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Hypomethylation of an Expanded *FMR1* Allele Is Not Associated with a Global DNA Methylation Defect

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Summary

The vast majority of fragile-X full mutations are heavily methylated throughout the expanded CGG repeat and the surrounding CpG island. Hypermethylation initiates and/or stabilizes transcriptional inactivation of the *FMR1* gene, which causes the fragile X–syndrome phenotype characterized, primarily, by mental retardation. The relation between repeat expansion and hypermethylation is not well understood nor is it absolute, as demonstrated by the identification of nonretarded males who carry hypomethylated full mutations. To better characterize the methylation pattern in a patient who carries a hypomethylated full mutation of ~60–700 repeats, we have evaluated methylation with the McrBC endonuclease, which allows analysis of numerous sites in the *FMR1* CpG island, including those located within the CGG repeat. We report that the expanded-repeat region is completely free of methylation in this full-mutation male. Significantly, this lack of methylation appears to be specific to the expanded *FMR1* CGG-repeat region, because various linked and unlinked repetitive-element loci are methylated normally. This finding demonstrates that the lack of methylation in the expanded CGG-repeat region is not associated with a global defect in methylation of highly repeated DNA sequences. We also report that de novo methylation of the expanded CGG-repeat region does not occur when it is moved via microcell-mediated chromosome transfer into a de novo methylation-competent mouse embryonal carcinoma cell line.

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Introduction

Fragile-X syndrome (MIM 309550) is an X-linked mental retardation disorder with an incidence of ~1/6,000 (Turner et al. 1996; de Vries et al. 1997; Morton et al. 1997). In most patients, amplification of an unstable CGG repeat, located in the 5′ untranslated region of the *FMR1* gene, is responsible for the syndrome (Kremer et al. 1991; Oberle et al. 1991; Verkerk et al. 1991; Yu et al. 1991). In the normal population, *FMR1* alleles contain 5–~55 repeats, are stable on transmission, and are not methylated except when located on an inactive X chromosome in females (Bell et al. 1991; Fu et al. 1991; Hansen et al. 1992; Hornstra et al. 1993). Premutation alleles with ~55–~220 repeats are found in unaffected carriers and, like normal alleles, are not methylated unless they are located on an inactive X chromosome. When transmitted by a woman, premutation alleles can expand to become disease-causing full mutations (Fu et al. 1991; Heitz et al. 1991; Oberle et al. 1991; Snow et al. 1993).

Full-mutation alleles contain from ~220 to >1,000 repeats and are almost always associated with extensive hypermethylation of the CGG repeat and a surrounding CpG island (Heitz et al. 1991; Oberle et al. 1991; Pieretti et al. 1991; Hansen et al. 1992; Sutcliffe et al. 1992; Hornstra et al. 1993). Hypermethylation of promoter elements (Pieretti et al. 1991; Sutcliffe et al. 1992) and histone deacetylation (Coffee et al. 1999) are associated with transcriptional silencing of the *FMR1* gene, presumably by interfering with transcription-factor binding (Schwemmle et al. 1997). The resulting lack of FMR1 protein (FMRP) is believed sufficient to cause the fragile X–syndrome phenotype (Pieretti et al. 1991; Devys et al. 1993; Siomi et al. 1993; Hammond et al. 1997). Although repeat expansions > ~220 triplets are typically accompanied by hypermethylation, unusual expansions have been described in which full-mutation alleles are unmethylated at diagnostic restriction sites (Loesch et al. 1993; McConkie-Rosell et al. 1993; Hagerman et al. 1994; Merenstein et al. 1994; Rousseau et al. 1994*b*; Smeets et al. 1995; de Vries et al. 1996; Lachiewicz et

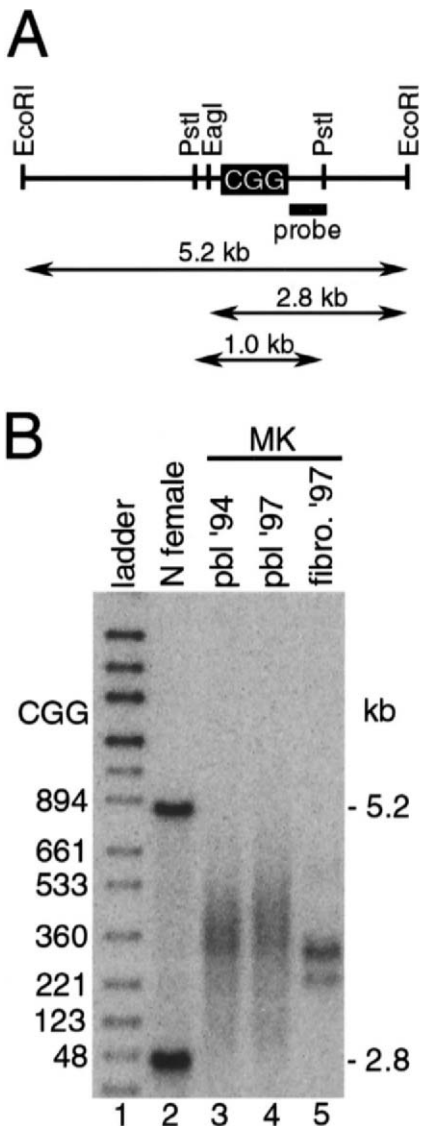

Figure 1 No methylation at *Eag*I site. *A,* Simplified restriction map of the region surrounding the *FMR1* CGG repeats. The size of the restriction fragments are determined on the basis of a normal allele with 30 repeats. The probe used was *pfxa3*. *B,* Southern blot of genomic DNA samples digested with *Eco*RI/*Eag*I and hybridized with *pfxa3*. A control female (lane 2) showing a methylated 5.2-kb fragment resistant to *Eag*I digestion (inactive X chromosomes) and an unmethylated 2.8-kb fragment that is digested with *Eag*I (active X chromosomes). Three DNA samples from the patient (lanes 3–5): two peripheral blood–lymphocyte samples collected in 1994 and 1997 (pbl'94 and pbl'97, respectively) and cultured fibroblasts established from a 1997 skin biopsy (fibro'97). The approximate CGG-repeat number, as a function of size for unmethylated alleles, is shown on the left.

al. 1996; Wang et al. 1996; Wohrle et al. 1998; Taylor et al. 1999). Male carriers of these mutations are often described as "high-functioning" and can have cognitive and physical phenotypes in the normal-to-mild range of affectedness. FMRP expression has been reported in many of these patients, although at levels reduced relative to that of normal controls. This reduction may be a result of translational suppression (Feng et al. 1995). *FMR1* methylation, in these individuals, has been studied with use of methylation-sensitive restriction endonucleases, which limit the number of methylation sites that can be analyzed. Although FMRP expression suggests that regulatory elements are also unmethylated, the methylation status of most sites in the CpG island, including the expanded CGG repeat, have not been described.

