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Methylation analysis of CGG sites in the CpG island of the human *FMR1* gene

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ABSTRACT

The fragile-X syndrome of mental retardation is associated with an expansion in the number of CGG repeats present in the *FMR1* gene. The repeat region is within sequences characteristic of a CpG island. Methylation of CpG dinucleotides that are 5′ to the CGG repeat has been shown to occur on the inactive X chromosome of normal females and on the X chromosome of affected fragile-X males, and is correlated with silencing of the *FMR1* gene. The methylation status of CpG sites 3′ to the repeat and within the repeat itself has not previously been reported. We have used two methylation-sensitive restriction enzymes, *Aci*I and *Fnu*4HI, to further characterize the methylation pattern of the *FMR1* CpG island in normal individuals and in those carrying fragile-X mutations. Our results indicate that: (i) CpG dinucleotides on the 3′ side of the CGG repeat are part of the CpG island that is methylated during inactivation of a normal X chromosome in females; (ii) the CGG repeats are also part of the CpG island and are extensively methylated as a result of normal X-chromosome inactivation; (iii) similar to normal males, unaffected fragile-X males with small CGG expansions are unmethylated in the CpG island; for affected males, the patterns of methylation are similar to those of a normal, inactive X chromosome; (iv) in contrast to the partial methylation observed for certain sites in lymphocyte DNA, complete methylation was observed in DNA from cell lines containing either a normal inactive X chromosome or a fragile-X chromosome from an affected male. Our data are consistent with the hypothesis that hypermethylation and silencing of the *FMR1* gene in affected fragile-X individuals result from the normal process of X-chromosome inactivation and the abnormal failure to reverse it prior to female meiosis.

INTRODUCTION

The fragile-X syndrome, a frequent cause of inherited mental retardation, has very unusual patterns of inheritance and expression [4, 40]. These patterns led to the proposal that the syndrome results from abnormal chromosome imprinting; the specific mechanism postulated to lead to imprinting is the failure to completely reactivate an inactive X chromosome that carries the fragile-X mutation [22, 23]. A number of molecular predictions of this 'X-inactivation imprinting' model have been verified: (i) an abnormal pattern of DNA hypermethylation at the fragile-X locus has been observed in affected individuals [2, 7, 17, 27]; (ii) this abnormal pattern of hypermethylation resembles the methylation pattern that is established for the normal allele at the fragile-X locus when an X chromosome is inactivated in females [2, 17, 50]; (iii) there is silencing of gene expression at the fragile-X locus (*FMR1* gene) in most affected males rather than production of an abnormal gene product [31]; (iv) cellular mosaicism for the hypermethylation at the fragile-X site is readily apparent in some affected individuals [27, 31, 38]. Recent data indicating that the chromosomal region containing the *FMR1* gene is abnormally late-replicating in affected fragile-X males [49, 52] are consistent with a central prediction of a related model—that fragile sites represent cis-acting mutations leading to delayed DNA replication [22].

An additional molecular observation that is accommodated but not predicted by the X-inactivation imprinting model is that there are two levels of instability of DNA sequences at the site of the fragile-X mutation [12, 20, 27, 47]. There is a low level of expansion of CGG repeats within the *FMR1* gene that represents, or is a consequence of, the fragile-X mutation; we refer to this as the primary CGG expansion. The primary expansion may lead to abnormal imprinting by blocking reactivation of an inactivated fragile-X chromosome, perhaps as a result of late DNA replication at this site [22]. In addition to this primary CGG expansion, there is a higher level of instability that is manifested as a large increase in the number of CGG repeats in somatic cells of affected individuals as well as in some unaffected females. We refer to this large increase as the secondary expansion. It is not yet clear whether this secondary expansion is transmitted through the germ line or is limited to somatic cells. The relationship between hypermethylation and the secondary expansion in affected individuals also needs to be examined in more detail.

Methylation data for only a few CpG sites in the *FMR1* CpG island have been reported, and these are all 5′ to the CGG repeat; no methylation data are available for sites within the CGG repeat. Hypermethylation patterns observed for affected males and normal females at two sites in the CpG island (*Bss*HII and *Eag*I) are consistent with the X-inactivation imprinting model [23]. The imprinting model has, however, been challenged by Sutherland and coauthors [45], who presented data indicating that a *Sac*II site 5′ to the CGG repeat is highly methylated on the fragile-X chromosome in lymphocytes but not in chorionic villus cells of male fetuses likely to be affected; this *Sac*II site is also inefficiently methylated on the inactive X chromosome in females [51]. In contrast, the nearby *Eag*I site is extensively methylated in chorionic villus and other tissues of such fetuses [6].

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Methylation analysis of more CpG sites in the region may therefore be useful in further molecular tests of the X-inactivation imprinting model.

Extensive characterization of methylation patterns may also be required to understand the relationship between methylation and expression of the disease. The importance of methylation in suppressing expression of the *FMR1* gene is suggested by reports of methylation and transcriptional mosaicism in fragile-X individuals [31]. In one case-report of fragile-X fetal material, cells that lacked methylation at a *Bss*HII site contained transcripts of the mutant *FMR1* gene [44].

We present data in this report on methylation patterns in the CpG island of the *FMR1* gene using the methylation-sensitive restriction endonucleases *Acl*I and *Fnu*4H1. Recognition sites are quite frequent for these enzymes and are present at all CpG's within the CGG repeat. We address four questions with our methylation analysis of the fragile-X locus: (i) Are CG dinucleotides on the 3′ side of the CGG repeat of the *FMR1* gene also part of the CpG island that is methylated during inactivation of the human X chromosome? (ii) Do CG dinucleotides within the CGG repeat become methylated during inactivation of a normal X chromosome? (iii) What is the methylation status of expanded CGG repeats and flanking *Fnu*4HI and *Acl*I sites within the CpG island? (iv) Do tissue-culture cells maintain the methylation patterns at the CpG island that are observed in lymphocytes?

