Absence of Expression of the FMR-1 Gene in Fragile X Syndrome

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Summary

We previously reported the isolation of a gene (FMR-1) expressed in brain at the fragile X locus. One exon of this gene lies within an EcoRI fragment that exhibits length variation in fragile X patients. This exon also contains the CGG repeat within the CpG island hyper-methylated in fragile X patients. To study the involvement of the FMR-1 gene in the fragile X syndrome, its expression was studied in lymphoblastoid cell lines and lymphocytes derived from patients and normal controls. FMR-1 mRNA was absent in the majority of male fragile X patients, suggesting a close involvement of this gene in development of the syndrome. Normal individuals and carriers all show expression. The methylation status of the BamHI site at the CpG island was also studied by Southern blot analysis of DNA from patients, carriers, and controls. The minority of fragile X affected males that show expression of FMR-1 demonstrated an associated incomplete methylation of the BamHI site.

Introduction

The molecular genetic events underlying the mutations leading to the fragile site in human Xq27.3 and the coincident fragile X (Fra(X)) syndrome have recently been uncovered (Vincent et al., 1991; Bell et al., 1991; Oberlé et al., 1991; Yu et al., 1991; Verkerk et al., 1991; Kremer et al., 1991). However, the mechanism of the mutation and its role in the development and transmission of the associated genetic disorder remain unclear.

Identification of very closely linked DNA fragments (Oostra et al., 1990; Rousseau et al., 1991; Hirst et al., 1991; Nelson et al., 1991), combined with the analysis of somatic cell hybrids containing breakpoints at the fragile site (Warren et al., 1990), facilitated the description of the region and the isolation of large-insert clones with which to characterize the site. Yeast artificial chromosome (YAC) clones spanning the fragile site have been identified by several groups (Heitz et al., 1991; Dietrich et al., 1991; Verkerk et al., 1991), resulting in the identification of the fragile site at a molecular level. A 5.1 kb EcoRI fragment contains the majority of hybrid breakpoints, a CpG island preferentially methylated in Fra(X) patients (Vincent et al., 1991; Bell et al., 1991), an exon of the fragile X mental retardation 1 (FMR-1) gene (Verkerk et al., 1991), and an unusual repetitive CGG sequence. This repeat demonstrates length variation in Fra(X) and normal individuals, with significant increases in affected Fra(X) patients (Kremer et al., 1991; Y.-H. F. et al., unpublished data).

Identification of an expressed FMR-1 gene at the Fra(X) locus (Verkerk et al., 1991) containing the CGG repeats responsible for genomic rearrangements in Fra(X) suggests that this gene is involved in the syndrome. The additional finding of Fra(X)-specific methylation at the CpG island proximal to the CGG repeats would indicate a possible regulatory mechanism of the mutation.

We have endeavored to ascertain the levels of FMR-1 mRNA in Fra(X) patients, carriers, and normal controls. Since the mRNA corresponding to this gene is found in leukocytes, we have addressed this question using mRNA derived from Epstein-Barr virus-transformed lymphoblasts as well as from fresh leukocytes from Fra(X) patients, carriers, and controls. To decrease the time of analysis and increase the sensitivity of the measurement, we have developed a polymerase chain reaction (PCR)-based assay in which total cellular RNA is reverse transcribed and the resulting cDNA is amplified with primers directed to the FMR-1 and hypoxanthine-guanine phosphorylase (HPRT) genes. HPRT serves as an internal standard for the PCR amplification.

We report here markedly reduced levels of FMR-1 mRNA in the majority of males Fra(X) patients, suggesting a close involvement of this gene in the development of the syndrome. Expression was observed in all normal males, normal members of Fra(X) pedigrees and carriers. Southern blot analysis of the 5.1 kb EcoRI fragment containing the fragile site revealed a complete correlation between total methylation at the BamHI site within the CpG island and absence of FMR-1 expression.