The association between repeat expansion and methylation is not well understood, and, in particular, it is unknown why some full-mutation alleles escape the methylation process. Reports of typically methylated full mutations in the grandsons of males bearing hypomethylated full mutations favor a role for *trans*-acting factors rather than a heritable *cis* effect (Smeets et al. 1995; Lachiewicz et al. 1996; Wohrle et al. 1998). The study of methylation variants at the fragile-X locus will help define the basic relation between repeat expansion and methylation and may also provide information about the potential efficacy of therapeutic approaches that utilize demethylating agents (Chiurazzi et al. 1994). Moreover, a description of *FMR1* methylation in hypomethylated full-mutation males is a necessary prerequisite for the study of the proposed influence of methylation on repeat stability (Wohrle et al. 1996).

The aims of the present study were to confirm that hypomethylation at the diagnostic *Eag*I site extends into the expanded CGG-repeat region and to ascertain whether this lack of methylation is indicative of a *trans* defect in de novo and/or maintenance methylation. We have assessed methylation at a large number of sites in the *FMR1* CpG island, including the CGG repeat, and at various repetitive DNA elements. Our data demonstrate an absence of methylation throughout the CpG island, despite normal methylation levels at flanking *Alu* elements and at other repetitive-element loci. The methylation deficit in the expanded CGG-repeat region was not corrected when the human X chromosome carrying this allele was transferred into a de novo methylation-competent mouse cell line. These results argue against a model in which hypomethylation of full mutation *FMR1* alleles is a result of a *trans* defect in DNA methylation.

Patient and Methods

Patient Description

M.K. is a 39-year-old male who was evaluated for a fragile-X mutation on the basis of a positive family history. M.K.'s sister, mother, and maternal aunt are known to be premutation carriers. The maternal aunt has two

sons who are both reported to carry fragile-X full mutations. One cousin is reported to be mentally retarded and to carry a typical methylated full mutation. The other cousin, like M.K., is reported to carry an unmethylated or partially unmethylated full mutation. In addition, M.K. has six other siblings, all of whom carry *FMR1* alleles in the normal size range.

Cognitively, M.K. appears to be quite "high-functioning." He graduated from high school and is raising a family with two sons. He has been in the military where he successfully completed specialized training. He is currently employed as a drug and alcohol counselor. M.K. has good eye contact and comfortably engages in conversation. On the basis of a short interview that did not include a physical evaluation or formal cognitive testing, he does not appear to be affected with the fragile-X syndrome either cognitively or behaviorally.

DNA Isolation and Analysis

Genomic DNA was isolated with use of either a standard phenol/chloroform extraction or the Puregene DNA Isolation Kit (Gentra Systems). Aliquots (10–30 μg) were digested with restriction endonucleases purchased either from Boehringer Manheim (*Eco*RI, *Pst*I, *Msp*I, and *Hin*dIII) or New England Biolabs (*Eag*I, McrBC, *Hpa*II, and *Bst*BI). *Eco*RI/*Eag*I double digests were performed simultaneously with both enzymes. All other double digestions were performed sequentially with an initial digestion using *Pst*I, *Eco*RI, or *Hin*dIII, followed by precipitation, and then a second digestion with *Mcr*BC, *Msp*I, *Hpa*II, or *Bst*BI. Digested DNA was precipitated with sodium acetate and ethanol, and then aliqouts of 1.5–2.5 μg were separated by elecrophoresis onto a 1% agarose/TAE gel and transferred to Biodyne B nylon membrane (Gibco BRL) with 5 × SSC. Hybridizations with a ^{32}P-radiolabelled probe (Boehringer Manheim; Random Prime Labelling Kit) were carried out at 65°C in Church Buffer with BSA (hybridization buffer II) (Strauss 1998), supplemented with 100 μg/ml heat-denatured herring sperm DNA. Probe *pfxa3* (a kind gift from David L. Nelson, Baylor College of Medicine, Houston), a 558-bp *Xho*I/*Pst*I fragment of pE5.1 (fig. 1*A*) (Fu et al. 1991; Verkerk et al. 1991), was used to detect restriction fragments containing the CGG repeat. Additional probes used to assess methylation at the repetitive-element loci are described below. Membranes were washed twice at room temperature in low-stringency wash buffer II (Strauss 1998) and then twice at 68.5°C in high-stringency wash buffer II (Strauss 1998), diluted to 60%. Membranes were exposed sequentially to a phosphorimaging screen (Molecular Dynamics) and then to X-ray film (Kodak; X-OMAT) at −70°C.