RESULTS AND DISCUSSION

Methylation sensitivity of *Acl*I

We have used two methylation-sensitive restriction endonucleases, *Acl*I and *Fnu*4HI, to characterize the methylation status of the CGG repeat and flanking regions that are within the human *FMR1* gene. *Fnu*4HI, with a recognition sequence of GCNGC, is inhibited by hemi- or symmetrical methylation at either cytosine [14, 19]. We demonstrate here that *Acl*I, an unusual enzyme with a nonpalindromic recognition sequence of CCGC:GCGG, is also methylation-sensitive.

We used target sequences present in the human phosphoglycerate kinase gene (*PGK1*) to examine the methylation sensitivity of *Acl*I. The methylation status of *Acl*I sites in the promoter regions of active and inactive *PGK1* genes has been determined by genomic sequencing [28, 29]. The sites are unmethylated on the active X and methylated on the inactive X. DNA from hybrid cells containing either an active or an inactive human X chromosome was digested with *Acl*I and then subjected to PCR. Two primer sets were used (C2/D2 and G2/J2), each amplifying across 6 *Acl*I sites. Ethidium-stained PCR products were absent from *Acl*I-digested DNA when it was derived from an unmethylated active X chromosome, whereas the appropriate product was present when DNA from a methylated, inactive-X template was digested and amplified (Fig. 1A).

CCGG sites in vertebrate genomes are known to be predominantly methylated because of the general resistance of DNA to *Hpa*II as seen on ethidium bromide-stained agarose gels [41]. *Acl*I, which also has a four base-pair recognition site containing a single CpG (GCGG), also fails to digest human DNA extensively, consistent with methylation at the majority of *Acl*I sites in the genome (Fig. 1B). *Acl*I was shown to be active in these reactions because extensive digestion of CpG-island sequences (*FMR1*, *PGK1* and reiterated sequences containing CGG repeats) was observed after reprobing membranes from these gels

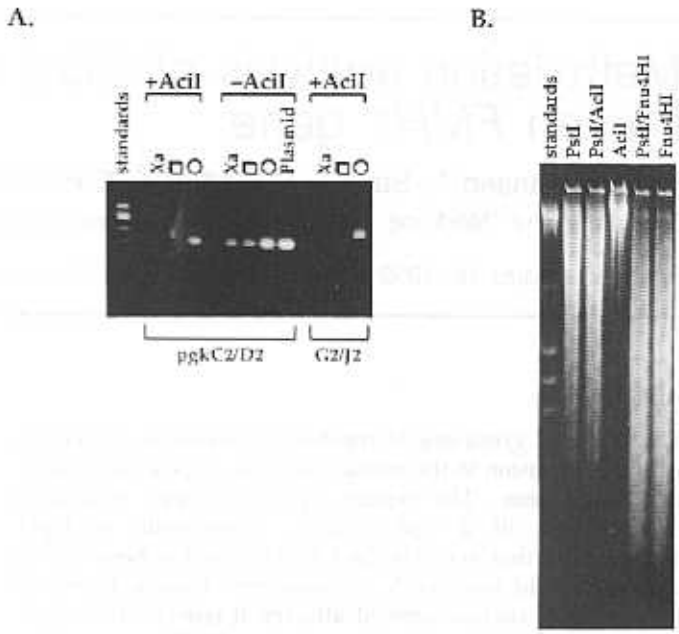

Figure 1. *Acl*I is methylation-sensitive. A. PCR analysis of *Acl*I sites in the CpG island of the *PGK1* gene. DNA was incubated in the presence or absence of *Acl*I and subsequently amplified with oligonucleotides specific for the 5′ CpG island of the *PGK1* gene (C2/D2 or G2/J2). The ethidium-stained PCR products were analyzed by agarose gel electrophoresis. Xa represents a hamster×human cell line (Y162-11C) carrying a normal, active human X chromosome; □ and ○ represent normal male and female blood lymphocytes, respectively. 'Plasmid' refers to RB0.8, which contains genomic sequence from the 5′ promoter of the human *PGK1* gene [16]. The largest of the size standards shown at the left of the gel is the 310 bp fragment of a standard mixture containing ϕX174 *Hae*III and λ *Hind*III fragments. B. Methylation analysis at *Acl*I sites in genomic DNA. Genomic DNA from normal male lymphocytes was digested with *Pst*I, *Acl*I or *Fnu*4HI in various combinations, as described above the gel lanes, and electrophoresed in an agarose gel containing ethidium bromide. The size standards that are most visible include the 23, 9.4, 6.6, 1.35, 1.1 and 0.87 kb fragments of the ϕX174 *Hae*III and λ *Hind*III fragment mixture.

(Fig. 3B, and data not shown). A large fraction of genomic DNA was digested by *Fnu*4HI because many of its recognition sites do not contain CpG's and are therefore unmethylated (Fig.1B; *Fnu*4HI sites lacking CpG are DGCWGCH, where D=A, G or T; W=A or T; H=A, C or T).

Methylation of CpG dinucleotides on the 3′ side of the CGG repeat on the normal X chromosome

Previous reports of methylation in the *FMR1* CpG island focused on a few sites 5′ to the CGG repeat [2, 17, 38, 45, 48]. The 571 bp 3′ to the repeat is also CpG-rich and contains multiple rare-cutter sites, which are often landmarks for CpG islands (Fig. 2). Examination of the published sequence of this region [12], reveals a base composition of 67% G + C, and a CpG dinucleotide frequency of 11%; these values are indicative of a CpG island [3]. Analysis of the region with *Fnu*4HI and *Acl*I, using the fmr9/10 probe, confirms this expectation: the region is generally unmethylated on the active X chromosome of either lymphocytes of males (Fig. 3A, lanes 11 and 12; Fig. 3B, lanes 2 and 4), or of hybrid cells containing an active X chromosome (Fig. 3A, lanes 2 and 3).

