Am. J. Hum. Genet. 71:923–932, 2002

Histone Modifications Depict an Aberrantly Heterochromatinized *FMR1* Gene in Fragile X Syndrome

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Fragile X syndrome is caused by an expansion of a polymorphic CGG triplet repeat that results in silencing of *FMR1* expression. This expansion triggers methylation of *FMR1*'s CpG island, hypoacetylation of associated histones, and chromatin condensation, all characteristics of a transcriptionally inactive gene. Here, we show that there is a graded spectrum of histone H4 acetylation that is proportional to CGG repeat length and that correlates with responsiveness of the gene to DNA demethylation but not with chromatin condensation. We also identify alterations in patient cells of two recently identified histone H3 modifications: methylation of histone H3 at lysine 4 and methylation of histone H3 at lysine 9, which are marks for euchromatin and heterochromatin, respectively. In fragile X cells, there is a decrease in methylation of histone H3 at lysine 4 with a large increase in methylation at lysine 9, a change that is consistent with the model of *FMR1*'s switch from euchromatin to heterochromatin in the disease state. The high level of histone H3 methylation at lysine 9 may account for the failure of H3 to be acetylated after treatment of fragile X cells with inhibitors of histone deacetylases, a treatment that fully restores acetylation to histone H4. Using 5-aza-2′-deoxycytidine, we show that DNA methylation is tightly coupled to the histone modifications associated with euchromatin but not to the heterochromatic mark of methylation of histone H3 at lysine 9, consistent with recent findings that this histone modification may direct DNA methylation. Despite the drug-induced accumulation of mRNA in patient cells to 35% of the wild-type level, FMR1 protein remained undetectable. The identification of intermediates in the heterochromatinization of *FMR1* has enabled us to begin to dissect the epigenetics of silencing of a disease-related gene in its natural chromosomal context.

Introduction

Fragile X mental retardation is caused by mutation of the *FMR1* gene (MIM 309550) located at Xq27.3. Of the cases of fragile X, >95% are due to the expansion of a CGG triplet repeat tract located at the 5′ end of *FMR1* (Warren and Sherman 2001). In the general population, there is a range of 6–54 repeats in this tract, with a mode of 30 (Fu et al. 1991; Snow et al. 1993). In patients with fragile X, the CGG repeat tract expands to >200 CGG repeats and can be as large as 1,000 repeats (Kremer et al. 1991; Verkerk et al. 1991). This expansion triggers methylation of cytosines in the CpGs in the repeat tract and in the flanking sequence, including the *FMR1* gene promoter, resulting in the silencing of *FMR1* transcription (Pieretti et al. 1991; Sutcliffe et al. 1992; Hornstra et al. 1993). Treatment of fragile X cells with the DNA methylation inhibitor 5-aza-2′-deoxycytidine (azadC) results in the loss of methylation at expanded *FMR1* DNA and reactivates transcription of the gene (Chiurazzi et al. 1998, 1999; Coffee et al. 1999). This result suggests that DNA methylation, not the expansion itself, is the major factor in the loss of transcriptional activity of *FMR1* in patients with fragile X.

DNA methylation has long been associated with the transcriptional repression of many genes (Eden and Cedar 1994; Li 1999). The effect of DNA methylation on gene silencing can be direct, by inhibition of transcription factor binding, or indirect, through the induction of changes in local chromatin structure at the site of methylation. One model suggests that DNA methylation induces changes in chromatin architecture through the recruitment of histone deacetylases (HDACs) by a methyl CpG binding (MBD) protein such as MeCP2 (Jones et al. 1998; Nan et al. 1998). Histone deacetylation is thought to result in a tighter association between the histone amino termini and DNA, resulting in a condensed chromatin structure that excludes critical transcription factors. In addition, it has been suggested that removal of the acetyl groups from the histones facilitates

Received April 12, 2002; accepted for publication July 15, 2002; electronically published September 13, 2002.

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* All editorial responsibility for this paper was handled by an associate editor of the *Journal*.

0002-9297/2002/7104-0019$15.00

nucleosome-nucleosome contacts, thus allowing stacking of the nucleosomes and formation of higher-order structures (Bestor 1998).