Analysis of Repetitive DNA–Element Methylation

Repetitive-element loci were identified in genomic DNA sequence with the RepeatMasker database. The *Alu* elements flanking the *FMR*1 CGG repeat, as well as the *L1* and *SVA* (sindbis virus) elements, are contained in the "HUMFMR1S DNA sequence" (Genbank accession number L29074). The positions of the *Alu, L1,* and *SVA* elements within the L29074 sequence are as follows: the *Alu* element upstream of *FMR1* CGG repeat (nucleotides 7984–8274), the *Alu* element downstream of CGG repeat (nucleotides 18523–18804), the *L1* element (nucleotides 84696–92586), and the *SVA* element (nucleotides 93455–95194). Hybridization probes flanking the elements were generated by PCR with the following primer pairs. The probe for the *Alu* element upstream of CGG repeat: forward primer (nucleotides 9881–9903) 5′-GAATGAGAGGTCATGGTTAAAGG-3′, and reverse primer (nucleotides 10137–10159) 5′-ATCTGATATTGGAATGATGCTTC-3′. The probe for *Alu* element downstream of CGG repeat: forward primer (nucleotides 18823–18849) 5′-AGGAGGGATATTTTACAATGCTGTAAG-3′, and reverse primer (nucleotides 19277–19301) 5′-TGTGATGAGAATCTTGGAATTTGAG-3′. The probe between *L1* and *SVA* elements: forward primer (nucleotides 92687–92710) 5′-CCTCCATCATCTCCTCTCTTAAAG-3′, and reverse primer (nucleotides 93216–93241) 5′-GAGGTTAGAATTTTGTTAGGGGAGAG-3′. PCR amplifications were carried out in 25-μl reactions with 50 ng genomic DNA, 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.8 μM each primer, 1 × PCR buffer II (PE Biosystems), and 1.25 U *Taq* DNA polymerase (Boehringer Manheim). Thermal cycling parameters were as follows (Stratagene Robocycler): 95°C for 4 min for 1 cycle; 95°C for 1 min, 62°C for 1 min, and 72°C for 2 min for 30 cycles; and 72°C for 10 min for 1 cycle. PCR products were purified from agarose gels (Qiagen; Qiaex II Gel Purification Kit) and cloned (Invitrogen; TA Cloning Kit). The cloned probes were isolated from the vector by standard methods.

The *Alu* element examined on the Y chromosome is contained within the "Homo Sapiens 5′ region *ZFY* gene sequence" (Genbank accession number U00242). This *Alu* element is located at nucleotides 4669–4954. The PCR-generated probe flanking this *Alu* was amplified with the following primer pair: forward primer (nucleotides 3594–3615) 5′-GCAGTGTCGGCTACGCTTTAGG-3′, and reverse primer (nucleotides 4638–4660) 5′-GCTACCTTCTTGATCATCCATCC-3′. PCR amplification conditions were the same as described except the annealing temperature was 66°C. This probe was also gel-purified, cloned, and then isolated from the vector.

Tissue Culture, Cell Lines, and Microcell-Mediated Chromosome Transfer

Human fibroblast cell lines were established, with informed consent, from skin biopsies of adult fragile-X full-mutation carriers (TC38-89, M.K.) referred for clinical fragile-X testing to either the Oregon Health Sciences University DNA Diagnostic Laboratory or the Kaiser Permanente Cytogenetics Laboratory. All human cell cultures were maintained at 37°C in 5% CO_2, in α-minimal essential media (α-MEM) supplemented with 20% FCS (JRH Biosciences), 2 mM L-glutamine, and 16 μg/ml gentamicin sulfate.

The DelTG3 mouse cell line is a thioguanine-resistant clone isolated form the mouse P19-derived embryonal carcinoma (EC) cell line H4D2 (Turker et al. 1989*a*, 1989*b*). The Dif6 cell line is a morphologically differentiated and thioguanine-resistant derivative of H4D2 (Turker et al. 1991). All mouse cell cultures were maintained at 37°C in 5% CO_2, in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% FCS and 5% serum plus (JRH Biosciences).

Whole-cell fusions were accomplished by mixing human fragile-X fibroblasts and mouse cell-line Dif6 to obtain ratios of 1:3, 1:1, and 3:1 with a total of 3×10^6 cells per 25-cm^2 flask. After mixing, the cells were incubated for ~6 h and fused, by treatment for 60 s or 80 s, with 1 ml of 50% polyethylene glycol solution (Sigma; PEG 1,450) in α-MEM. The cultures were rinsed three times with PBS, incubated overnight in nonselective media, and split the next day into 150-mm dishes at a concentration of $1 - 2 \times 10^5$ cells/dish. Selection for fusion clones was applied ~48 h post-fusion with 10 μg/ml hypoxanthine (Sigma), 10 μg/ml azaserine (Sigma), and 1 mg/ml geneticin (Gibco BRL). Individual fusion clones were isolated 11–14 d after initiating selection and were then maintained in media supplemented with hypoxanthine and azaserine to retain the human X chromosome. Microcell fusions were performed essentially as described by Fournier (1981). Microcells were isolated from a whole-cell hybrid clone and fused to DelTG3 by treatment for 60 s with a 50% PEG solution in α-MEM. Selection for the human X chromosome was accomplished with azaserine and hypoxanthine. Individual microcell hybrid clones were isolated 16 d after fusion and were expanded through ~24 population doublings until the cells were harvested for DNA isolation.

Results

A Full Mutation with No CpG-Island Methylation in DNA Isolated from Patient M.K.

Analysis of methylation at the *FMR1* locus was initially performed by Southern blot with *Eco*RI/*Eag*I–digested genomic DNA derived from patient M.K.'s peripheral blood lymphocytes and cultured skin fibroblasts. Hybridization of the membrane with the probe *pfxa3* resulted in the expected 2.8-kb and 5.2-kb fragments in a control female (fig. 1*B*, lane 2). The hybridization band, at 2.8 kb, represents normal unmethylated alleles with ~30 repeats. The 5.2-kb band represents the same normal alleles that have a methylated *Eag*I site due to X chromosome inactivation (fig. 1*A*). Peripheral blood DNA from the patient showed an unusual hybridization pattern with a large, highly diffuse smear ranging in size from ~60–700 repeats (fig. 1*B*, lanes 3 and 4; see also fig. 2*A*, lane 18). DNA derived from M.K.'s cultured skin fibroblasts produced a hybridization pattern with less smearing, two major bands at 230 and 330 repeats, and a third, faint band at 600–650 repeats (fig. 1*B*, lane 5; see also fig. 2*A*, lane 20). Despite most of the alleles in both tissue types falling well within the full-mutation size range, digestion with *Eag*I appeared complete, which is consistent with a complete lack of methylation at this site located 282 bp upstream

