Deletion of All CGG Repeats Plus Flanking Sequences in FMR1 Does Not Abolish Gene Expression

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Summary

The fragile X syndrome is due to the new class of dynamic mutations. It is associated with an expansion of a trinucleotide repeat (CGG) in exon 1 of the fragile X mental retardation gene 1 gene (FMR1). Here we present a fragile X family with an unique female patient who was rendered hemizygous for the FRAXA locus due to a large deletion of one X chromosome. In addition, the other X had a microdeletion in FMR1. PCR and sequence analysis revealed that the microdeletion included all CGG repeats plus 97 bp of flanking sequences, leaving transcription start site and translation start site intact. Despite this total lack of CGG repeats in the FMR1 gene, Western blot analysis showed expression of FMRP, and the patient's phenotype was essentially normal. X-inactivation studies of the androgen-receptor (AR) locus and haplotype determination of microsatellite markers gave evidence that the deletion probably originated from regression of a fully mutated FMR1 gene. Although the minimal number of CGG repeats hitherto reported in FRAXA is six, and at least four other genes associated with CGG repeats are known, suggesting an as yet unknown function of these repeats, our study clearly demonstrates that the absence of CGG repeats does not abolish expression of the FMR1 gene in lymphoblastoid cells.

Introduction

The fragile X syndrome is the most frequent form of inherited mental retardation (Oostra et al. 1995). The clinical symptoms include mental retardation ranging from moderate to profound, macroorchidism, and minor dysmorphic facial features often combined with autism-like behavior (Oostra et al. 1995).

In the vast majority of fragile X patients, the molecular mechanism of the syndrome is based on a large expansion of a CGG trinucleotide repeat (to >200 repeats) located in the 5′-UTR of the FMR1 gene (fragile X mental retardation gene 1) (Verkerk et al. 1991). This full mutation is associated with hypermethylation of both the CGG repeat and an upstream CpG island, resulting in repression of expression of the FMR1 gene (Pieretti et al. 1991). The transition is strictly maternal, although some minor expansions and regressions of repeat number in the premutation range (50–200 repeats) have been observed in father-to-daughter transmissions (Fisch et al. 1995; Väisänen et al. 1996).

Transmission of premutations is associated with a risk for an increase in repeat size when transmitted by a female, but occasionally a reduction in repeat number is seen, from premutation to a smaller premutation (Rousseau et al. 1991; Mornet et al. 1996) and from premutation to normal size allele (Vits et al. 1994; Brown et al. 1996; Mornet et al. 1996). Regression from a full mutation to a premutated allele has also been described (Fu et al. 1991; Rousseau et al. 1991; Malzac et al. 1996).

Several deletions in FMR1 have been characterized; some are large deletions removing all of the FMR1 gene plus flanking sequences (Gedeon et al. 1992; Tarleton et al. 1993; Quan et al. 1995; Birot et al. 1996), and some are deletions of part of FMR1 (Wörhle et al. 1992; Gu et al. 1994; Meijer et al. 1994; Trottier et al. 1994; Hart et al. 1995; Hirst et al. 1995; Quan et al. 1995), all associated with the fragile X syndrome in males. In addition to these deletions, a few females have been found with large X-chromosome deletions at Xq27-28. Two of these had both their FMR1 and iduronatesulfatase genes deleted, and they were both mentally retarded (Clarke et al. 1992; Schmidt et al. 1992).

Besides this group of fragile X patients, whose phenotype is due to deletions of all or part of FMR1, another group of patients with deletions in FMR1 are known. These patients have relatively small deletions, all located in the 5′-UTR surrounding the CGG repeat of FMR1, and the patients are often mosaics for a full mutation and the deletion (de Graaff et al. 1996; Milà et al. 1996; Mannermaa et al. 1996) or a normal allele and a deletion (Hirst et al. 1995). Protein expression studies have so far only been reported for one of these mosaic patients