In addition to lysine acetylation, other modifications of histones, such as methylation of lysine and arginine, ubiquitination, and phosphorylation, have been identified (Strahl and Allis 2000; Jenuwin and Allis 2001). In addition to a direct influence on the structure of chromatin, these modifications may also serve as "marks" for interactions with other chromatin proteins (Strahl and Allis 2000; Jenuwin and Allis 2001). For example, the transcription factors PCAF and $TAF_{II}250$ selectively interact with acetylated histone tails through their bromodomains (Dhalliun et al. 1999; Jacobson et al. 2000). Likewise, proteins containing chromodomains, such as HP1, interact with histone H3 tails methylated at lysine 9, a mark associated with heterochromatin (Bannister et al. 2001; Lachner et al. 2001). HP1 has been shown to dimerize in solution, leaving the chromodomains at the ends of flexible linkers, free for association with histone H3 methylated at lysine 9, providing an attractive model for how higher-order chromatin structure can be organized in regions marked by histone H3 methylation at lysine 9 (Brasher et al. 2000; Jenuwin and Allis 2001). Interestingly, a recent report shows that disruption of methylation of histone H3 at lysine 9 perturbs DNA methylation in *Neurospora crassa,* indicating that histone methylation can direct DNA methylation (Tamaru and Selker 2001). Methylation of histone H3 at lysine 4, on the other hand, is a conserved euchromatic mark that is associated with transcriptionally active genes but not with inactive facultative heterochromatin (Strahl et al. 1999; Litt et al. 2001; Boggs et al. 2002; Peters et al. 2002). Observations such as these have led to the "histone code" hypothesis, according to which a specific constellation of modified histone residues are thought to regulate unique biological outcomes through specific interactions with other components of chromatin (Strahl and Allis 2000; Jenuwin and Allis 2001).

Elsewhere, we demonstrated that there is a loss of acetylation of histone H3 and H4 at *FMR1* in fragile X cells compared with normal cells (Coffee et al. 1999). Here, we demonstrate that naturally occurring fragile X alleles, which carry various numbers of CGG repeats, possess a level of H4 acetylation that is inversely proportional to their CGG repeat tract length and to the cells' delayed responsiveness to azadC treatment. However, increased acetylation of histone H4 associated with these *FMR1* alleles, either naturally occurring or induced by the HDAC inhibitor trichostatin A (TSA), was not sufficient to relax the condensed chromatin structure at the locus. We also identify fragile X–specific alterations in two additional histone modifications at *FMR1:* methylation of histone H3 at lysine 4 (a mark for euchromatin) and methylation of lysine 9 (a mark for heterochromatin). Histone H3 at *FMR1* in fragile X cells is methylated at lysine 9 and undermethylated at lysine 4. The modification is similar to the code that is found in heterochromatic regions of the genomes of model organisms and to the code that is imprinted both on human genes and on the inactive X chromosome in all mammals (Strahl et al. 1999; Litt et al. 2001; Xin et al. 2001; Boggs et al. 2002; Peters et al. 2002). Treatment of fragile X cells with HDAC inhibitors has little, if any, effect on these modifications, suggesting that histone H3 methylation at lysine 9 blocks the acetylation of this residue. Finally, we show that *FMR1* DNA methylation, altered by treatment with azadC, is tightly coupled to changes in histone H3 and H4 acetylation, and to methylation of histone H3 at lysine 4, all marks for euchromatin. However, methylation of histone H3 at lysine 9, a mark for heterochromatin, did not follow drug-induced changes in DNA methylation, indicating that it may operate independently of DNA methylation.

Material and Methods

Cell Lines, Cell Culture, and Drug Treatment

Epstein-Barr virus (EBV)–transformed lymphoblastoid cell lines E3 and E4 derived from male patients with fragile X and carrying *FMR1* alleles of 230 CGG repeats or 410 CGG repeats (data not shown), respectively, were a generous gift from G. Neri (Universita Cattolica, Rome) (Chiurazzi et al. 1999). The EBV-transformed lymphoblastoid cell line J-1, carrying an unmethylated 30–CGG repeat allele, was derived from an unaffected male and GM3200A (available from Coriell Cell Repositories), carrying a methylated 530–CGG repeat allele, was derived from a male patient with fragile X. The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100μg/ml streptomycin.