In contrast, the female pattern of DNA fragments after digestion with *Pst*I and either *Acl*I or *Fnu*4HI indicates significant but heterogeneous methylation at these sites. For example, the

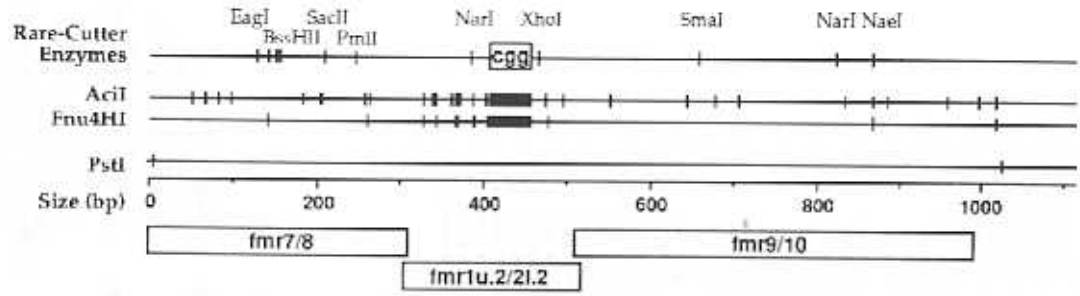

Figure 2. Map of the *FMR1* CpG island. Shown are cleavage sites of the specified restriction enzymes, indicated by vertical lines, the CGG repeat region (cgg) and locations of genomic probes fmr7/8, fmr9/10 and fmru1.2/fmr12.2.

prominent *Fnu*4HI/*Pst*I products at 520 bp and 620 bp indicate that the *Fnu*4HI site at nucleotide 865 appears to be >90% methylated (Fig. 3A, lane 9; Fig. 3B, lane 9). These fragments map to the 3′ region of the *Pst*I fragment containing *Fnu*4HI site 865 because they do not hybridize to the 5′ fmr7/8 probe (data not shown), and because about 60% of their 3′ termini result from *Pst*I cleavages (Fig. 3B, compare lane 10 vs. lane 9; also note the appearance of a 2.4 kb band without *Pst*I). The remaining 40% of 3′ termini are probably at *Fnu*4HI site 1014, which is 11 bp 5′ to the *Pst*I site (Fig. 2). This result indicates the existence of cellular mosaicism at the 1014 site in which 60% of the cells are methylated at this site in one chromosome, presumably the inactive X chromosome. The 390 bp *Fnu*4HI fragment is probably derived from the active X chromosome that is unmethylated at sites 475 and 865, as it is common to both male and female DNA (Fig. 3A, lanes 9 and 12; Fig. 3B, lanes 4, 5, 9 and 10). The relative amounts of the 520 bp and 620 bp fragments indicate a methylation mosaicism in which about 40% of the lymphocytes are methylated at *Fnu*4HI site 475. Similar results were obtained from two other female lymphocyte DNAs. Because these sites appear to be completely unmethylated in DNA from males, we conclude that it is the inactive X chromosome of normal females that contains the hypermethylated sites at the CpG island. This conclusion is supported by analysis of the pattern of methylation on the inactive X chromosome of a hybrid cell line, to be discussed below.

DNA digestion data are also consistent with partial methylation of the *Aci*I sites in this region of normal female X chromosomes when they become inactivated. These sites are not methylated in DNA from males or in DNA from tissue culture cells containing an active human X chromosome. The 520–600 bp bands observed in the *Pst*I/*Aci*I double digest of DNA from three normal females (Fig. 3A, lane 8; data not shown), appear to reflect complete methylation in some cells of all *Aci*I sites in the region 3′ to the CGG repeat. The proportion of DNA in this region of the gel, compared with shorter, female-specific DNA fragments, suggests that about 25% of the inactive X chromosomes in a normal female have complete methylation of at least 7 *Aci*I sites in this region (sites 549 through 884), represented by the 520 bp and 600 bp fragments. This conclusion is supported by the diminished amount of 520–600 bp fragments, and the appearance of large fragments (about 6 kb), when *Aci*I alone is used to digest female DNA (Fig. 3B, lane 8).

Analysis of the region 5′ to the CGG repeat with the fmr7/8 probe yielded similar results (data not shown). *Pst*I/*Fnu*4HI digestion of male DNA resulted in small fragments that were apparently derived from unmethylated *Fnu*4HI sites 139 and 257. Digestion of female DNA gave several additional bands greater than 290 bp in size, including a strong 370 bp band. The 370 bp band was not present after digests with only *Fnu*4HI. Therefore, the inactive-X allele appears to be predominantly methylated at sites 139 and 257, and is probably also methylated at sites 324 and 339. Digestion of normal male DNA indicates that *Aci*I sites in the region are unmethylated (no bands greater than 150 bp detected; the largest fragment expected if DNA in this region were completely unmethylated at *Aci*I sites is 85 bp). Cellular mosaicism for methylation at these sites on the inactive X chromosome of females is indicated by the *Pst*I/*Aci*I fragments ranging in size from 240 bp to 370 bp.

Cellular mosaicism with respect to methylation has also been indicated for another CpG island on the inactive, human X chromosome, that of the *PGK1* gene. Genomic sequencing and methylation-sensitive restriction enzyme analysis of this gene indicated that 25% of CpG sites in the 5′ CpG island are partially methylated in lymphocytes [16, 30]. As previously noted for the *PGK1* gene [30], the sites of partial methylation in the *FMR1* gene contain the trinucleotide sequence GCG. The unmethylated state of the trinucleotide appears to require additional determinants in flanking sequences, such as a very high GC content [30], because the GCG motif also appears in sites that are fully methylated in both genes.

We conclude that many CpG dinucleotides in the 570 bp 3′ to the CGG repeat of the *FMR1* gene show partial to complete methylation resulting from X chromosome inactivation in a female. In addition, we have observed a *Nar*I site 3′ to the CGG repeat (site 821) that is about 50% methylated (data not shown). The extent of methylation at these 3′ sites lies within the range observed for CpG-containing sites 5′ to the CGG repeat. Other sites that have been examined 5′ to the repeat on the inactive X chromosome include *Bss*HII (sites 141, 149 and 151) and *Eag*I (site 125), which are over 90% methylated [2, 38, 48]; the nearby *Sac*II site 204 is only about 20% methylated [45]. Regions both 3′ and 5′ to the CGG repeat are thus part of a CpG island within which partial to complete methylation is correlated with X-chromosome inactivation.