Results

Absence of Expression of the FMR-1 Gene In Lymphoblastoid Cell Lines from Fra(X) Patients

To survey for the presence of FMR-1 mRNA in a rapid, simple, and reliable manner, we developed a PCR-based method that allows the simultaneous detection of the transcripts of the FMR-1 and HPRT genes. A total of 14 normal controls, 20 affected males, and 10 carrier females were analyzed. Two sets of primers, derived from the FMR-1 cDNA sequence (Verkerk et al., 1991) at positions 297–320 and 420–442 (numbered 4625 and 4654, respectively) and at positions 989–1009 and 1112–1134 (numbered 4687 and 4747, respectively) (Figure 1), were used to amplify cDNA. The primers do not amplify genomic DNA (data
not shown), demonstrating that the amplification products bridge exon–intron boundaries. In combination with each pair of FMR-1 primers, a pair of oligonucleotides (numbered 243 and 244) derived from the HPRT cDNA sequencing (Gibbs et al., 1989) was used to detect the transcription product of the X-linked HPRT gene as an internal control.

Figure 2 shows the reverse transcriptase (RT)-PCR products from Fra(X) males, carriers, and normal cell lines visualized on a 3% agarose gel stained with ethidium bromide. The larger band (450 bp) corresponds to the amplification product of the HPRT gene, which is present in all the lanes. The PCR product corresponding to amplification of the FMR-1 transcript with primers 4924 and 4925 is 146 bp in length. This product is absent in lanes g, k, m, and n, corresponding to samples from Fra(X) patients. DNA from two normal males and normal females (lanes a, b, c, d, and i) yields an FMR-1 amplification product. Females heterozygous at the 5.1 kb EcoRI fragment, as defined by Southern blot analysis (lanes e, f, j, and l), also show an amplification product for the FMR-1 gene. Lane h represents a Fra(X) male patient (1412) who demonstrates expression (see below). Primers 4987 and 4747 gave identical results (data not shown).

To better evaluate the amount of FMR-1 PCR product relative to the HPRT transcript, we limited the number of amplification cycles to 28. Furthermore, the PCR products were quantitated on a laser scanner apparatus (Gene Scanner), allowing more precise evaluation of FMR-1 and HPRT transcript amounts. Figure 3 illustrates the gel quantitation using the Gene Scanner, with the amplification products of four unrelated Fra(X) male patients and two normal male controls. No peak corresponding to the FMR-1 transcript is seen in the four patients, while the HPRT peak is observed in all cases. The two normal samples yielded both amplification products. We have previously reported Southern blot analysis of DNA from these four patients using probe pE5.1 (Verkerk et al., 1991) and demonstrated an EcoRI fragment of increased size from all four patients. Digestion of these DNAs with BspHI shows complete resistance, suggesting total methylation of the CpG island in these patients’ cell lines. Both control DNAs are completely cleaved by BspHI into the expected 2.7 and 2.4 kb fragments, indicating a completely unmethylated BspHI site (Southern blot analysis of DNA from one of the patients and one of the controls is reported in Figure 6, lanes 1 and 3, respectively).

FMR-1 Expression and Genomic Rearrangements in a Fra(X) Pedigree
The pedigree of one Fra(X) family studied in detail is shown in Figure 4. Cytogenetics analysis had shown Fra(X) chromosomes for individuals 1405, 1406, 1410, 1411, and 1412, with 3%, 6%, 12%, and 10% expression, respectively. However, individual 1406, whose mental retardation was clinically thought to be due to Fra(X) syndrome, failed to demonstrate the presence of the fragile site at Xq27.3.

Southern blot analysis with probe pE5.1 reveals the DNA pattern at the Fra(X) site for all the members of the pedigree (Figure 4). EcoRI digestion shows a normal 5.1 kb band for individuals 1405, 1406, 1407, and 1408. Double digestions (EcoRI and BspHI) of DNA from these individuals reveal the unmethylated status of the BspHI restriction site, since bands of 2.7 and 2.4 kb are observed. In female 1405, partial methylation of the BspHI site is observed, as expected because of random X inactivation. Females 1408 and 1410 are heterozygous at the Fra(X) site, showing a 5.1 kb band together with a band of increased size. The 5.1 kb band has a partially methylated BspHI site; the double digestion reveals 2.7 and 2.4 kb fragments but also some undigested 5.1 kb fragment. It is clear from these results that male 1406 does not carry the Fra(X) mutation present in the rest of the family. It is difficult to ascertain the degree of methylation of the bands of altered size in individuals 1409 and 1410. We have found that the BspHI digestion of the altered fragment, in those cases where this fragment is unmethylated, often results in the production of a constant 2.4 kb band and of a smear of hybridization above 2.7 kb that is hard to visualize (see Figure 6, lane 2). The 2.7 kb fragment contains the CGG repeat, and the smear of bands therefore represents a heterogeneity of fragments in a given patient. This phenomenon is not easily distinguishable for individuals 1409 and 1410.

