1995a] were we able to detect the pattern previously reported in lymphoblasts [Devys et al., 1993; Verheij et al., 1993]. To understand this striking finding, we processed in parallel monkey neocortical homogenates. The latter have previously provided valuable information about protein stability in human cerebral cortex, removing the potential influence of postmortem delay [Kaufmann et al., 1997a]. FMRP-ir immunoblotting patterns in monkey homogenates were identical under both lysing conditions (Fig. 1). Apparently, FMRP in rat cerebral cortex is also stable under standard conditions as recently shown by Feng et al. [1997] in subcellular fractionation studies. As we used the 1a antibody, which recognizes the N-terminal region of the protein [Devys et al., 1993; Verheij et al., 1995], we could conclude that the absence of the major 80 kDa band and the appearance of lower MW bands under nondenaturing conditions represents instability of the C-terminal region of FMRP. The functional significance of this feature is uncertain, but most likely reflects differences in protease content among tissues. Interestingly FMRP-ir epitopes of ~45 kDa, which were the most prominent in our nondenatured-leukocyte sample, have been reported as FMRP isoforms in visceral organs, particularly kidney and liver of several species including humans [Verheij et al. 1995].

To our knowledge, the present investigation is the most comprehensive survey of FMRP expression in subjects with different FMR1 mutation patterns yet reported. Previous reports have focused on PM carriers

**TABLE I. β-coefficients, and the Associated Probabilities, From Two Stepwise/Hierarchical Regression Analyses Assessing the Correlations Between Subject IQ and Corresponding Parental IQ and Molecular Measures.**

<table>
<thead>
<tr>
<th>Subgroups</th>
<th>Controls* (N = 17)</th>
<th>All FraX (N = 26)</th>
<th>FraX w/ AR&gt;0 (N = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable (in child)</td>
<td>Regression step</td>
<td>Independent variable(s)</td>
<td>β</td>
</tr>
<tr>
<td>(1) Full-scale IQ</td>
<td>1</td>
<td>Mean parental IQ</td>
<td>.591</td>
</tr>
<tr>
<td>(2) Full-scale IQ</td>
<td>2</td>
<td>Activation ratio</td>
<td>b</td>
</tr>
<tr>
<td>(1) Full-scale IQ</td>
<td>1</td>
<td>Mean parental IQ</td>
<td>.591</td>
</tr>
<tr>
<td>(2) Full-scale IQ</td>
<td>2</td>
<td>FMRP-ir</td>
<td>.123</td>
</tr>
</tbody>
</table>

*In the first analysis, Activation Ratio was used as the molecular predictor variable while in the second, FMRP-ir was utilized in the same manner.

ns, not significant.

*Includes four subjects with the FraX PM (see text)

bNot applicable as AR has no variability in controls who by definition have AR = 1.
Clarification of this issue is essential considering previous studies that have emphasized disturbances in $FMR1$ transcription linked to hypermethylation [Pieretti et al., 1991], and deficits in translation related to large CGG expansions [Feng et al., 1995b] in males with the FM. In spite of the fact that some level of FMRP-ir was detected in males with the FM, and in other males with FM associated with shorter (<2.8-kb) DNA fragments, as a group, males with the FM appeared to be significantly different, on a molecular level, from females with the FM and from individuals with normal and PM alleles. Of note, the immunoblotting assay described here demonstrated a sensitivity comparable with the most sensitive Southern blotting method as demonstrated in the FMRP-ir patterns of the three males with mosaicism who were originally labeled as having only FM alleles.