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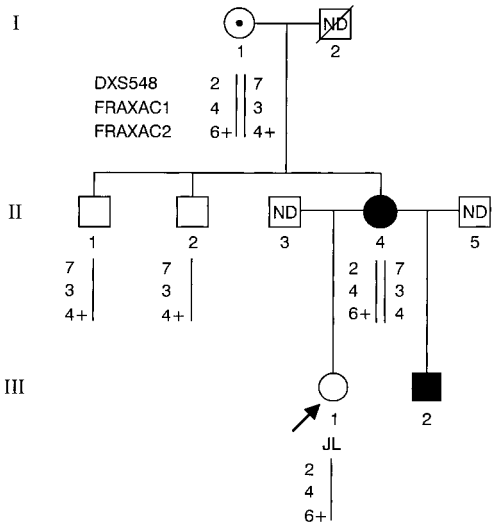

Figure 1 Pedigree of family, showing normal males (open squares), carrier females (circles with a dot), affected males (filled squares), and affected females (filled circles). JL is marked by an arrow. Males not investigated are shown by an open square marked "ND." Genotypes for DXS548, FRAXAC1, and FRAXAC2 are shown below symbols.

and revealed expression of FMRP in 28% of his lymphoblasts, corresponding well with the percentage of cells carrying the deletion (patient 1 in fig. 3*B;* de Graaff et al. 1996).

In this article, we present a female patient who was found to possess a large cytogenetically visible presumably terminal deletion on one X chromosome (46,X,del[X][q24]), eliminating the FRAXA locus, and a microdeletion on the other X chromosome. The microdeletion was located in exon 1 of the FMR1 gene, removing all of the CGG repeats and some flanking sequences on both sides. Despite the total lack of CGG repeats, Western blot analysis showed expression of FMRP comparable to normal controls. The data presented show that the CGG repeats and the immediate flanking sequences located in the 5′-UTR of FMR1 may not be necessary for expression of the gene in lymphoblastoid cells, but further experiments are necessary to elucidate whether they have an additional function. From haplotype analysis and X-inactivation studies, we presume that the deletion originated as a regression of a full mutation. This deletion is the largest known so far in which protein expression also has been investigated.

Patients, Material, and Methods

Clinical Case Report

Patient JL (subject III-1) was referred to us for FRAXA analysis because of her fully mutated mother (II-4) and brother (III-2) (fig. 1). The premutation carrier was the grandmother (I-1). The mother had two brothers (II-1 and II-2) who were both healthy and had normal CGG repeat numbers.

JL was an 11-year-old (10 years 10 mo) girl when investigated by us. She was the first of two children born to a fully mutated fragile X mother. The pregnancy was uneventful, but delivery was complicated by intrauterine and perinatal asphyxia. Her birth weight was 2,500 g, and the Apgar score was low. Requiring respirator treatment, she was transferred to a neonatal intensive care unit. Discharged in good health at 6 wk of age, she was followed as outpatient for 5 years with social problems only and was readmitted to hospital 9 years old because of suspicion of precocious puberty (thelarche since 7 years 6 mo). She was a normally developed girl in terms of psychomotor skills, with height at the 50% percentile. Her phenotype was essentially normal, without Turner syndrome stigmata. A hearing loss had been diagnosed, and she wore hearing aids. Sexual pubertal development corresponded to Tanner stage 2–3 without menarche. Ultrasound investigation showed the presence of normal uterus and ovaries. Conventional cytogenetic analysis (Q-banding) revealed a terminal deletion of the X chromosome, karyotype 46,X,del(X)(q24).

Southern Blot Analysis

Genomic DNA was isolated from blood leukocytes with the standard salting-out method (Miller et al. 1988). Seven micrograms of DNA were digested to completion with *Eco*RI and *Eag*I. After gel electrophoresis on a 0.7% agarose gel, the DNA was transferred to Hybond N+ blotting membrane. The membrane was prehybridized in 0.5 M NaP_i pH 7.2, 7% SDS, 1 mM EDTA, and hybridized with ^{32}P-labeled pPX6 probe at 65°C overnight. The pPX6 is a 600-bp *Pst*I-*Xho*I fragment that detects the CpG island and the $(CGG)_n$ repeats. pPX6 was labeled using the random-primed DNA-labeling kit from Boehringer Mannheim. After hybridization, filters were washed in 40 mM NaP_i pH 7.2, 1% SDS and in 20 mM NaP_i pH 7.2, 1% SDS and were analyzed either using a phosphorimager from Pharmacia or by standard autoradiography.