Expression of the FMR-1 gene can be observed for all the individuals just described (1405, 1406, 1407, 1408, 1409, and 1410) (Figure 5). Ratios of HPRT to FMR-1 coamplified cDNA were determined by laser quantitation and were found to be 1.5, 1.4, 1.36, 2.4, 3.6, and 2.3, respectively. On the basis of the Southern blot analysis, we conclude that individuals 1409 and 1410 are carriers of the
Figure 2. Agarose Gel Separation of RT-PCR Products
RT-PCR products from Fras1 patients (lanes a, g, h, k, m, and n), carriers (lanes e, f, i, and j), and normal (lanes b, o, d, and f; female, lane e) cell lines were visualized on an ethidium bromide-stained 3% agarose gel. The larger band (450 bp) corresponds to the amplification product from the HPRT gene transcript. The PCR product corresponding to the amplification of the FMR-1 transcript with primers 4624 and 4625 is 146 bp in length.

Figure 3. Gene Scansmaster Chromograms of RT-PCR Products
RT-PCR chromatograms of four unrelated Fras1 (X) male patients (cell lines GM 03900, 06912, 07294, 08617; panels 1, 4, 6, and 8, respectively) and of two male controls (cell lines RJK 1283 and 1322; panels 1 and 2, respectively). Primers 4624 and 4625 from the FMR-1 cDNA sequence were used in combination with primers 243 and 244 derived from the HPRT cDNA sequence. No peaks corresponding to FMR-1 can be observed for the four Fras1 (X) patients, while in panels 1 and 2, HPRT to FMR-1 ratios of 1.8 and 1.6, respectively, can be calculated.

Figure 4. Pedigree and Southern Blot Analysis of a Fras1 (X) Family (BS)
The pedigree shows two mentally retarded Fras1 (X)-positive males (black squares), one Fras1 (X)-negative intracellularly retarded male (stippled square), two carrier females (hatched circles), and other normal members of the family (empty squares and circles). Southern blot analysis with probe pE5.1 was performed on DNA from fresh leukocytes for all individuals. In the case of 1412, fresh leukocyte DNA (a) and lymphoblastoid cell line DNA (b) are shown. EcoRI digestion is shown in the upper panel; a normal 5.1 kb fragment is seen in individuals 1405, 1406, 1407, and 1408. Individuals 1409 and 1410 show both the normal-sized fragment and a larger fragment. An increased-sized band is seen in 1411, while 1412 shows both the increased-sized band and a near-normal-sized band. Double digestions with EcoRI and BsmHI are shown in the lower panel (see text for detailed explanation). Brackets indicate the fragments of altered size that appear as smear distribution between 6.0 and 7.5 kb.

Fras1 (X) mutation. Higher than normal HPRT to FMR-1 ratios are observed for 1409, 1410, and 1408. While the carrier status could correlate with the higher ratios for the two females, suggesting diminished expression of FMR-1, the ratio of 2.4 in the normal male remains unexplained.

The results from the index Fras1 (X) cases in this family are most interesting. The male 1412, who is mentally retarded, has a cytogenetically demonstrable Fras1 (X) site (10%). Expression studies of the cDNA from a lymphoblastoid cell line derived from this patient demonstrate a normal level of expression of the FMR-1 gene (HPRT to FMR-1 ratio of 1.6). However, his similarly affected brother (1411) shows no FMR-1 expression. Southern blot analysis of DNA from these individuals demonstrates a clear difference between them. A band of near-normal size is found in DNA from patient 1412. This is true in both a lymphoblastoid cell line and fresh blood leukocytes. A larger fragment in the Fras1 (X) mutation size range is also observed. The ratios between the bands vary in the two DNA sources, with an almost 1:1 ratio in the lymphoblastoid line and less of the near-normal band in fresh-blood DNA. This suggests mosaicism, as 1412 has a single X chromosome by cytogenetic criteria. The near-normal band is clearly unmethylated; it can be digested completely with BsmHI. The
Figure 5. Gene Scanner Chromatograms of RT-PCR Products from a Fra (X) Family (BS)