TSA (330 nM; Sigma) or sodium butyrate (10 mM; Sigma) was added to the media for 24 h; azadC (1 μM; Sigma) was added to the media and was replenished every 48 h by replacement of half the conditioned media with fresh media and drug. In the azadC withdrawal experiment, cells were synchronized with two 8-h blocks of thymidine prior to addition of azadC (Chiurazzi et al. 1998).

Chromatin Immunoprecipitation

We performed chromatin immunoprecipitation as described elsewhere (Coffee et al. 1999). The chromatin solution was used for immunoprecipitation with antibodies directed against histone H4 acetylated at lysines 5, 8, 12, and 16 (Upstate Biotech), histone H3 acetylated

at lysines 9 and 14 (Upstate Biotech), histone H3 dimethylated at lysine 9 (Upstate Biotech), and histone H3 dimethylated at lysine 4 (Upstate Biotech). Prior to PCR analysis, the IP-DNAs were digested with *Xho*I to separate the CGG repeat tract, which interferes with amplification. The PCR conditions and primers used were as described elsewhere (Coffee et al. 1999). The PCR products were electrophoresed, and the image was captured using the Eagle Eye imaging system and was analyzed with Molecular Dynamics ImageQuant software.

RT-PCR and LightCycler RT-PCR

Total RNA was isolated using Trizol (Gibco BRL) or RNeasy (Qiagen), according to the manufacturers' instructions. Conventional RT-PCR was performed using the GeneAmp RNA PCR kit (Perkin Elmer), as described elsewhere (Coffee et al. 1999). LightCycler RT-PCR was performed using the LightCycler RNA amplification kit (Roche), according to the manufacturers' suggested conditions for one-step RT-PCR. Total RNA (100 ng) was used for each sample. The PCR conditions were as follows: RT at 55°C for 10 min, followed by an initial denaturation at 95°C for 30 s, then 45 cycles of 95°C for 1 s, 60°C for 10 s, and 72°C for 30 s. SYBR Green I (Roche) was used as the fluorescent marker to monitor DNA accumulation. The primers used were 5′-GATGAAGATACCTGCACATTC-3′ and 5′-TAGCTCCAATCTGTCGCAACTGC-3′, yielding a 593-bp product. Melting-curve analysis of the reactions was done using the LightCycler software (Roche).

Western Blot Analysis

Protein was isolated and quantified using the Bradford reagent (BioRad) and amounts specified in figure 1 were loaded onto a 7.5% SDS polyacrylamide gel. The resolved protein was transferred to Hybond-P membrane (Amersham), was stained with PonceauS (Sigma) to confirm transfer, and was then blocked in 10% milk (Carnation) in PBS/0.2% Tween-20 for 1 h. The membrane was probed with the anti-FMR1 protein (FMRP) monoclonal antibody 1FM 1AC hybridoma supernatant at 1:10 (Devys et al. 1993) for 3 h, washed, and then probed with an anti-mouse Ig, Fc-specific-antibody coupled to HRP (Sigma) for 1 h. After four 15-min washes, the blot was developed using a chemiluminescent substrate (Amersham) and was then exposed to film.

Nuclease Accessibility

Approximately 10^7 EBV-transformed lymphoblastoid cells were washed with PBS and permeabilized with L-α-lysophosphatidylcholine, as described elsewhere (Eberhart et at. 1996). The cells were collected and suspended in NEB2 restriction enzyme buffer (New England Biolabs), and the indicated amounts of *Msp*I were