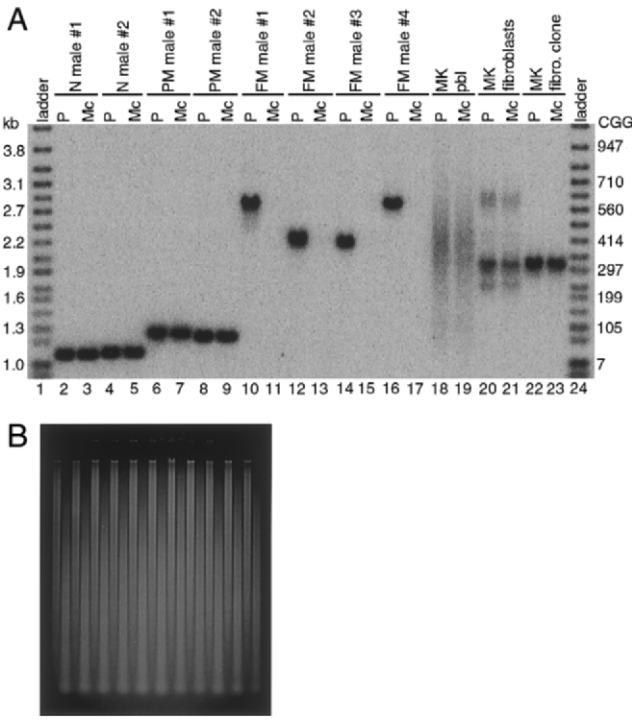

Figure 2 No methylation detected with the McrBC endonuclease. *A*, Southern-blot analysis of genomic DNA samples digested with either *Pst*I alone (P) or *Pst*I followed by McrBC (Mc) and hybridized with probe *pfxa3*. Genomic DNA samples were derived from two normal males (N male), two premutation males (PM male), four full-mutation males (FM male), and patient M.K.'s peripheral blood lymphocytes (M.K. pbl), fibroblasts (M.K. fibroblasts), and fibroblast clone (M.K. fibro. clone). *B*, Photograph of the ethidium bromide–stained gel, taken prior to Southern blotting.

of the CGG repeat (fig. 1*A*). Methylation at the *Eag*I site would have produced a hybridization pattern of ⩾5.2 kb. Analysis of DNA samples from 37 clonal fibroblast lines derived from M.K.'s skin culture also revealed no evidence of methylation at the *Eag*I site (data not shown). The allele sizes observed in these clones occurred at frequencies proportionate to their relative abundance in the mass culture. Most clones contained alleles of 230–330 repeats, and some contained large alleles of 600–650 repeats.

To assess more fully methylation levels throughout the CGG expansion and flanking regions, DNA preparations were digested with the McrBC endonuclease combination. The recognition sequence of the McrBC enzyme pair consists of two half-sites, each composed of a purine followed by methylcytosine. Since only cytosines within a CpG dinucleotide are eligible for methylation in mammals, an McrBC half-site can be either A^mCG or G^mCG. Digestion of DNA occurs when two half-sites are separated by 32 bp–2 kb, with optimal separation at 55–103 bp (Stewart and Raleigh 1998). There are 48 potential half-sites and an additional half-site at each CGG repeat within the *Pst*I-restriction fragment that contains the repeat and most of the CpG island. Analysis of methylation at the McrBC sites was performed by Southern blot of DNA samples digested with either *Pst*I alone or with *Pst*I followed by McrBC. Hybridization was again carried out with probe *pfxa3*. DNA samples derived from two control males and two premutation males showed identical hybridization patterns in samples digested with *Pst*I alone or with *Pst*I + McrBC (fig. 2*A,* lanes 2–9). The failure of McrBC to digest these DNA samples is consistent with an absence of methylation. In contrast, DNA preparations from four full-mutation males were digested completely by McrBC, as demonstrated by the absence of hybridization bands in these lanes (fig. 2*A,* lanes 11, 13, 15, and 17). Although it is not possible to determine which specific sites are methylated and serving as half-sites in these samples, the absence of a hybridization signal in the *Pst*I + McrBC lanes is consistent with substantial methylation. A photograph of the ethidium bromide–stained gel taken before Southern blotting shows that DNA content in these lanes was indistinguishable from the other *Pst*I + McrBC lanes (fig. 2*B*). DNA samples from the patient were not digested by McrBC and produced hybridization patterns identical to those observed in the samples digested with *Pst*I alone (fig. 2*A,* lanes 18–23). This result indicates that the McrBC half-sites throughout the CpG island and within the CGG repeat are largely or completely free of methylation, although it is possible that a single half-site is methylated, or that methylation occurs at two or more sites separated by <32 bp. We note that some of the *Pst*I + McrBC lanes have a slightly diminished hybridization signal compared to the lanes containing samples digested with *Pst*I alone. We attribute this to underloading of the *Pst*I + McrBC samples as a consequence of GTP in the McrBC digestion buffer, which interfered with spectrophotometric quantitation of the samples post-digestion.

Hypomethylation of the Expanded CGG-Repeat Region Occurs in the Presence of Normal Methylation of Repetitive DNA Elements

Most repetitive elements are heavily methylated in mammalian somatic cells, and it has been suggested that methylation of expanded *FMR1* CGG repeats is due to their resemblance to these elements (Bestor and Tycko 1996). To determine whether *FMR1* hypomethylation in patient M.K. is associated with decreased methylation at repetitive DNA elements, methylation was assessed at five repetitive-element loci. These loci were selected on the basis of one of the following: (1) proximity to the *FMR1* CGG repeat (two *Alu* elements), (2) high CpG density (an X chromosome *SVA* element), or (3) timing of de novo methylation during gametogenesis and early development (a Y chromosome *Alu* element and an X chromosome *L1* element). Methylation at each of the loci was ascertained by Southern-blot analysis of genomic DNA samples digested with a methylation-sensitive restriction enzyme. Hybridization probes flanking the elements were generated by PCR as presented in the Patient and Methods section.