RT-PCR chromatograms of all the members of the BS pedigree are shown. Primers 4604 and 4626 from the FMR-1 cDNA sequence were used in combination with primers 243 and 244 derived from the HPR7 cDNA sequence. Ratios between the two chromatographic peaks were calculated and are as follows: 1405, 1.5; 1408, 1.4; 1407, 1.35; 1409, 2.4; 1408, 3.8; 1410, 2.9; 1412, 1.6. No ratio can be calculated for 1411, in which no FMR-1 peak is detected.

The presence of the near-normal band in fresh leukocyte DNA shows that this mosaicism is already present in the patient's blood and is enhanced during the clonal selection that occurs in lymphocyte transformation. Patient 1411, on the other hand, shows only an increased-sized EcoRI band that is resistant to BssHII digestion, demonstrating a genomic pattern very similar to that observed in the majority of patients so far examined. Thus, FMR-1 expression in patient 1412 correlates with an unmethylated, near-normal-sized EcoRI fragment at the fragile site.

Correlation between FMR-1 Expression and Genomic Rearrangements in Other Fra (X) Patients

Four of twenty Fra (X) patients demonstrated expression of FMR-1 by RT-PCR, similar to that observed in 1412. In three of the four cases, the presence of FMR-1 expression can be correlated with the observation of a mosaic pattern including normal or near-normal-sized EcoRI fragments and partial methylation of the BssHII site on the near-normal-sized band. In one of these patients, expression of the FMR-1 gene was demonstrated by RT-PCR from fresh leukocytes. The fourth Fra (X) patient, among those who show FMR-1 expression, is represented by a cell line from the Human Genetic Mutant Cell Repository (GM 06897), for which Southern blot analysis is shown in Figure 6 (lane 2). Partial methylation of the BssHII site is present, but no EcoRI fragment close to the normal 5.1 kb length can be observed in this patient's cell line. The hybridization in this DNA appears as a smear mainly distributed between 6.2 and 7.0 kb. Double digestions with EcoRI and BssHII reveal that the mutant band in GM 06897 (lanes 2) is incompletely methylated, since BssHII cleavage produces a constant band (2.4 kb) and one variable ( smear of hybridization) band. Some of the altered-sized material remains, suggesting that a portion of these fragments is methylated. No BssHII digestion can be observed in GM 06817 (lanes 1).

Discussion

We report a study of the expression of the FMR-1 gene, which is adjacent to the Fra (X)-related CpG island and contains one of its exons, using a CCG repeat thought to be involved in the Fra (X) mutation (Verkerk et al., 1991; Kramer et al., 1991; Y.-H. F. et al., unpublished data). The FMR-1 gene expresses an mRNA found in a variety of tissues, and our study utilized RT-PCR analysis of leukocyte and lymphoblastoid cell line mFNA from Fra (X) families, Fra (X) unrelated patients, and normal controls.

The data presented above demonstrate the involvement of the FMR-1 gene in the Fra (X) phenotype. The majority
Table 1. Ratios of FMR-1 to HPRT cDNA Values from RT-PCR Products

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number</th>
<th>HPRT/FMR-1 (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fra (X) males</td>
<td>16</td>
<td>1.50 ± 0.44</td>
</tr>
<tr>
<td>Fra (X) males</td>
<td>4</td>
<td>2.46 ± 0.68</td>
</tr>
<tr>
<td>Fra (X) carriers</td>
<td>10</td>
<td>2.18 ± 1.59</td>
</tr>
<tr>
<td>Normal males</td>
<td>10</td>
<td>1.54 ± 0.36</td>
</tr>
<tr>
<td>Normal females</td>
<td>4</td>
<td>1.54 ± 0.30</td>
</tr>
</tbody>
</table>