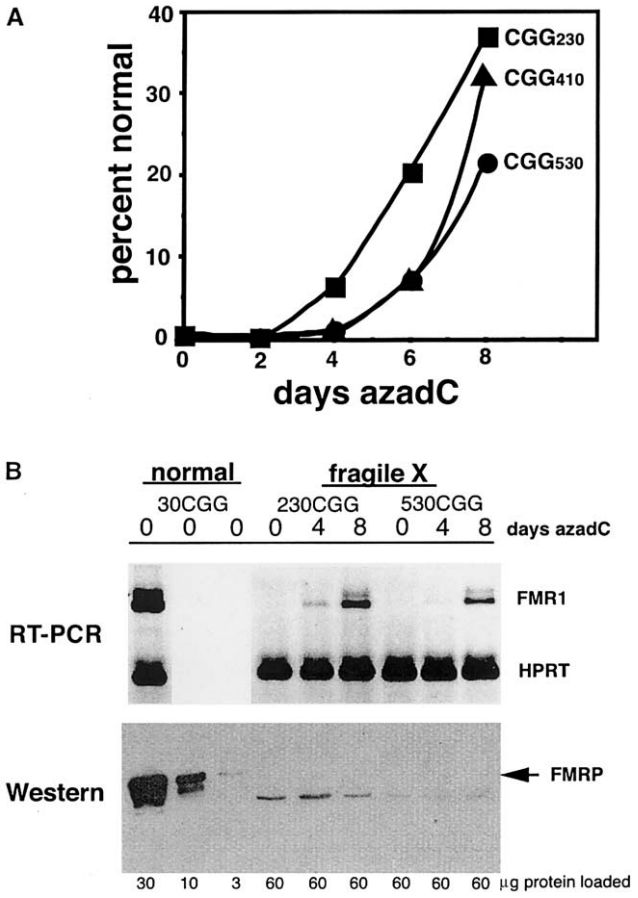

Figure 1 Fragile X cell lines treated with azadC. *A*, Light cycler RT-PCR quantitation of *FMR1* transcript following azadC treatment of fragile X cell lines harboring 230 CGGs, 410 CGGs, or 530 CGGs. The amount of *FMR1* transcript in 100 ng of total RNA (expressed as percent of transcript from a normal cell line) is plotted against the time of azadC treatment. *B*, RT-PCR and western blot analysis of azadC-treated normal control cells and fragile X cells with either 230 CGGs or 530 CGGs. Cells were treated with azadC for the indicated times, and samples were collected for conventional RT-PCR and western blot analysis. In the western analysis, the indicated amounts of total cell lysate were loaded on the gel. A cross-reacting band of unknown identity is observed when large amounts of protein (60 μg) from all cell lines, regardless of treatment, are loaded in a lane.

added. The samples were incubated for 60 min at 37°C and treated with proteinase K, and the DNA was isolated, digested with *Hin*dIII, and analyzed by Southern blot hybridization as described elsewhere (Pieretti et al. 1991).

Southern Blot Analysis

DNA for Southern blot analysis was isolated from the cells by use of a salting-out procedure (Miller et al. 1988). Genomic DNA (10 μg) was digested with the restriction enzyme pair of *Hin*dIII and *Bss*HII. The products of the digestion were separated by electrophoresis

on an 0.8% agarose gel and transferred to Hybond N^+ membrane (Amersham). The membrane was hybridized with a ^{32}P-labeled 5.2-kb fragment of *FMR1* DNA isolated from plasmid pE5.1 (Pieretti et al. 1991).

Results

Histone H4 Acetylation at FMR1 Is Proportional to CGG Repeat Tract Length and to Responsiveness to azadC Treatment

Patients with fragile X harbor CGG repeat expansions that vary from ~200 repeats to >1,000 repeats. Previous work suggested that fragile X cell lines carrying shorter CGG repeat tracts were more responsive to azadC (Chiurazzi et al. 1999). Using light-cycler RT-PCR, we carefully measured the amount of *FMR1* transcript induced by azadC over time in three different male fragile X cell lines harboring alleles with ~230, ~410, or ~530 CGG repeats. These alleles are fully methylated at the diagnostic *Eag*I and *Bss*HII restriction sites located in the *FMR1* promoter (data not shown). *FMR1* transcripts became readily detectable in the 230-CGG repeat cell line at day 4, with the longer-repeat cell lines taking until day 6 to produce an equivalent amount of *FMR1* transcript (fig. 1). *FMR1* mRNA continued to accumulate in all the cell lines throughout this course of treatment, with the amount of *FMR1* transcript present at day 8 being inversely proportional to CGG repeat tract length (fig. 1).