PCR and Sequencing

Amplification was performed using 100 ng genomic DNA in the presence of 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 100 μg/ml BSA, 10% DMSO, 0.1 mM dGTP, 0.2 mM dATP, dCTP, and dTTP each, 0.1 mM 7-deaza dGTP, 0.8 μM of each primer FMRG (sense primer) (5-AGTGCGACCTGTCACCGCCCTTC-3′) and FMR1B (antisense primer) (5′-AGGGCGAAGATGGGGCCTGC-3′) and 1.25 units cloned *pfu* polymerase, in a total volume of 25 μl. The DNA was denatured for 5 min at 98°C, followed by 35 cycles of denaturing at 98°C for 1 min and annealing and exten-

sion at 65°C for 3 min. The amplification was ended by a final extension at 72°C for 7 min.

DNA sequencing was performed on the PCR fragment using the same primers, which were end labeled by T4 kinase with γ-^{32}P-ATP. The Thermo Sequenase cycle sequencing kit (Amersham Life Science) was used following the manufacturer's instructions.

Haplotype PCR Analysis

The polymorphic markers (DXS548, FRAXAC1, and FRAXAC2) in the region surrounding the FMR1 gene were determined. Primers for FRAXAC1 and FRAXAC2 were those described by Richards et al. (1991), and primers for DXS548 were described by Verkerk et al. (1991). PCR of FRAXAC1 was performed using 100 ng genomic DNA in the presence of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM dATP, dCTP, dGTP, and dTTP each, 0.25 μM primers (FRAXAC1F was radioactive end labeled by T4 kinase), and 0.5 units *Taq* polymerase. The DNA was denatured for 5 min before the rest of the components were added (hot start); then 10 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 1.5 min, and extension at 72°C for 1.5 min was performed, followed by 25 cycles with the annealing temperature lowered to 55°C. The protocol was ended by a final extension step at 72°C for 7 min. PCR of FRAXAC2 and DXS548 were performed using 100 ng genomic DNA in the presence of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.2 mM $MgCl_2$, 0.2 mM dATP, dCTP, dGTP and dTTP each, 5% glycerol, 0.27 μM each primer (of which FRAXAC2F and DXS548-1 were radioactive end labeled) and 0.5 units *Taq* polymerase. A hot start was performed as described for FRAXAC1. The DNA was denatured at 93°C each for 8 min followed by 28 cycles of 45 s denaturing at 93°C, annealing for 1 minute at 52°C, and extension for 2 min at 72°C. A final extension step at 72°C for 7 min ended the protocol.

X-Inactivation Analysis

For studying the X inactivation in the AR locus, 2 μg of genomic DNA was digested to completion with *Hpa*II, and in parallel 2 mg of genomic DNA was incubated in the same buffer without enzyme. One half microliter of digested/undigested DNA was used for a PCR reaction in the presence of 10 mM Tris-HCL, 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 μM each primer (La Spada et al. 1991) (AR2 end labeled), and 0.5 units *Taq* polymerase. The DNA was initially denatured at 94°C for 5 min, followed by 25 cycles of denaturing at 94°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 45 s. The amplification was ended by a final extension at 72°C for 5 min.

Western Blot Analysis

Cytoplasmic proteins were isolated from Epstein-Barr virus–transformed lymphocytes. Ten milliliters of culture was centrifuged at 1,200 rpm for 5 min, and the pellet was washed twice in cold PBS. The pellet was resuspended in 100 μl of PBS with 2% SDS, 1 mM EDTA, 0.5 mg/ml, AEBSF, 10 μg/ml aprotenin, and 10 μg/ml leupeptin. The sample was boiled for 5 min, put on ice, and new inhibitors were added. Insoluble material was pelleted by centrifugation at 15,000 *g* for 10 min.

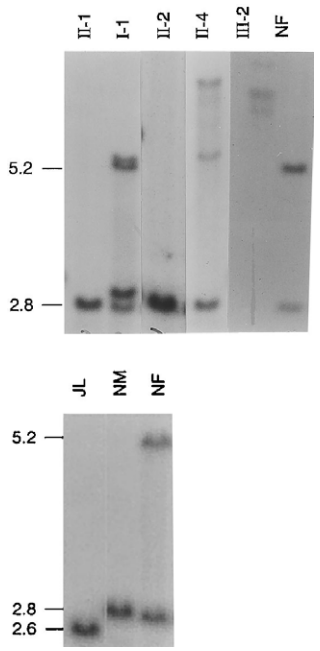

Figure 2 Southern blot analysis using probe pPX6 hybridized to *Eco*RI- and *Eag*I-digested DNA as described in Patients, Material, and Methods. The position and length of the normal fragments are indicated on the left side of the figure (2.8 and 5.2 kb), and, in addition, the position and size of the deleted fragment in JL (2.6 kb) are shown on the lower figure. The subjects are indicated above lanes. NM and NF represent a normal male and normal female, respectively.