A total of 20 Fra (X) males were examined, of which only four showed detectable FMR-1 expression. All 10 carrier females showed FMR-1 gene expression, although to a different extent, with a mean HPRT to FMR-1 ratio of 2.18 and a standard deviation of 1.59. In a total of 14 normal cell lines, FMR-1 was always expressed and the mean HPRT to FMR-1 ratio was 1.56.

of Fra (X) patients (16 out of 20) do not express FMR-1 in leukocytes, while normal males and females, normal members of Fra (X) pedigrees, and heterozygous females all express the transcript. These data are summarized in Table 1. The absence of expression of FMR-1 in males correlates with the presence of an EcoRI band of decreased size, as ascertained with probe pE5. The BshHII site present within this EcoRI fragment is completely methylated in all these FMR-1-deficient patients. Methylation of the rare-cutting enzyme sites present in this CpG island in Fra (X) patients was previously reported by Vincent et al. (1991) and by Bell et al. (1991).

We conclude that the absence of FMR-1 expression and the presence of an increased-sized EcoRI band carrying a methylated BshHII site at the Fra (X) site are linked events. A number of hypotheses for the mechanism by which one event might cause the other can be considered. Amplification of the transcribed CGG repeats found in Fra (X) patients could directly alter mRNA expression from FMR-1. This could result in transcriptional termination or production of an unstable mRNA. Alternatively, an increased number of CGG repeats could result in increased methylation. Associated methylation at the adjacent CpG island could result in diminished transcription if the CpG island represents a regulatory region of the FMR-1 gene.

The observation of FMR-1 mRNA in 20% of Fra (X) male patients provides some insight into mechanisms. All four individuals demonstrating expression of FMR-1 are Fra (X) patients in whom the degree of mental retardation is not significantly different from that in the other 16 patients studied. All four patients, however, show an atypical Southern blot pattern at the Fra (X) locus with pE5.1 as a probe. Three of the four are mosaic for an apparently normal band and a smear of increased-sized fragments. The BshHII site associated with the near-normal-sized band in these patients is methylated to different degrees. In the fourth patient of this group (GM 06937), no normal-sized band can be detected, although partial methylation of the altered fragment is observed. The same patient's cell line had also been studied by pulsed-field gel electrophoresis with probe D038 by Vincent et al. (1991), and an incomplete methylation of the BshHII, EagI, and SacII sites was reported. We conclude, therefore, that the presence of the apparently normal band and, perhaps more importantly, incomplete methylation of the CpG island correlate with the presence of FMR-1 mRNA in these four patients.

If lack of expression of FMR-1 is the mechanism accounting for the Fra (X) phenotype, then the four patients demonstrating near-normal levels of mRNA should not be affected. Several explanations could account for this discrepancy. It is possible that the level of expression of FMR-1 in these patients did not reach the necessary threshold to provide normal levels of the protein in the relevant tissue(s) at the appropriate time in development. It may be the case that FMR-1 levels and DNA methylation observed in leukocytes in these patients are not representative of the situation in other body tissues or at other developmental stages. In addition, the detection of FMR-1 mRNA does not assure normal expression and function of the FMR-1 protein. Since the Fra (X) mutation is in the CGG repeat within the coding region, any increase would be expected to produce a truncated protein, as stop codons are encountered frequently in the other two frames. Even with the proper open reading frame maintained, a massive amplification of the CGG codon would dramatically change the charge and the structure of the encoded protein, modifying its function. Analysis of FMR-1 protein in these four patients will be highly informative.

The situation for heterozygous females is more complex, since 30% show the Fra (X)-associated phenotype. We observed that the degree of expression of FMR-1 in heterozygous females was somewhat reduced, but we were unable to correlate this reduction with the degree of mental retardation. The methylation status of the BshHII site was more difficult to assess because of the inactivation of one of the two X chromosomes. In all carrier females studied, however, some BshHII cleavage was always present, revealing a nonmethylated status of this restriction site in at least one of the two fragments; this can account for the FMR-1 expression observed in the cells examined. Once again, since lymphoblastoid cell lines and peripheral blood leukocytes were studied, we have not measured the methylation and FMR-1 expression in the appropriate tissue and at the stage crucial for the development of the Fra (X) phenotype. In affected females, we can only speculate that FMR-1 expression is impaired by preferential inactivation of the normal allele in the relevant tissue and stage of development. This is the case in some other X-linked recessive disorders, such as Fabry's disease, ornithine transcarbamylase deficiency, Lesch–Nyhan syndrome, and Duchenne muscular dystrophy, where female carriers demonstrating symptoms can be found (McKusick, 1990).