Methylation within the CGG repeat of a normal *FMR1* allele

*Aci*I and *Fnu*4HI recognition sites are present at every monomer of the CGG repeat. To analyze restriction fragments from this region, it was necessary to use probes flanking the CGG repeat because CGG repeats were found to be reiterated in the genome (data not shown). The normal inactive-X alleles in lymphocytes

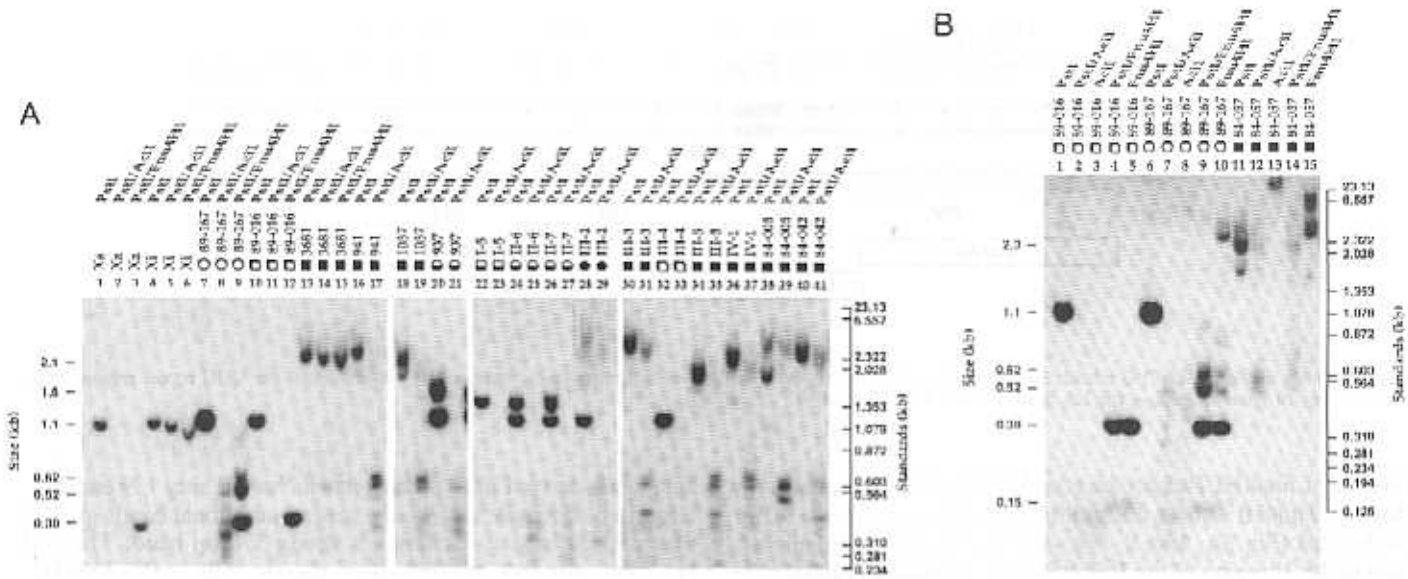

Figure 3. Methylation analysis of CGG sites in normal and mutant *FMR1* alleles by Southern hybridization. **A.** Analysis of methylation at *Acil* and *Fnu*4HI sites within the *Pst*I fragment of the *FMR1* CpG island. *Pst*I digests were compared to *Pst*I/*Acil* or *Pst*I/*Fnu*4III double-digests in adjacent lanes for each individual or cell line, as described at the top of each lane. Numerals above the symbols identify specific individuals or cell lines. Xi represents a hamster×human cell line (X8-6T2) carrying a normal, inactive human X chromosome; Xa represents a hybrid cell line (Y162-11C) carrying a normal, active human X chromosome; □ and ○ represent normal male and female blood lymphocytes, respectively; ◧ and ◐ represent lymphocytes of males and females, respectively, who are unaffected fragile-X carriers; ■ and ● represent lymphocytes of affected fragile-X males and females, respectively except for 3681, which represents a lymphoblastoid cell line derived from the lymphocytes of individual 941. Membranes were probed with radiolabeled fmr9/10. Familial relationships include: carrier female 937 (lane 20) is the mother of affected male 941 (lane 16) and aunt to affected male 1037 (lane 18); male carrier I-5 (lane 22) is the father of carrier females II-6 and II-7 (lanes 24 and 26, respectively); carrier II-6 is the mother of affected female III-2 and affected male III-3 (lanes 28 and 30, respectively); affected female III-2 is the mother of affected male IV-1 (lane 36); carrier II-7 is the mother of affected male III-5 (lane 34) and normal male III-4 (lane 32). Sizes for selected bands, indicated on the left side of the figure, were calculated from standards (φX174 *Hae*III and λ *Hin*dIII fragments) whose migration is indicated on the right side of the figure. The 3′ border of the CGG repeat is 571 base pairs from the 3′ *Pst*I site. The size of the 0.62 kb *Fnu*4HI/*Pst*I fragment observed in normal female DNA was confirmed by comparison with the internal *Xho*I/*Pst*I fragment at 0.56 kb (data not shown). According to sequence data [12], the 0.52 kb *Fnu*4HI/*Pst*I fragment should actually be 0.55 kb in size. Appropriate controls were performed to ensure that there had been complete digestion with *Acil* and *Fnu*4HI (see Methods and Materials section). **B.** *Fnu*4HI analysis of mutant *FMR1* alleles and further analysis of *Acil* and *Fnu*4HI sites. Symbols and other illustrations were as described in A. *Fnu*4HI or *Acil* digestions were performed in the presence or absence of *Pst*I. Faint bands present near the 0.125 kb standard represent *AciI*-*AciI* (lanes 2, 3, 7 and 8) or *Fnu*4III-*Fnu*4III (lanes 4, 5, 9 and 10) cleavage products derived from a normal male and a normal female. These bands are absent in digests of DNA from affected fragile-X males (lanes 11–15).