Thirty micrograms of protein extract (measured by standard Lowry method) were run on a 8% SDS-PAGE and were blotted onto Hybond ECL membrane. The membrane was blocked in 5% milk powder in PBST (PBS, 0.1% Tween 20) and incubated with a monoclonal anti-FMR1 antibody (1C3-1a) diluted in PBST 1:2,500 or a monoclonal anti–retinoblastoma (Rb) antibody (1F8) diluted 1:20. The enhanced chemiluminescence system (Amersham, Life Science) was used as detection system following the manufacturer's instructions.

Results

DNA Analysis

We performed Southern blot analysis using the pPX6 probe on genomic DNA double digested with *Eco*RI and the methylation-sensitive enzyme *Eag*I (fig. 2). Patient JL showed hybridization to only one fragment, which was

smaller than the expected 2.8-kb fragment and estimated to have a size of 2.6 kb. No smear corresponding to a repeat size in the fully mutated range appeared on the Southern blot, not even after overexposure (authors' unpublished data). This was confirmed on Southern blot analysis using DNA isolated from the lymphoblastoid cell line used for Western blot analysis (data not shown). The Southern blot analysis revealed that subject I-1 carried a premutation, which had expanded to a full mutation in II-4. Both II-1 and II-2 appeared as normal males, whereas III-2 was a fully mutated male.

PCR using primers FMRG and FMR1B (see Patients, Material, and Methods), which would give a band size of 618 bp in an individual with 30 CGG repeats, gave a fragment of ~450 bp in JL (authors' unpublished data), indicating that a deletion was present. In order to determine the exact breakpoints of the deletion, the PCR fragment was sequenced. This revealed that JL had no CGG repeats and, furthermore, that between 63 and 67 bp immediately 5′ of the repeat and between 30 and 34 bp directly 3′ of the repeat were deleted, leaving both transcription start site and translation start site intact (fig. 3).

Protein Expression Studies

Western blot analysis using protein extracts isolated from lymphoblastoid cells and a monoclonal anti-FMR1 antibody showed several bands in the normal control, ranging in size from ~70 kDa to 85 kDa, corresponding to different alternatively spliced forms (fig. 4*A*). JL showed expression of the same bands in the same amounts as the normal control, in contrast to a fragile X patient who did not show any expression of FMRP (fig. 4*A*). Using the Rb protein as a control, it was seen that slightly more cell extract was loaded for JL than for the controls (fig. 4*B*).

Investigation of X-Chromosome Segregation and X Inactivation

As an attempt to differentiate between the paternally and maternally inherited X chromosome in JL, we determined the haplotypes for DXS548, FRAXAC1, and FRAXAC2 of I-1, II-1, II-2, II-4, and JL (results shown in fig. 1) (unfortunately, JL's father was unavailable for investigation). From this analysis, it was obvious that grandmaternal haplotype 2 4 6+ was the one segregating with the mutation. This haplotype was found in JL.

Investigation of the AR locus polymorphism showed that JL had one allele in common with her mother and one allele different. The paternal allele was exclusively inactivated, as evidenced after digestion with *Hpa*II (authors' unpublished data).