It has been reported that CGG repeat expansions, of the size usually found in patients, in the *FMR1* transcript can impair translation by impeding the migration of the 40S subunit along mRNA (Feng et al. 1995). However, FMRP has been detected after azadC treatment of fragile X syndrome cells (Chiurazzi et al. 1998). Since we achieved such high levels of *FMR1* transcript during reactivation of the short allele–containing cells with azadC, we used western blot analysis to determine whether FMRP also accumulated. In normal cells, FMRP could be detected with as little as 3 μg of total cell protein lysate loaded on the gel (fig. 1*B*). However, in fragile X cells, FMRP could not be detected, even when 20-fold more protein was analyzed. Therefore, in the 230-CGG repeat cell line in which transcription was reactivated to a level >35% of normal after 8 d of azadC treatment, FMRP remained undetectable (<5% of normal), indicating that FMRP translation is inhibited by CGG expansion.

The finding of differential reactivation led us to test the idea that histone acetylation at *FMR1* differed as a function of repeat size. Using chromatin immunoprecipitation (ChIP), we found that histone H3 was significantly hypoacetylated and was barely detectable in the three fragile X cell lines (fig. 2). The average ratios of histone H3 acetylation, relative to the constitutively expressed, X-linked *G6PD* gene, for the fragile X cell lines carrying repeats of 230 CGGs, 410 CGGs, or 530 CGGs were 0.07, 0.03, and <0.01, respectively, versus 1.14 for normal cells (fig. 2). The average ratios of histone H4 acetylation at *FMR1* were 0.63, 0.23, and 0.13, respectively, versus 1.11 for normal cells. Therefore, there was a graded loss of histone acetylation, especially histone H4, that was proportional to CGG repeat length and to the cell line's responsiveness to azadC, suggesting that alleles with longer CGG repeat tracts are further down a pathway of transcriptional repression.

Acetylation of Histone H4 Alone Does Not Relax the Condensed Chromatin Structure at Fragile X FMR1 Alleles

Relative to normal cells, chromatin at *FMR1* in permeabilized fragile X cells is resistant to *Msp*I digestion, indicating that it is organized into a condensed state (Eberhart et al. 1996). The availability of cell lines harboring *FMR1* alleles with a spectrum of histone H4 acetylation led us to test whether the higher levels of histone H4 acetylation are a reflection of a more open chromatin structure. Using the *Msp*I nuclease accessibility assay (Eberhart et al. 1996), we gauged the compaction of chromatin at *FMR1* associated with various levels of histone H4 acetylation. In permeabilized normal cells, cleavage at the 16 *Msp*I sites at the *FMR1* promoter (fig. 3*A*) was readily observed at all *Msp*I concentrations (fig. 3*B, lanes 1–4*). The same sites were much less accessible to *Msp*I in permeabilized fragile X cells with 530 CGG repeats, where histone H4 was almost completely deacetylated (fig. 3*B, lanes 9–12*). *FMR1* DNA associated with an increased level of histone H4 acetylation at the naturally occurring 230-CGG repeat allele, where H4 acetylation was ~50% of normal, was also highly resistant to *Msp*I digestion (fig. 3*B, lanes 5–8*). Finally, TSA treatment of the 530-CGG repeat cell line, which restores H4 acetylation to normal levels but has little or no effect on H3 acetylation (Coffee et al. 1999; also see fig. 4), did not observably increase accessibility of *FMR1* DNA to *Msp*I (fig. 3*B, lanes 13–16*). This indicates that even a wild-type level of histone H4 acetylation is not sufficient to open the condensed heterochromatin-like structure at *FMR1* in patients with fragile X, which may explain, in part, why TSA does not efficiently reactivate transcription of *FMR1* alleles in fragile X cells (Chiurazzi et al. 1999; Coffee et al. 1999).