are sufficiently methylated at the two *Fnu*4HI sites 3′ to the CGG repeat (sites 475 and 865) to determine, using the fmr9/10 probe, whether or not there is methylation of the CGG repeat. After digestion with *Pst*I and *Fnu*4HI, a 390 bp fragment derived from cleavages at unmethylated *Fnu*4HI sites 475 and 865 is observed that is also seen in male DNA and is assumed to be derived from *FMR1* alleles on the active X chromosome (Fig. 3A, lane 9; Fig. 3B, lane 9). A number of fragments greater than 390 bp are also observed that must contain methylated *Fnu*4HI sites and are probably derived from the inactive X chromosome. A 620 bp band is observed in an amount corresponding to about 40% of inactive-X DNA. This fragment contains at least a large portion of the CGG repeat because cleavage at the 3′ border of the repeat would result in a 570 bp *Pst*I/*Fnu*4HI fragment. Therefore, about 50 bp of the CGG repeat must be extensively methylated and contained within the 620 bp fragment. The size of the 620 bp *Fnu*4HI/*Pst*I fragment was confirmed by comparison with an internal *Xho*I/*Pst*I fragment at 560 bp (data not shown).

Additional evidence for extensive methylation of the CGG repeat in normal inactive-X alleles is obtained from observations of minor bands at about 1 kb in *Pst*I/*Fnu*4HI double digests of female lymphocyte DNA (Fig. 3A, lane 9 and Fig. 3B, lane 9). All *Fnu*4HI sites between the *Pst*I sites at nucleotides 6 and 1025, with the possible exception of either site 139 or 865, must be methylated in such fragments. (The possible exclusion from methylation of sites 139 or 865 is indicated by the somewhat faster mobility of the approximately 1 kb band in the *Pst*I/*Fnu*4HI digest compared with that of the *Pst*I fragment.) It thus appears that in a small fraction of normal female lymphocytes, estimated by densitometry to be about 15% for the female whose DNA is represented in Figure 3, methylation of the CGG repeat on the inactive-X chromosome is so extensive that no *Fnu*4HI cleavage occurs within the repeat or flanking regions.

The recognition sequence of *Fnu*4HI spans two potentially methylatable cytosines, (GCNGC, or GCGGC:GCCGC for the repeat); as mentioned previously, both cytosines must be unmethylated for *Fnu*4HI digestion to occur [14, 19]. Therefore, the 620 bp and 1 kb *Fnu*4HI/*Pst*I fragments must be methylated at least at every other CpG within their CGG repeats. Together, the 620 bp and 1 kb bands represent 48, 48 and 46% of the inactive-X *FMR1* fragments in three normal females whose DNA has been analyzed. Extensive methylation of the CGG repeat may also exist on the inactive X chromosome in the remaining cells, but such methylation would have escaped our detection because methylation of the 3′ flanking *Fnu*4HI sites is necessary for our assay to be informative. Extensive methylation of the CGG repeat in a normal inactive-X allele may also be inferred from results with a human×hamster hybrid cell line. In this analysis, complete

methylation of the CGG repeat is indicated by the resistance of the *Pst*I fragment to digestion by *Aci*I, which should independently recognize each CpG of the repeat (Fig. 3A, lane 5).

With an assay based on complete enzymatic digestion, the assessment of methylation within the CGG repeat cannot be done for the active X chromosome because of the lack of methylation in the 5′ and 3′ flanking regions. Partial *Aci*I digests of normal male DNA, however, indicate that the CGG repeat region is predominantly or completely unmethylated on the active, normal X chromosome (data not shown).

We conclude that methylation of CpG dinucleotides in the CGG repeat of normal *FMR1* alleles is similar to that of CpG sites within most of the known CpG islands on the human X chromosome: they are extensively methylated when on inactive X chromosomes in female cells, and poorly methylated when on active X chromosomes.

Methylation of the CGG repeat and flanking *Fnu*4HI and *Aci*I sites in mutant *FMR1* alleles

The pattern of methylation observed for fragile-X males carrying the primary (short) CGG expansion resembles that of normal males: there is no significant methylation at the *Aci*I sites that are 3′ to the CGG repeat (compare lanes 23 with lanes 11 and 33, Fig. 3A). Similarly, *Aci*I/*Pst*I digests from several heterozygous females carrying a fragile-X allele with a primary expansion resemble those of normal females (compare lanes 21, 25, and 27 with lane 8, Fig. 3A, and lane 7, Fig. 3B). This result indicates that the state of the mutation in which there is a small expansion of the CGG repeat is not associated with a major increase in methylation of the *Aci*I sites 3′ to the CGG repeat beyond that which is normally associated with X chromosome inactivation.

*Pst*I digestion of DNA from affected males reveals large fragments that we term secondary expansions of the CGG repeat (Fig. 3). The *Aci*I and *Fnu*4HI digestion patterns indicate extensive methylation in the 3′ region of the CpG island. These patterns resemble those derived from the inactive X chromosome of normal females. More extensive methylation of some *Aci*I and *Fnu*4HI sites, however, is inferred to exist for the affected males compared with the normal inactive-X allele of females. For example, in the *Pst*I/*Aci*I double digests of DNA from affected males, up to six prominent bands between 300 bp and 600 bp are observed. Bands of these sizes are also observed from DNA of normal females, but the distribution of fragments in this size range is shifted to the smaller fragments for normal females (compare lanes 8 with 17 and 19, Fig. 3). Similarly, *Fnu*4HI/*Pst*I double digests result in bands at 520 and 620 bp for DNA from both affected males and normal females. Affected male DNA, however, also exhibits larger fragments in the range of 620 bp to the size of the *Pst*I fragment (Fig. 3b, lane 14). This result indicates that some of the cells from affected males have more extensive methylation at sites 3′ to the CGG repeat than does DNA from the inactive X chromosome of normal females (compare lanes 9 and 14, Fig. 3B).