Discussion

The function of the CGG repeats is unknown, but several observations suggest that they may have a function. The smallest number of CGG repeats found in FMR1 in the normal population is six (Fu et al. 1991), and four other folate-sensitive fragile sites associated with CGG or CCG repeats are known (FRAXE, FRAXF, FRA16A, and FRA11B) (Nancarrow et al. 1994; Parrish et al. 1994; Jones et al. 1995; Gecz et al. 1996; Gu et al. 1996), of which two (FRAXE and FRA11B) have been associated with a gene (FMR2 and CBL2) (Jones et al. 1995; Gecz et al. 1996; Gu et al. 1996). Furthermore, CGG repeats are also found in the 5′-UTR of other genes such as the breakpoint cluster region gene (Riggins et al. 1994), and the core-binding factor beta subunit gene (Hajra et al. 1995). Several groups have tried to identify proteins binding to the CGG repeats to elucidate

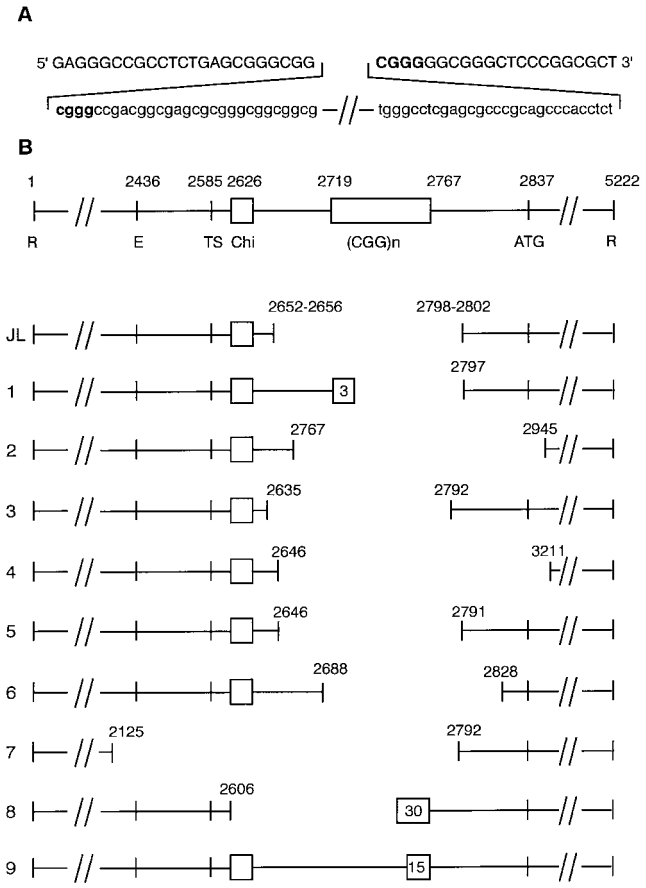

Figure 3 *A,* Sequence of the region surrounding the breakpoints of the deletion found in JL. The shown sequence starts at nt 2626 and ends at 2820 (numbering according to Fu et al. [1996] EMBL accession no. X61378). The CGGG sequence that could be the first or the last four bases deleted is shown in bold. *B,* Schematic representation of the deletion in exon 1 of the FMR1 gene of JL (not drawn to scale). All CGG repeats were deleted, and, in addition, 63–67 bp immediately 5′ to the CGG repeat and 30–34 bp 3′ of the repeat were deleted. Some of the other known deletions in this area are shown for comparison and reference: 1 (de Graaff et al. 1996), 2–5 (de Graaff et al. 1995), 6 (Mannermaa et al. 1996), 7 (Hirst et al. 1995), and 8–9 (Milà et al. 1996).

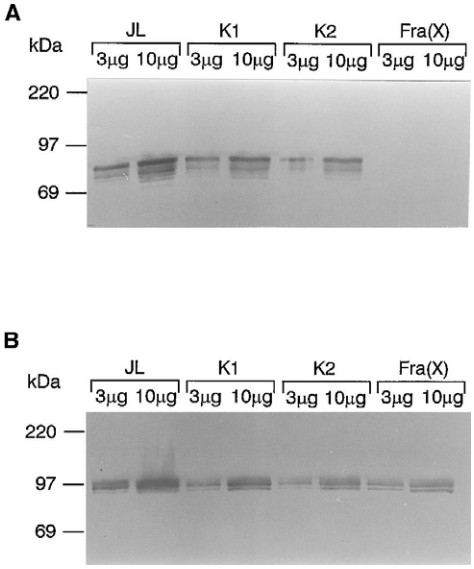

Figure 4 Western blot analysis using proteins extracted from lymphoblastoid cells. *A,* Western blot analysis using an anti-FMRP antibody (1C3-1a). The amount of total protein extract loaded is indicated above lanes. K1 and K2 are normal male controls, and Fra (X) is a fully mutated fragile X male. *B,* Western blot analysis using an anti-Rb antibody (1F8), performed to confirm the amount of loaded protein extract. The conditions were the same as in figure 4*A*.

the function of the CGG repeats (if any), but so far no final conclusions have been made (Richards et al. 1993; Yano-Yanagisawa et al. 1995; Deissler et al. 1996).