Alu elements closest to the *FMR1* CGG repeat are located 4.7 kb downstream (in the middle of intron 1) and 5.6 kb upstream. These elements each contain at least one *Msp*I/*Hpa*II restriction site at which methylation can be assessed (fig. 3*A* and *C*). DNA preparations were digested with *Eco*RI alone, with *Eco*RI + *Msp*I, or with *Eco*RI + *Hpa*II. Hybridization with a probe flanking the downstream *Alu* showed no apparent *Hpa*II digestion, demonstrating a high degree of methylation at these sites in peripheral blood–lymphocyte DNA samples from normal controls (fig. 3*B,* lanes 4 and 7) and four fragile-X carriers (data not shown). The hybridization pattern in M.K.'s lymphocyte and fibroblast DNA samples are indistinguishable from that of the controls (fig. 3*B,* lanes 10 and 13), indicating that this *Alu* element is methylated normally in the patient. Although most *Alu* elements are heavily methylated in somatic cells (Schmid 1991), these elements are largely hypomethylated in sperm cells (Hellmann-Blumberg et al. 1993; Kochanek et al. 1993). As a control for probe specificity and *Hpa*II digestion, DNA isolated from the sperm of a normal control was analyzed. Hybridization in the sperm DNA *Eco*RI + *Hpa*II lane demonstrated significant *Hpa*II digestion, consistent with markedly reduced methylation of both *Msp*I/*Hpa*II sites (fig. 3*B,* lane 16). Analysis of the upstream *Alu* was accomplished

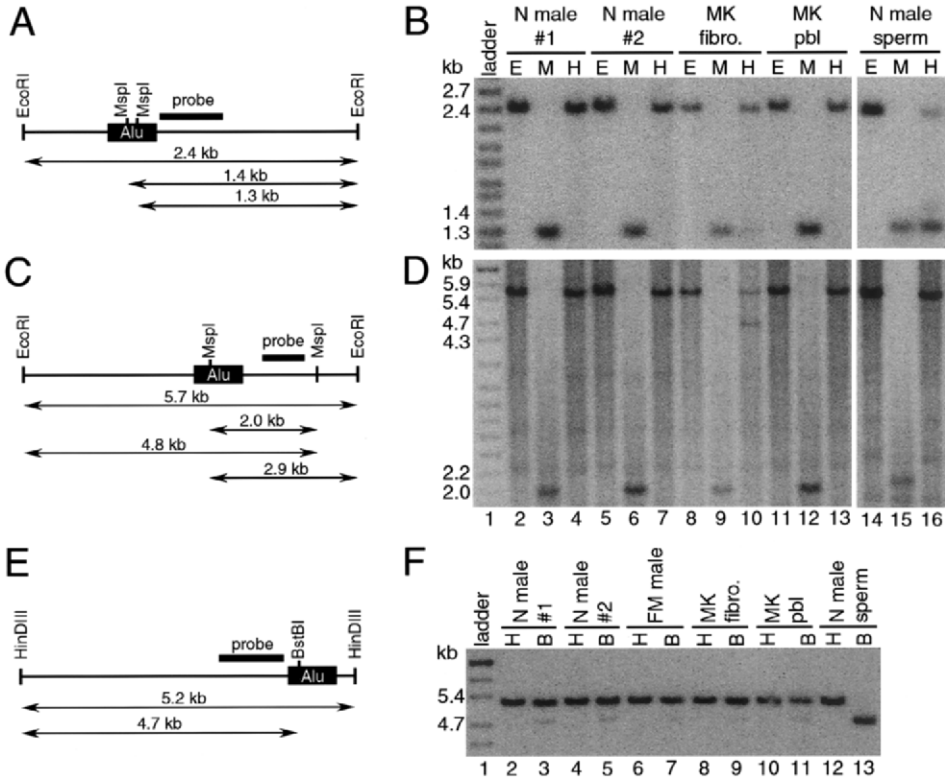

Figure 3 Analysis of *Alu* methylation. *A,* Restriction map of region surrounding an *Alu* element 4.7 kb downstream of the *FMR1* CGG repeats. *B,* Southern-blot analysis of methylation at the downstream *Alu,* done with a PCR-generated probe and genomic DNA samples digested with *Eco*RI alone (E), *Eco*RI and *Msp*I (M), or *Eco*RI and *Hpa*II (H). Genomic DNA samples were derived from two normal males (N male), patient M.K.'s fibroblasts (M.K. fibro.) and peripheral blood lymphocytes (M.K. pbl), and sperm from a normal male (N male sperm). *C,* Restriction map of region surrounding an *Alu* element located 5.4 kb upstream of the *FMR1* CGG repeat. *D,* Southern-blot analysis of methylation at the upstream *Alu,* shown in *C,* done with a PCR-generated probe and the same Southern-blot membrane used in *B*. *E,* Restriction map of the region surrounding a Y chromosome *Alu* near the *ZFY* gene. *F,* Methylation of the Y chromosome *Alu,* shown in *E,* done by Southern blot of DNA samples digested with *Hin*dIII alone (H) or with *Hin*dIII and *Bst*BI (B), and hybridized with a PCR-generated probe. DNA samples were derived from two normal males (N males), a full-mutation male (FM male), patient M.K.'s peripheral blood lymphocytes (M.K. pbl) and fibroblasts (M.K. fibro.), and sperm from a normal male (N male sperm).

with use of the same Southern-blot membranes, which were stripped and hybridized to a probe flanking the upstream element (fig. 3*C*). The hybridization pattern again showed a high degree of methylation for this element in the peripheral blood–lymphocyte controls (fig. 3*D,* lanes 4 and 7) and the M.K. samples (fig. 3D, lanes 10 and 13). Partial methylation of an*Hpa*II site outside the *Alu* sequence was observed in M.K.'s fibroblast-derived DNA (fig. 3*D,* lane 10), but complete methylation at this site was observed in his lymphocyte DNA (fig. 3*D,* lane 13). Unlike the downstream *Alu,* hybridization with the upstream *Alu* probe revealed a high degree of methylation in the sperm-derived DNA (fig. 3*D,* lane 16).

Alu elements are inherited from the female parent in the methylated state and from the male parent in the unmethylated state (Schmid 1996; Yoder et al. 1997). The paternally inherited *Alu* elements are methylated de novo during early embryogenesis (Yoder et al. 1997). To study methylation at an *Alu* element likely to be subject to de novo methylation during M.K.'s development, we assessed methylation at a Y chromosome *Alu,* located upstream of the *ZFY* gene. DNA samples were digested with *Hin*dIII alone or with *Hin*dIII + *Bst*BI (fig. 3*E*). Digestion with *Bst*BI did not occur in somatic controls or in DNA from M.K.'s lymphocytes and fibroblasts (fig. 3*F,* lanes 3, 5, 7, 9, and 11). The inability of *Bst*BI to digest these samples is consistent with a high degree of methylation. Sperm DNA was completely digested with *Bst*BI, indicating this site is unmethylated in this tissue (fig. 3*F,* lane 13).