Analysis of the region 5′ to the CGG repeat with probe fmr7/8 provides conclusions similar to the 3′ analysis. Like normal active-X alleles, the mutant allele with a primary expansion was unmethylated; DNA from affected males was methylated in a pattern similar to that of the inactive-X but with some bands of increased size indicative of more extensive methylation (data not shown). For example, strong 390 bp *Pst*I/*Fnu*4HI bands were observed in affected males, indicating methylation of *Fnu*4HI sites 5′ to the CGG repeat (sites 139, 257, 324, 339, 363, 366). Most affected males also gave prominent *Pst*I/*Aci*I bands at 390 bp, indicating that many of the 19 *Aci*I sites 5′ to the CGG repeat are methylated in a large fraction of lymphocytes.

Complete methylation of the *Aci*I and *Fnu*4HI sites in the CpG island, including those present in virtually every CGG repeat, can also be inferred for DNA from some cells of affected males. *Pst*I fragments from some of these individuals show little or no decrease in size after double-digestion with *Aci*I (compare lanes 38 and 39, Fig. 3A). The *Aci*I-resistant fragments represent up to about 50% of the fragments hybridizing to the fmr9/10 probe (Fig. 3A, lanes 40 and 41 illustrate 52% *Aci*I-resistant fragments; lanes 34 and 35 illustrate less than 10%). Individuals who exhibit the largest *Pst*I fragments, and who thus appear to have the largest secondary expansion of CGG repeats, have the largest proportion of their cells with fully methylated *Aci*I sites within the *Pst*I fragment. This correlation is strengthened by the observation that fully methylated fragments from affected individuals with *Pst*I size-mosaicism appear to be derived from the largest of the multiple *Pst*I fragments (a densitometric comparison of lanes 38 and 39 in Fig. 3A reveals that about 75% of the largest band is resistant to *Aci*I and is therefore extensively methylated). Extensive methylation across the CGG repeat is also observed for DNA from females carrying an allele with a secondary expansion. *Pst*I fragments from such an individual (III-2) were heterogeneous in size when derived from the fragile-X chromosome; only the larger fragments showed extensive methylation throughout the *Pst*I fragment, including the CGG repeat (Fig. 3A, lanes 28 and 29).

We conclude that the state of a fragile-X allele such as that present in affected males is characterized by a hypermethylation pattern resembling that present on normal inactive X chromosome, but is augmented in the frequency of methylation at certain sites.

Stability of methylation patterns in DNA of tissue culture cells

Our limited data for tissue-culture DNA indicate that unmethylated *Fnu*4HI and *Aci*I sites of the 5′ region of the *FMR1* gene from the active X chromosome are maintained in tissue-culture cells (Fig. 3A, lanes 1–3). In contrast, patterns of methylation augmented relative to those observed in female lymphocytes were present in a hybrid cell line containing a normal inactive X chromosome (Fig. 3A, lanes 4–6), and in a lymphoblastoid cell line from an affected male (Fig. 3A, lanes 13–15). In the latter case, we directly compared tissue culture DNA with blood lymphocyte DNA from the same affected male. His lymphocyte DNA (Fig. 3A, lanes 16 and 17) showed markedly less methylation than did DNA from an established line of lymphoblastoid cells (Fig. 3A, lanes 13 and 14); the lymphocyte pattern of methylation resembled that of an inactive X chromosome in normal females, whereas the tissue culture DNA indicated that most cells had virtually complete methylation of *Aci*I and *Fnu*4HI sites in the *Pst*I fragment, including those in the CGG repeat. Essentially complete methylation at *Fnu*4HI sites was also observed in DNA from three other lymphoblastoid cell lines derived from affected fragile-X males (data not shown). *Fnu*4HI/*Pst*I digestion patterns of DNA from two lymphoblastoid cell lines derived from normal females were also examined. The lymphoblastoid fragment patterns were similar to the lymphocyte patterns of normal females that were described earlier except that methylation appeared to be augmented on the inactive X chromosome (data not shown). The majority of methylated

fragments from lymphoblastoid DNA were 620 bp or greater and the completely methylated band corresponded to about 30% of the inactive-X fragments for each of the two cell lines.

These data are consistent with an augmentation of methylation during passage of tissue culture cells at the CpG island of both normal and mutant *FMR1* alleles. The increased methylation could have occurred either by *de novo* methylation or by a loss of cellular mosaicism that occurred as a result of clonal outgrowth of the lymphoblastoid cell line and cloning of the hybrid cell line. Analogous results have been reported for the CpG island of the *PGK1* gene, in which partial methylation of the inactive-X allele was observed in lymphocytes while essentially complete methylation was observed in a hybrid cell line [16, 30]. Other CpG islands are also known to have increased methylation in cells maintained in culture [1, 18]. The augmented patterns of *FMR1* methylation seen for tissue-culture cells and for lymphocytes of affected individuals as compared to patterns for normal female lymphocytes may occur by similar mechanisms, perhaps by a spreading of methylation [46].

Concluding remarks

Our data support the following answers to the questions posed in the introduction.

(i) CpG dinucleotides on the 3′ side of the CGG repeat are part of the CpG island of the *FMR1* gene that is methylated during inactivation of a normal X chromosome in females.

(ii) The CGG repeat, also included within the CpG island, contains CpG dinucleotides that become extensively methylated in a large fraction of cells during inactivation of a normal X chromosome.

(iii) The *FMR1* CpG islands of fragile-X carriers with primary expansions resemble that of a normal, active X chromosome in that the tested *Aci*I and *Fnu*4HI sites were unmethylated. For alleles with secondary expansions, the *Aci*I and *Fnu*4HI digestion patterns have features in common with those observed for a normal inactive-X allele. Some potentially methylated sites are, however, methylated at a higher frequency in DNA from alleles with secondary expansions compared with inactive-X DNA from normal or primary expansion alleles. In particular, the proportion of cells in which all *Aci*I sites are methylated in the *Pst*I fragment, including those in every CGG repeat, is increased in cells that contain the largest number of CGG repeats.