Histone H3 Methylation Is Increased at Lysine 9 and Is Decreased at Lysine 4 at FMR1 in Fragile X Cells

Recently, it has been shown that methylation of histone H3 at lysine 4 or lysine 9 is selectively enriched in euchromatic and heterochromatic regions of chromosomes, respectively (Strahl et al. 1999; Rea et al. 2000; Boggs et al. 2002; Peters et al. 2002). We used ChIP

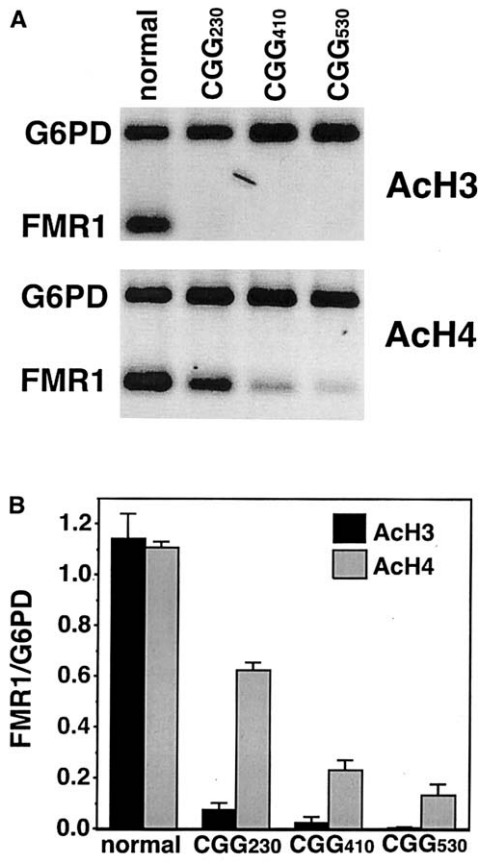

Figure 2 Histone H3 and histone H4 acetylation at *FMR1* in fragile X cell lines harboring 230 CGGs, 410 CGGs, or 530 CGGs. *A,* A representative multiplex PCR analysis of DNAs immunoprecipitated with either anti-acetyl histone H3 or anti-acetyl histone H4 antibodies from a normal cell line or three fragile X cell lines. The top band is specific for the constitutively active, X-linked glucose 6-phosphate dehydrogenase (G6PD) gene and the bottom band specific for *FMR1*. *B,* Quantitation of the level of histone H3 or H4 acetylation associated with *FMR1* in the normal and the three fragile X cell lines. Amount of *FMR1* DNA immunoprecipitated, relative to the internal control G6PD DNA, is averaged from three independent experiments. Error bars = 1 SD.

analysis to determine whether these chromatin marks were altered at *FMR1* in fragile X cells. Histone H3 methylation at lysine 4 was significantly reduced at *FMR1* in fragile X cells (fig. 4, "MeH3-K4," *lanes 1* and *2*). Conversely, the amount of methylation of histone H3 at lysine 9 at *FMR1* in fragile X cells was significantly greater than the amount observed at *FMR1* in normal cells (fig. 4, "MeH3-K9," *lanes 1* and *2*). As expected, methylation of histone H3 at lysine 9 was not observed at the constitutively active G6PD gene. These results demonstrate a complementary pattern of histone H3 methylation at *FMR1* in normal and fragile X cells, in which the euchromatic marks in normal cells are replaced with heterochromatic marks in fragile X cells.

An individual lysine 9 of histone H3 can be acetylated or methylated but cannot possess both groups simultaneously. To determine whether methylation of histone H3 at *FMR1* changed in a coordinated fashion with acetylation, we used ChIP to assess alterations in these modifications at *FMR1* in fragile X cell lines after treatment with the HDAC inhibitors sodium butyrate or TSA. After drug treatment, histone H4 acetylation at *FMR1* increased to wild-type levels, as shown previously (fig. 4, "AcH4") (Coffee et al. 1999). There was a small increase in methylation of histone H3 at lysine 4 with sodium butyrate (fig. 4, "MeH3-K4"). Methylation of H3 lysine 9 was not reduced, and may slightly increase after the drug treatment (fig. 4, "AcH3" and "MeH3-K9"). These data suggest that methylation of histone H3 at lysine 9 blocks its acetylation, preventing the transition of *FMR1* from a heterochromatic-like state to a euchromatic-like state.