We found higher levels of FMRP-ir in females with the FM than anticipated; this result is in contrast with a recent study that demonstrated that FMRP expression is in the low to intermediate level in this population [Willemsen et al., 1997]. Several factors could potentially contribute to these results. First, the technique used by Willemsen and colleagues [1997] quantifies FMRP-ir in terms of the percentage of positive leukocytes, without taking into consideration the FMRP-ir levels of each cell. As our blotting method is applied to homogenates, FMRP-ir is measured in terms of average level per cell. Second, these authors selected their population of females with the FM on the basis of mental retardation. No information about age, pattern of methylation, or IQ score is provided in this report. Therefore, it is difficult to ascertain the molecular and clinical similarity of the groups of females with the FM utilized in the two studies. Age also is a variable that should be considered in any study involving females with the $FMR1$ FM. As it has been mentioned above, Rousseau et al. [1991b] have shown that females with the FM have an age-dependent bias in X chromosome inactivation with selection for cells with activation of the normal $FMR1$ allele. Nevertheless, our data on females with the FM showed only a significant correlation between age and AR, but not between age and FMRP-ir. Moreover, AR and FMRP-ir in individuals with intermediate AR values, mainly females with the FM, are only modestly correlated. FMRP-ir levels are quite variable in individuals carrying active $FMR1$ alleles; they can range from slightly more than 50% to nearly 200% of standard levels. Females with the FM showed two distinctive qualitative and quantitative patterns of FMRP-ir expression. Eleven out of 14 had normal levels, and typical multiple-band pattern of FMRP-ir, while only three out of 14 had the two-band pattern and less than 50% of standard FMRP-ir levels. Although the number of subjects is small, these results suggest that FMRP expression in females with FM does not occur in a continuous manner. In summary, our data stress the complexity of the relationship between genotype and molecular phenotype in FraX. Future studies utilizing younger females with the FM and more stable cell populations (e.g., fibroblasts) are needed to clarify to what extent AR, as compared with FMRP level, is a reliable measure of $FMR1$ transcription and translation.
Our analyses support the idea that AR and FMRP-ir are important predictors of cognitive functioning in individuals with the FMR1 mutation. These two putatively related molecular variables were found to have comparably strong correlations with subject FSIQ in both FraX groups (all FraX subjects and FraX individuals with AR > 0) after the effect of MPIQ had been accounted for and removed from the regression model. This finding is consistent with previous work investigating FMRP [Abrams et al., 1994; de Vries et al., 1996; Reiss et al., 1995] as well as FMRP [Tassone et al., 1997] correlating to cognitive functioning in FraX subjects. Emphasizing the role of FMR1 expression in cognition, our control population (normal and PM alleles) showed no significant correlation between FMRP-ir and global cognitive level. This latter finding is in agreement with a recent study that also demonstrated no correlation between CGG repeat length and IQ in normal subjects [Mazzocco et al., in press]. In spite of the fact that there are molecular to clinical correlations in FraX individuals, the residual plots also indicate that there remains considerable variability in IQ that is not explained by the models utilizing MPIQ and AR or FMRP-ir as predictor variables. Some of this unexplained variability may stem from the use of multiple IQ tests in our population or the fact that in 27 cases, MPIQ was based only on maternal IQ. Additionally, it is possible that our results are influenced by a subject ascertainment bias that selects for exceptionally low- and/or high-functioning individuals. Finally, despite the existence of statistical associations between AR or FMRP-ir and IQ, it is important to emphasize that FMRP expression in leukocytes does not necessarily depict FMR1 expression in the central nervous system (CNS). In a recent investigation [Abrams et al., submitted], we demonstrated that there is a high degree of correspondence in the patterns of FMRP-ir of leukocytes and olfactory neuroblasts in normal controls and males with the FM. Olfactory neuroblasts, which are obtained by nasal biopsies, are CNS-derived neuronal elements that would exemplify gene expression in other CNS regions [Wolozin et al., 1993]. Similar analyses using olfactory neuroblasts or other CNS samples, including correlations with FMR1 peripheral expression, in the FraX spectrum are necessary to elucidate the cognitive significance of FMRP levels. In addition, cognitive measures other than FSIQ should be used in these studies. FraX, as well as other developmental disorders [Bellugi et al., 1990], is characterized by a distinctive cognitive profile, with impairment of visuo-spatial and attentional-organizational domains, that is relatively independent of IQ [Mazzocco et al., 1993; Reiss et al., 1995]. The effect of specific gene mutations, as those involving FMR1, upon these syndrome-specific cognitive profiles deserves further exploration.

We conclude that it is possible to quantify FMRP expression by direct assays of peripheral leukocytes. These analyses may become important tools for the understanding of the physical and neurobehavioral consequences of FMR1 mutations. FMRP immunoblotting might also be valuable for monitoring gene therapies that are being developed for FraX [Chiurazzi et al., 1997]. The possibility of incomplete transcriptional/translational silencing in males with FM, raised by our results, requires follow up investigations directed at sequencing the 70 kDa FMRP-ir band. These studies, currently ongoing, will help to elucidate the complex relationships between genotype and molecular phenotype in FraX.

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REFERENCES