It is not known whether de novo methylation of *Alu* elements and fragile-X full-mutation alleles share any mechanistic similarities. Although both can potentially form secondary structures, which may serve as signals for de novo methylation, expanded fragile-X repeats are

significantly larger and more CpG dense than are *Alu* elements. To assess methylation at a site that more closely resembles an expanded fragile-X repeat, we searched for large repetitive elements with high CpG density near the *FMR1* gene. Approximately 80 kb downstream of the *FMR1* CGG repeat is a region of high CpG density contained within an *SVA* element. The *SVA* element spans 1.7 kb and contains 86 (9.9%) CpG dinucleotides, 21 of which are located in *Msp*I/*Hpa*II–recognition sites. Adjacent to the *SVA* element is a large *L1* element that contains two additional *Msp*I/*Hpa*II sites (fig. 4*A*). Methylation of the *L1* and *SVA* CpG sites was assessed with the same Southern-blot membranes that were used for analysis of the X chromosome *Alu* elements. Hybridization patterns in the normal and fragile-X peripheral blood–lymphocyte controls were consistent with complete methylation of both sites in the *L1* element and with near-complete methylation at the sites in the *SVA* element (fig. 4*B,* lanes 4 and 7). The hybridization pattern in M.K.'s lymphocyte-derived DNA was indistinguishable from the controls' (fig. 4*B,* lane 13). M.K.'s fibroblast-derived DNA sample was substantially less methylated at sites in both the *L1* and *SVA* elements (fig. 4*B,* lane 10). However, the same methylation pattern was also observed in fibroblast-derived DNA from six controls, indicating that the difference between the lymphocyte and fibroblast cells is tissue specific and not attributable to the specific conditions in M.K.'s cells (data not shown). Sperm-derived DNA from a normal control was completely methylated at both *L1* sites and was substantially less methylated at at least some of the *SVA* sites compared to the somatic controls (fig. 4*B,* lane 16).

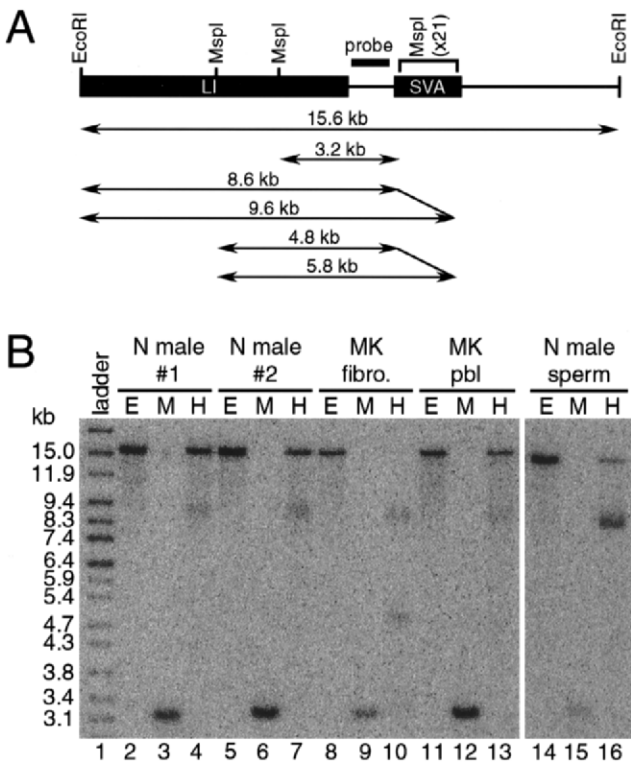

Figure 4 Analysis of methylation at X chromosome *L1* and *SVA* elements. *A,* Restriction map of region surrounding *L1* and *SVA* elements located ~80 kb downstream of the *FMR1* CGG repeats. *B,* Southern-blot analysis was accomplished with a PCR-generated probe and the same Southern-blot membranes used in figure 3*B* and *D*. Partial methylation of the 21 recognition sites located in the *SVA* element produces a smear, which is denoted on the restriction map by a diagonal line.

Microcell-Mediated Chromosome Transfer into Mouse EC Cells Does Not Induce De Novo Methylation of Hypomethylated Expanded CGG Repeats

To test whether the methylation deficiency in M.K.'s cells could be complemented in *trans,* an X chromosome bearing an unmethylated full mutation was transferred by microcell fusion into a de novo methylation-competent mouse EC cell. EC cells are capable of methylating certain DNA substrates de novo, including proviral genomes (Stewart et al. 1982) and mammalian sequences that direct the formation of methylation patterns (Turker et al. 1991; Mummaneni et al. 1993, 1995). These sequences include *B1*-repetitive elements (P. A. Yates, R. W. Burman, P. Mummaneni, S. Krussel, and M. S. Tucker, unpublished data) which are homologous to the human *Alu* elements (Schmid 1996). The unmethylated full-mutation allele was transferred initially by whole-cell fusion to a differentiated and 6-thioguanine resistant (HPRT−) mouse cell line that lacks the capacity for de novo methylation of transfected DNA (Turker et al. 1991). Retention of the human X chromosome was selected on the basis of expression of the *HPRT* locus, located ~19 Mb centromeric of the *FMR1* gene. A whole-cell hybrid containing an unmethylated expansion of 350 repeats was selected as the microcell donor. The repeat region remained unmethylated in the differentiated background, which is indicated by resistance to McrBC digestion (fig. 5, lane 9). Microcells were isolated from the donor clone by standard procedures and fused to DelTG3, a thioguanine-resistant derivative of the P19 EC cell line. Nine fusion clones, each of which exhibited the undifferentiated morphology characteristic of EC cells, were isolated. All of the microcell clones retained the human *HPRT* locus, but only four of the nine retained the *FMR1* region indicated by Southern-blot analysis and PCR amplification of markers located between the loci (data not shown). Despite propagation of the chromosomes through ~24 population doublings, the *FMR1* CpG island remained unmethylated by both *Eco*RI/*Eag*I digestion (data not shown) and *Pst*I +