(iv) The unmethylated state of testable *Aci*I and *Fnu*4HI sites in the CpG island of the *FMR1* gene from the normal active-X chromosome was maintained in tissue culture. However, in contrast to the partial methylation observed in lymphocyte DNA, methylation was essentially complete in tissue-culture lines containing either a normal inactive X chromosome or a fragile-X chromosome.

Our results are therefore consistent with the hypothesis that the patterns of *FMR1* methylation observed for alleles from affected individuals and for normal inactive-X alleles have a common origin, as predicted by the X-inactivation imprinting model [23]. The origin of persistent methylation observed in affected individuals is likely to be a consequence of a pattern originating in X-inactivation rather than in the mutation itself. The fragile-X mutation, characterized by the primary CGG expansion, is neither necessary nor sufficient for extensive methylation of the CpG island of the *FMR1* gene. Mutant fragile-X alleles can be transmitted for several generations without expression of the phenotype and without methylation of CpG sites adjacent to the CGG repeat [27, 38]. We demonstrate here that a large fraction of normal lymphocytes contains extensively methylated CGG repeats and flanking *Fnu*4HI sites on the inactive X chromosome; complete methylation of *Aci*I sites was also observed in a normal *FMR1* allele that had been maintained in tissue culture cells on an inactive X chromosome.

It is likely that hypermethylation of the CpG island of the *FMR1* gene is sufficient to lead to transcriptional repression [13, 31, 33, 44]. It is not known, however, whether the methylated state is the primary imprinting signal, or whether it is a consequence of another imprinting mechanism such as a switch from early to late replication [35]. Evidence presented for primary imprinting factors other than methylation include observations of X-chromosome inactivation of the HPRT gene in early mouse embryos prior to methylation [24] and of the absence of methylation in the inactive *PGK1* gene and other X-linked CpG islands in human oogonia [26, 42]. Analysis of *FMR1* methylation in the human germ line will be important for understanding the mechanism by which the imprinted state of the fragile-X mutation is transmitted by females.

More detailed examination of fetal DNA by methods we have described should help to determine the patterns of methylation establishment and their developmental progression in both normal and fragile-X individuals. The evaluation of fragile-X status with respect to methylation should be more conclusive with the assays described here compared with assays relying on only one or two sites [45, 51]. Examination of only a few sites may be misleading because of partial and mosaic patterns of DNA methylation.

Because extensive methylation of the CpG island of the *FMR1* gene can occur on the inactive-X chromosomes for both normal and mutant alleles with primary expansions, it is reasonable to ask whether or not an abnormal persistence of hypermethylation in embryoblasts could lead to the secondary expansion of CGG repeats. Methylation of cytosine is known to increase the strength of GC base pairing [5] and could induce abnormal events that lead to the secondary expansion during replication or repair of the CGG repeat. Direct tests of this possibility will be necessary to distinguish it from an alternative view that the expanded CGG repeats lead directly to enhanced methylation [27, 51]. Methylase activity, for example, is markedly greater toward substrates with unusual DNA structures such as those containing an unpaired or mismatched C in the CpG recognition sequence [43]. Such substrates might arise in the expanded CGG repeat from single-stranded loopouts [37] or from other unusual structures induced by the high GC content of the region. Methylation could also have resulted from an oogonial-specific monitoring system that marks abnormal sequences for inactive chromatin formation [8]. Although we cannot rule out this alternative view, our results indicate that factors other than X-chromosome inactivation are not necessary to explain the patterns of hypermethylation observed for the *FMR1* gene in affected individuals.

MATERIALS AND METHODS

Probes

Plasmid pE5.1, containing the 5′ region of the human *FMR1* gene, was obtained from Dr. David Nelson, Institute for Molecular Genetics, Baylor College of Medicine. Gel-purified 1 kb *Pst*I fragments were used as a template for PCR probe production. The following oligonucleotides were used as primers: FMR7 (5′-CTGCAGAAATGGGCGTTCT-3′), FMR8 (5′-GTGAAACGAAACGGAGCTGAG-3′), FMR9 (5′-CAGGGCTGAAGAGAAGATG-3′), FMR10 (5′-CTCCTCCACAACTACCCAC-3′), FMR1U.2 (5′-TTCACTTCCGGTGGAGGGCCGCCT-3′), and FMR2L.2 (5′-TCAGCCCTGCTAGCGCCGGGAGC-3′). CGG6 is an oligonucleotide used to probe genomic CGG repeats (5′-CGGCGGCGGCGGCGGCGG-3′). PCR was performed in an Ericomp water-

cooled thermocycler. For fmr7/8 and fmr9/10 synthesis, 100 μl reactions contained AmpliTaq reaction buffer (Perkin-Elmer Cetus), 0.2 mM each deoxynucleotide triphosphate, 0.2 μM each primer (FMR7 and FMR8 or FMR9 and FMR10), about 20 pg template DNA and 3 units of Taq enzyme. Initial denaturation was for 5 min at 95°, followed by 27 cycles of 1 min at 95°, 1 min at 55°, 2 min at 72°, and a final extension at 72° for 7 min. The reaction mixture used for fmr1u.2/2l.2 synthesis was 10 mM Tris/HCl, pH 8.9, 40 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate, 0.25 μM each primer (FMR1U.2 and FMR2L.2), 0.01% gelatin, about 20 pg template DNA and 10% DMSO. The reaction mixture was denatured for 7 min at 99° and held at 80° until addition of 3 units of Taq enzyme, after which 36 cycles of 1 min at 95°, 30 sec at 72° and 30 sec at 77° were performed, with a 7 min final extension at 77°. PCR-generated probes were purified by agarose gel electrophoresis. The probes were radiolabeled with ^{32}P-dCTP using the random hexanucleotide priming method [11] or by PCR [39]. CGG6 was end-labeled using ^{32}P-γ-ATP and polynucleotide kinase [25].