We conclude, on the basis of these studies, that FMR-1 gene expression is not detected in the majority of Fra (X) patients and therefore accounts for at least part of the Fra (X) syndrome. This would appear to be the simplest explanation, since the Fra (X) mutation sequence resides within the FMR-1 coding domain. One cannot formally exclude the possibility that regulation of more than one gene at this location is altered owing to methylation of the region.

Experimental Procedures

Cell Lines

The lymphoblastoid cell lines GM 03200, 04025c, 06937, 06912.
07264, and 08317 were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ); all the other lymphoblastoid cell lines were developed at Baylor College of Medicine and at Emory University by Epstein-Barr virus transformation of peripheral blood lymphocytes.

cDNA Synthesis

Lymphoblastoid cell cultures were harvested from T-75 flask, pelleted, and washed in PBS, total cellular RNA was extracted with RNAzol I (Citra/ Richmond). Lysases were separated from total blood, pelleted, and washed in PBS, and total RNA was similarly extracted. Five micrograms of total cellular RNA was incubated at 37°C for 1 hr in a total volume of 40 μl containing 1.2 μg of random hexamer primer (dN12), 1.2 μg of oligo(dT) primer (dT12), 1 μl each dNTP, 1 mM dithiothreitol, 50 mM Tris·HCL (pH 8.3), 75 mM KCl, 5 mM MgCl2, 10 μg of RNase inhibitor, and 1 μl of SuperScript RNAase H·RT (BRL, 200 U/μl). After this first-strand synthesis, the cDNA was collected by ethanol precipitation and resuspended in 20 μl of H2O, of which 2 μl was used for the subsequent PCR.

In Vitro DNA Amplification

PCR was carried out using 10% of the RT reaction in a total volume of 50 μl containing 1× Cetus Taq DNA polymerase buffer, 1 mM each primer, 250 mM each dNTP, and 2.5 U of Taq DNA polymerase (Perkin-Elmer/Cetus). After an initial 5 min at 94°C, the PCR consisted of 28 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min 45 sec at 72°C. The FMR-1 primers used were 4924 (5′-AGCTGACGCCAGCTCTGATGGAGCT-3′) and 4925 (5′-AACTCCGTCGCCGCTTATGGAGGGTTCA-3′), derived from positions 4929-4932 and 4942-4945 of the published FMR-1 cDNA sequence (Verkerk et al., 1991), or 4687 (5′-TTGAAGAAAGACACAGCTCCATG-3′) and 4747 (5′-TACACCAACAGGACTCCATGTGGG-3′), from positions 989-1009 and 1123-1134 of the published FMR-1 sequence. The HPR7 primers used were 243 and 244 (Gibbs et al., 1988). PCR products were visualized on a 3% (1.5% regular LE agarose [BRL], 1.5% NuSieve GTG [FMC]) agarose gel stained with ethidium bromide.

Quantification of the Ratio between the PCR Products

PCR products were quantitated by electrophoresis in a Model 362 Gene Scanner fluorescent-fragment analyzer (Applied Biosystems). Samples of 2.5 μl from each PCR reaction were assayed on 6% SequiPaque (FMC) agarose gels containing 0.5% (w/v) Ficoll 400. The buffer was 89 mM Tris base, 59 mM boric acid, 2 mM EDTA, and 40 μg/ml ethidium bromide. After electrophoresis at 100 V over 4 cm path, the fluorescence emitted from laser excitation of each PCR product stained with ethidium bromide was recorded and converted to chromographic peaks by software supplied by the manufacturer. Ratios (HPR7 to FMR1) were calculated using the relative areas of the two peaks.

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