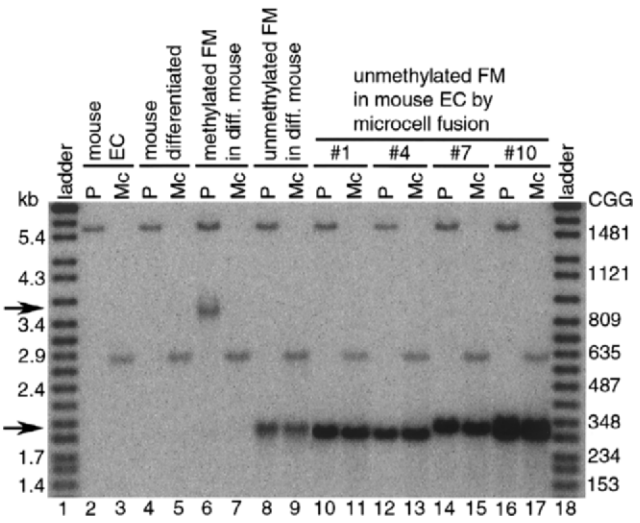

Figure 5 Microcell-fusion clones. Methylation was analyzed by Southern blot of DNA samples digested with *Pst*I alone (P) or with *Pst*I followed by McrBC (Mc), and hybridized with probe *pfxa3*. Nonpolymorphic mouse-specific bands were observed at 5.6 kb and at 2.7 kb in *Pst*I- and *Pst*I + McrBC–digested DNA, respectively. The positions of human *FMR1*-specific hybridization bands with ~850 or ~350 repeats are indicated by arrows. Mouse DNA, derived from cultured EC (mouse EC) or from differentiated cells (mouse differentiated), shows only the presence of the mouse-specific bands (lanes 2–5). Introduction of a human fragile-X chromosome into the differentiated cells by whole-cell fusion results in an additional human-specific band in the fusion clones (lanes 6 and 8). When the human X chromosome contains a methylated expansion (methylated FM in differentiated mouse), it is sensitive to digestion with McrBC (lane 7). An unmethylated expansion (unmethylated FM in differentiated mouse) is resistant to digestion in the mouse background, indicating that the human locus remains free of methylation in the differentiated mouse cells (lane 9). Microcell-fusion clones in which an unmethylated full mutation is replicated in an EC cell background (unmethylated FM in mouse EC, by microcell fusion) are also resistant to McrBC digestion and are thus not methylated (lanes 11, 13, 15, and 17).

McrBC digestion in each of the microcell clones (fig. 5, lanes 11, 13, 15, and 17).

Discussion

The patient examined in this study is one of a small group of individuals that carries *FMR1* full-mutation alleles lacking significant methylation at upstream sites (Loesch et al. 1993; McConkie-Rosell et al. 1993; Hagerman et al. 1994; Merenstein et al. 1994; Rousseau et al. 1994*a,* 1994*b*; Feng et al. 1995; Smeets et al. 1995; de Vries et al. 1996; Lachiewicz et al. 1996; Wang et al. 1996; Wohrle et al. 1998; Taylor et al. 1999). Characterization of methylation in these individuals has, in most cases, been made on the basis of Southern-blot analysis of genomic DNA samples digested with methylation-sensitive restriction endonucleases (usually *Eag*I, *Bss*HII, and *Nru*I). Although each of these enzymes assesses methylation at only a small percentage of the CpG dinucleotides that comprise the *FMR1* CpG island, the production of FMRP and normal protein DNA–footprinting interactions (Schwemmle 1999) suggest that sites throughout the promoter are also unmethylated. Furthermore, analysis of epigenetic-methylation patterns with bisulfite sequencing has demonstrated that *FMR1* methylation patterns are exclusive (i.e., a particular DNA molecule will be either hypo- or hypermethylated) (Stoger et al. 1997). We observed that M.K. carries a mix of premutation and full-mutation alleles that are completely unmethylated at the *Eag*I restriction site. Moreover, analysis with McrBC-digested DNA demonstrates that most if not all CpG dinucleotides, both within and surrounding the CGG repeats, are likewise free of methylation. Although we cannot rule out a low level of methylation at M.K.'s *FMR1* locus, the large majority of his DNA is clearly resistant to McrBC digestion, as was detected with use of probe *pfxa3*. Methylation restricted to the CGG repeat is unlikely to impact transcription of the *FMR1* gene, since the known regulatory elements are located upstream (Hwu et al. 1993; Schwemmle et al. 1997). Methylation of these repeats is, however, relevant to both the proposed relationship between repeat stability and methylation (Wohrle et al. 1996, 1998) and to the proposal that the expanded repeats are to serve as the initial target for de novo methylation (Bestor and Tycko 1996).

Most of the methylated cytosines in human DNA are thought to reside in the 35% of the genome that is composed of transposable elements, such as the *Alu*, *L1,* and retroviral elements (Smit 1996). Although the biological roles of cytosine methylation are subjects of debate, one proposed function is the suppression of the transcription of transposable or parasitic sequence elements (Liu and Schmid 1993; Yoder et al. 1997). Therefore, these elements may act as specific targets for de novo methylation. This model predicts that the fragile-X CGG repeat might become a target for de novo methylation when expanded sufficiently to resemble a parasitic element. It has been speculated that the de novo methylation specificity of the mammalian DNA methyltransferase is dependent on DNA secondary structures (Bestor and Tycko 1996; Bender 1998). Numerous types of secondary structures are observed in disease-causing triplet repeats (Sinden 1999), and, in the case of CGG repeats, three-way hairpin–slippage structures are efficient substrates for de novo methylation in vitro (Chen et al. 1995, 1998). The demarcation that exists in the fragile-X syndrome between unmethylated premutation alleles and methylated full mutations might then represent a threshold at which the CGG repeat reaches a length that allows the formation of secondary structures resembling parasitic sequence elements. Alternatively, methylation of expanded fragile-X repeats may occur randomly or in

association with the switch to very late DNA replication that occurs in the *FMR1* region of cells that carry full mutations (Hansen et al. 1997). A selective disadvantage during early development for the cells that contain an unmethylated expansion (Hansen et al. 1997) could explain the appearance of only methylated full mutations in the cells of most adult patients with fragile-X syndrome.