Cell culture

Standard growth conditions for cultured cells have been described [15, 36]. X8-6T2 is a 6 thioguanine-resistant human×hamster hybrid cell line that contains a human inactive X chromosome [9, 10, 15]. Y162-11CS3 is a human×hamster hybrid cell line containing an active human X chromosome. The lymphoblastoid cell line 3681 was derived from a fragile-X male (941) and was generously provided by Dr. W. T. Brown, Department of Human Genetics, New York State Institute for Basic Research in Developmental Disabilities.

DNA analysis

DNA from donated blood lymphocytes and cultured cells were isolated as described [16, 32]. DNA samples from individuals 937, 941 and 1037 were provided by Dr. W. T. Brown. DNA concentrations were determined by fluorescent dye binding [21].

Digestion of genomic DNA with *AciI* (New England Biolabs) and/or *PstI* (BRL) was carried out in the same reaction buffer (50 mM Tris/HCl, pH 8, 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mg/ml BSA, 1 mM DTT, 1 mM spermidine, 9 units each enzyme, 3 μg DNA) at 37° overnight. Digestion with *Fnu*4HI (New England Biolabs) or *Fnu*4HI and *PstI* was carried out similarly in 20 mM Tris-acetate, pH 7.9 , 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, 9 units of each enzyme, and 3 μg DNA. Other digestion conditions were as described [16]. Digested samples were concentrated by ethanol precipitation in the presence of 7 μg yeast tRNA and 1.9 M ammonium acetate. Washed pellets were dried and dissolved in 10 μl of electrophoretic sample buffer [16]. Electrophoresis was performed on 2% agarose or 7% acrylamide gels in 89 mM Tris-borate, pH 8.3, 25 mM EDTA.

Agarose gels were transferred to nylon membranes (Bio-Rad Zetaprobe) under alkaline conditions essentially as described [34]. Native DNA fragments were electrophoretically transferred from polyacrylamide gels to nylon membranes, fixed and denatured as described [16, 34]. Hybridizations with PCR-generated probes were carried out at 42° in a hybridization oven (Robbins Scientific) under conditions similar to those previously described [34]. Filters were washed successively for 15 min each in: 2×SSC , 0.1% SDS at 42°C; 0.5×SSC, 0.1% SDS at 65°C; 0.5×SSC, 0.1% SDS at 65°C; and 0.1×SSC, 0.1% SDS at 65°C. For the fmr7/8 probe, the last wash was in 0.2×SSC instead of 0.1×SSC. Hybridization with CGG6 was carried out at 55° in 5×SSC, 20 mM NaH_2PO_4, pH 7, 7% SDS, 10× Denhardt's, 0.1 mg/ml denatured salmon testis DNA. Filters were washed in 3×SSC, 5% SDS at 55° and three times in 1×SSC, 1% SDS at 60° for 15 min each.

Completeness of *AciI* or *Fnu*4HI digestion was monitored by reprobing membranes with RB0.8, a probe for the *PGK1* promoter [16], and/or CGG6 and/or fmr1u.2/fmu2l.2. Densitometry of autoradiograms was performed on a Quick Scan Jr. apparatus (Helena Laboratories). Fragment intensities were compared directly because i) the bands compared are of similar size and should transfer from the gel to the membrane with similar efficiencies and ii) the fragments are complementary to essentially the same regions of the probe and should hybridize with similar efficiencies. These assumptions are supported by the finding that for lymphocyte DNA from three normal females, the ratio of intensity of the 390 bp band, assigned to the active-X allele, to the sum of intensities of larger, methylated bands, assigned to the inactive X chromosome, is close to the value of 50% expected for X-chromosome inactivation. Computer-generated restriction maps were derived from published sequences [12, 20] using SEQ, a sequence analysis program from Intelligenetics. Restriction-enzyme sites are identified according to the nucleotide position 5' to the cleavage site within the *PstI* fragment containing the CGG repeat, here numbered 6 to 1024. The sequence of the *PstI* fragment is included in the data reported by Fu and coworkers [12].

For testing the methylation sensitivity of *AciI* by PCR, DNA was digested as above, the enzyme was heat inactivated at 67° for 15 min, and 0.3 μg DNA was used directly in a PCR reaction. The conditions used were: 10 mM Tris/HCl, pH 8.9, 40 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 1 μM of each primer, 3 units Taq polymerase. The reaction mixture was denatured for 5 min at 95°, after which 25 cycles of 1 min at 95°, 2 min at 66° and 3 min at 76° were performed, followed by a 7 min final extension at 76°. The primer pairs used were C2/D2 and G2/J2. The sequences of these primers and the regions of the *PGK1* gene that they amplify have been published previously [30].

The use of methylation-sensitive restriction endonucleases to determine patterns of cytosine methylation is well-described. Our application of this methodology to the CpG island of the *FMR1* gene introduces no additional variables, except possibly the presence of a very large region of CGG repeats in abnormal alleles. Under normal digestion conditions, the unusual CGG repeat apparently does not to affect endonuclease cleavage in the region other than through CpG methylation because: i) unmethylated alleles with secondary expansions have been reported to be cleaved by methylation-sensitive enzymes [45], ii) methylation-insensitive endonucleases are not inhibited by the secondary CGG expansion (*PstI*, *PvuII*, *BamHI*, and *AvaII* for example [20]), iii) several *AciI* and *Fnu*4HI sites are cleaved in DNA from affected male lymphocytes but not in derived lymphoblastoid cell lines (Fig. 3A: compare 941 with 3686) and iv) that methylation is responsible for inhibition of *AciI* and *Fnu*4HI digestion is evident when comparing the digestion patterns of a normal active-X allele present in a hybrid cell line (Y162-11C: extensive digestion) with those of a normal inactive-X allele also present in a hybrid cell (X8-6T2, completely undigested).

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ABBREVIATIONS

FMR1, gene associated with Fragile-X Syndrome; *PGK1*, X-linked phosphoglycerate kinase gene; kb, kilobase pair(s); bp, base pair(s).

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