Acetylation of Histone H3 and H4 Is Tightly Coupled to DNA Methylation

Elsewhere, we and others have demonstrated that treatment of fragile X cells with azadC resulted in transcriptional reactivation of *FMR1* (Chiurazzi et al. 1998, 1999; Coffee et al. 1999). This reactivation is accom-

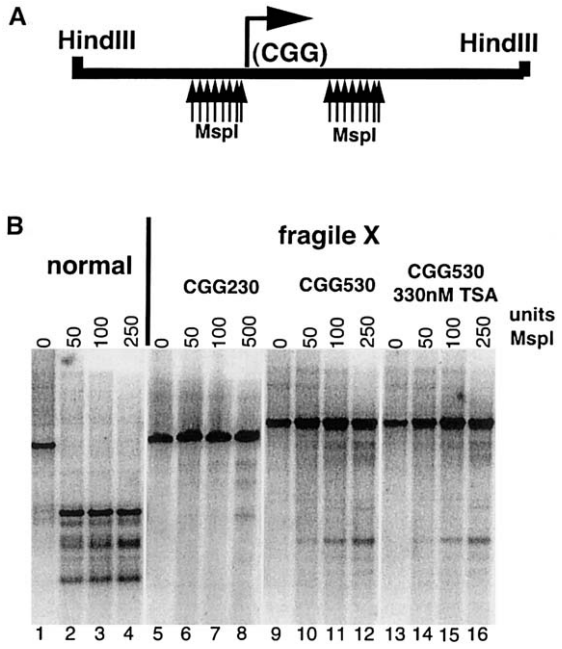

Figure 3 Nuclease accessibility analysis of *FMR1* DNA associated with various levels of acetylated histone H4. *A,* Map of the 5′ end of *FMR1,* showing the location of the relevant restriction sites, the transcriptional start site, and the CGG repeat tract. *B,* Southern analysis of *FMR1* DNA recovered after nuclease accessibility analysis from a normal cell line (*lanes 1–4*), the 230-CGG repeat fragile X cell line (*lanes 5–8*), the 530-CGG repeat fragile X cell line (*lanes 9–12*), and the 530-CGG repeat cell line treated with 330 nM TSA for 24 h (*lanes 13–16*).

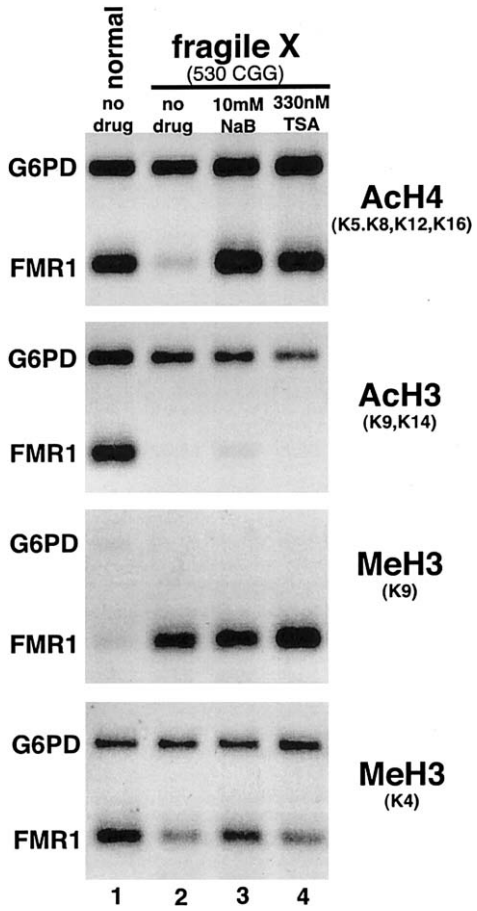

Figure 4 ChIP analysis of histone H4 and H3 acetylation, H3 methylation at lysine 9 and histone H3 methylation at lysine 4 in a normal and a fragile X cell line carrying a 530-CGG repeat allele without HDAC inhibitor treatment (*lanes 1* and *2*) and the fragile X cell line treated with 330 nM TSA or 10 mM sodium butyrate for 24 h (*lanes 3* and *4*). The antibodies used and the lysine modifications they are directed against are indicated for each panel.