In this article, we describe a unique female patient who lacks all CGG repeats in the FMR1 gene. On one X chromosome she has a large cytogenetically visible deletion from q24 to qter, and on the other X chromosome she has a microdeletion that removes all the CGG repeats in exon 1 of the FMR1 gene plus 97 bp of flanking sequences dispersed as 63–67 bp 5′ to the CGG repeat in addition to 30–34 bp 3′ to the repeat. The reason for the uncertainty for the exact breakpoints is the fact that a CGGG sequence is either the first or the last four bases deleted (see fig. 3*A*). Western blot analysis demonstrated that FMRP expression was not blocked in lymphoblastoid cells from JL. Since JL did not display any mosaicism (which is in contrast to the other patients known to have deletions in this region), it can be concluded that the CGG repeats in the 5′-UTR of the FMR1 gene are not necessary for expression of the gene. Furthermore, there are no regulatory elements located 63 bp 5′ to the repeat and 30 bp 3′ to the repeat. These conclusions only apply to the cells studied, that is, lymphoblastoid cells, and further studies are necessary to investigate whether the lack of repeats has functions elsewhere.

The instability of the CGG repeats in the FMR1 gene is a well-known phenomenon. The presence of both a full mutation and a deletion in the CGG region in the mosaic patients again demonstrates the instability of the region, and it can be speculated that these deletions originated from regression of the full mutation, but this has not been documented in any of the former cases. Here we present data showing that the deletion originated as a deletion of a full mutation. Genotyping of three microsatellite markers (DXS548, FRAXAC1, and FRAXAC2) in the region surrounding the FMR1 gene revealed that JL inherited the allele that originally carried the full mutation. Furthermore, the inherited maternal allele was active with totally nonrandom inactivation of the paternal allele, as evidenced by Southern blot (no band corresponding to inactive X) and AR locus analysis (paternal allele exclusively inactive). This is the first unequivocal demonstration that a deletion in the 5′-UTR of the FMR1 gene arose from regression of a full mutation.

The time and mechanism of the CGG expansion are not fully understood. One hypothesis states that expansion is a mitotic event taking place postzygotically. This is supported by the fact that somatic variation is seen within an individual (Wörhle et al. 1993). The other hypothesis states that expansion takes place meiotically only during oogenesis, which is consistent with the observation that expansion is never seen in offspring of premutated males (so-called normal transmitting males). Fully mutated males carry only a premutation in their gametes (Reyniers et al. 1993), which has been taken as evidence for a mitotic expansion; however, experiments showing that testicular cells of a 13-wk full-mutation fetus contain only fully mutated alleles and no premutated alleles support the hypothesis that expansion takes place during maternal germ-line development or very early in embryogenesis (Malter et al. 1997). Perhaps the repeats are unstable both in meiosis and mitosis, but this is pure speculation. Since JL inherited an unaltered maternal haplotype for markers surrounding FMR1, we can conclude that regression did not take place as part of a meiotic crossover event in the CGG repeat. The deletion event probably took place relatively early, since no mosaicism was detected in JL; however, it cannot be ruled out that JL showed mosaicism in other tissues.

It is generally accepted that fragile X syndrome is caused by the absence of FMRP. The phenotype of JL was essentially normal. The problems this patient had (hearing problems and some deficits in perceptual spatial skills) could be ascribed to the problems in connection to the delivery or be due to the large deletion on the other X. From a review of published cases with Xq deletions, it was concluded that the phenotypic effect of Xq− is highly variable, presumably because of variable inactivation (Geerkens et al. 1994). Thus, an essentially normal phenotype is compatible with the large deletion; furthermore, the del(X)(q24) chromosome in JL was preferentially inactivated.

In this article, we present results showing that the CGG repeats located in the 5′-UTR of the FMR1 gene plus 63 bp immediately 5′ to the repeat and 30 bp directly 3′ to the repeat do not contain any regulatory elements necessary for expression of the FMR1 gene.

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