The above discussion suggests that one explanation for rare hypomethylated full-mutation alleles is that they have escaped the process of de novo methylation. To test this possibility, we have examined methylation levels at repetitive-sequence elements that were likely to have been subject to de novo methylation during M.K.'s early development. Most *Alu* elements inherited from the male parent are initially hypomethylated and achieve the highly methylated state characteristic of adult somatic cells during the wave of de novo methylation that occurs after implantation (Yoder et al. 1997). Conversely, *L1* elements are inherited from the female parent in the unmethylated state and, like *Alu* elements, are subject to de novo methylation during embryogenesis (Yoder et al. 1997). We observed that both a paternally inherited *Alu* element and a maternally inherited *L1* element are methylated in M.K.'s somatic cells. The presence of methylation in these elements indicates that de novo methylation occurred successfully at these sites during M.K.'s early development. On the basis of these findings, we have concluded that if the *FMR1* methylation abnormality in M.K. is due to a defect in *trans,* then *Alu* and *L1* methylation are likely to be mediated by pathways other than that responsible for methylation of the *FMR1* CGG-repeat region. If de novo methylation of different genomic sequences occurs with distinct enzymology, repetitive elements that share characteristics in common with the fragile-X CGG repeat might be more likely to be methylated with the same pathway. To locate such an element, we searched for regions with high CpG density and identified a 1.7-kb *SVA* element, 80 kb downstream of the *FMR1* CGG repeat that contains 86 (9.9%) CpG dinucleotides. Methylation of this element appears to have occurred normally in M.K.'s cells, further indicating that a generalized methylation deficiency is not present at the elements tested.

Another possible explanation for the absence of *FMR1* CpG-island methylation in M.K. is that a failure of maintenance methylation has occurred specifically in the *FMR1* region. To assess this possibility, we examined methylation levels at *Alu* elements flanking the expanded CGG-repeat region. These *Alu* elements were inherited maternally with high levels of methylation that are believed to persist during the early embryonic period characterized by dynamic changes in global methylation patterns. The possibility of perturbed maintenance methylation as an explanation for M.K.'s methylation deficiency is suggested by the *Arabidopsis thaliana* mutant, *ddm1,* that exhibits a reduction in genomic cytosine methylation, which occurs first in repeated sequences (Vongs et al. 1993; Kakutani et al. 1996; Jeddeloh et al. 1998). One model of DDM1 function is that it acts as a part of a nucleosome-remodeling complex that increases accessibility of the DNA to the maintenance methyltransferase (Jeddeloh et al. 1999). This is a particularly attractive model when applied to the fragile-X CGG repeats because it predicts that preferential hypomethylation of sequences that exist in highly condensed chromatin, such as fragile-X full mutations (Luo et al. 1993; Eberhart and Warren 1996; Godde et al. 1996; Wang and Griffith 1996), can result from a mutation in *trans*. However, it seems unlikely that an explanation for M.K.'s unmethylated full mutation will be found at the level of an *FMR1* region-specific abnormality in maintenance methylation, because the two *Alu* elements flanking the CGG repeat, at a distance of ~5 kb, are maintained with a high level of methylation.

Alternatively, M.K.'s full mutation may have escaped the methylation process, not on the basis of a failure of de novo or maintenance methylation, but, rather, as a result of the timing of repeat expansion during early development. The ability to de novo methylate DNA substrates is primarily a characteristic of embryonic cells (Jaenisch 1997). It is possible that, during the embryonic period characterized by cellular de novo methylation competence, M.K. carried an allele that failed to serve as a substrate for methylation due to its size in the upper-premutation range. If this allele expanded after the period of de novo methylation was complete, then it might thereafter remain unmethylated despite attaining the size of a typical full mutation (Wohrle et al. 1998). This possibility is supported by studies that have demonstrated that unmethylated alleles are unstable in cultured fibroblasts (Wohrle et al. 1998; Glaser et al. 1999; Burman et al., in press).

To date, the only inherited defect in genomic methylation identified is the ICF (immunodeficiency, centromeric instability, and facial anomalies) syndrome (MIM 242860). The ICF syndrome is a rare autosomal recessive condition that is characterized by chromosomal abnormalities and reduced methylation in repeated satellite regions and in some *Alu* elements (Miniou et al. 1997*a,* 1997*b*). Cell-fusion experiments have shown that this defect can be at least partially complemented in *trans* (Schuffenhauer et al. 1995). To test directly whether the *FMR1*-specific methylation deficiency in patient M.K. could be complemented in *trans,* we introduced the chromosome bearing this allele into mouse EC cells. These cells have a high capacity to de novo methylate transfected DNA (Turker et al. 1991) and also have the ability

to methylate target sequences introduced by microcell-mediated chromosome transfer (P. A. Yates and M. S. Turner, unpublished data). One target for de novo methylation is the mouse *B1* element (P. A. Yates, R. W. Burman, P. Mummaneni, S. Krussel, and M. S. Tucker, unpublished data), which is homologous to the human *Alu* element (Schmid 1996). We found no evidence of methylation by using either the *Eag*I-restriction assay or the McrBC assay in DNA prepared from microcell hybrids, which had replicated patient M.K.'s X chromosome ~24 times. Several explanations can be invoked to describe this result. One possibility is that expanded CGG repeats do not serve as a methylation signal in the EC cells utilized and/or they have not formed the secondary structure that is required for de novo methylation to occur. Alternatively, M.K.'s chromosome may contain some type of variation in *cis* that renders his repeat unrecognizable as a target or that is otherwise inherently resistant to de novo methylation. However, the presence of a cousin with a methylated expansion of presumably the same allele argues against these latter possibilities, unless a distinct genetic alteration occurred in patient M.K. Finally, the lack of methylation at the transferred allele may simply reflect an inherent difference in the way mouse and human cells maintain/metabolize large CGG repeats. Additional work will be required to sort through these possibilities.

To summarize, we have described a patient with a fully expanded fragile-X mutation lacking *Eag*I-site methylation. Analysis with the McrBC endonuclease demonstrated further that the CGG repeat and ~1 kb of surrounding DNA are also free of methylation. Methylation patterns are formed normally at *Alu* elements within 5 kb of the repeat and at other repetitive-element loci on the X and Y chromosomes. These results suggest that hypomethylation of the expanded *FMR1* CGG-repeat region is not due to global or regional defects in de novo or in maintenance methylation processes. Whether this deficit represents a rare stochastic event or a rare heritable alteration remains to be determined.

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Electronic-Database Information

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

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for fragile-X syndrome [MIM 309550] and ICF syndrome [MIM 242860])

RepeatMasker database, http://ftp.genome.washington.edu/RM/RepeatMasker.html (for repetitive-element loci)

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