panied by an increase of both histone H3 and H4 acetylation at the 5′ end of the gene (Coffee et al. 1999; and shaded portion of fig. 5). To determine whether the changes in DNA methylation and the concomitant changes in histone acetylation and transcription are stable without the continual presence of azadC, the drug was removed from the media, and the cells were passaged for an additional 30 d (unshaded portion of fig. 5). Samples were collected at various intervals and were assayed for DNA methylation, by digestion with methylation-sensitive restriction enzymes, histone acetylation, and *FMR1* mRNA levels (fig. 5). Immediately following withdrawal of azadC, there was an increase in DNA methylation with a parallel decrease in histone acetylation. Steady-state *FMR1* mRNA levels began to decrease several days later. Twenty days after the removal of azadC, *FMR1* DNA became completely remethylated at the diagnostic *Bss*HII site in the *FMR1* promoter, histone acetylation returned to pretreatment levels, and *FMR1* transcript became undetectable (fig. 5). Thus, the changes in DNA methylation and histone acetylation induced by azadC at *FMR1* are not maintained without the continuous presence of the drug, and the heterochromatic nature of *FMR1* in fragile X cells is tightly coupled to DNA methylation.

Methylation of Histone H3 at Lysine 9 Does Not Follow Drug-Induced Loss of DNA Methylation and Transcriptional Reactivation of FMR1

Methylation of histone H3 at lysine 9 and methylation of cytosines in CpG dinucleotides are features of heterochromatin (Jenuwin and Allis 2001). The molecular link between these two epigenetic changes, however, remains to be defined. To determine whether histone H3 methylation is coupled to DNA methylation at *FMR1*, we used the azadC-mediated demethylation of *FMR1* DNA as a model to assess the changes in histone H3 methylation that occurs during the switch from a transcriptionally inactive heterochromatin-like state to a transcriptionally active euchromatin-like state. DNA demethylation became detectable (as assessed by cleavage at the methyl-sensitive *Bss*HII restriction sites located in the *FMR1* promoter) as early as 2 d after addition of

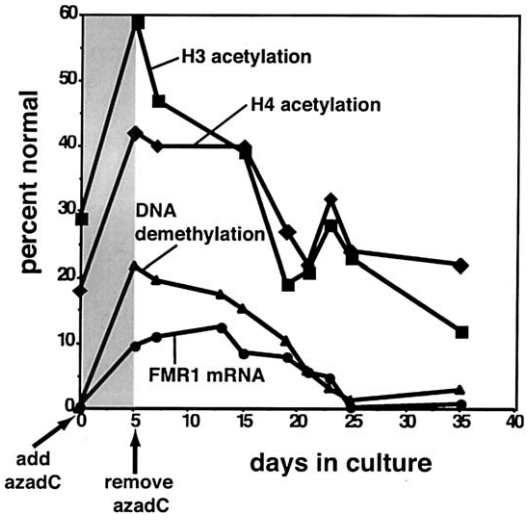

Figure 5 DNA methylation, ChIP, and RT-PCR analysis of *FMR1* after addition and withdrawal of azadC. The fragile X cell line harboring 530 CGG repeats was treated with 1 μM azadC for 5 d (shaded area), followed by removal of the drug from the media. The treated cells continued to be passaged for an additional 30 d. Histone H3 and H4 acetylation is plotted as a percentage of untreated normal, the amount of demethylation is plotted as percent of *FMR1* DNAs cleaved with the methyl-sensitive restriction enzyme *Bss*HII, and the amount of *FMR1* transcript is plotted as the percent of *FMR1* transcript found in untreated normal cells.

azadC, and it continued to increase until approximately day 8, when it plateaued at ~13%–14% (fig. 6). *FMR1* transcript became detectable by RT-PCR at approximately day 6 and increased steadily throughout the remainder of the treatment (fig. 6). Like the euchromatic marks of histone H3 and H4 acetylation, histone H3 methylation at lysine 4 increases at *FMR1* steadily throughout the treatment, doubling from 45% of the normal level in the untreated fragile X cell to 90% of normal after 16 d of azadC treatment (data not shown). Methylation of histone H3 at lysine 9 decreased initially at *FMR1,* dropping to about half the amount seen in untreated cells after 2 d of treatment, but it reproducibly recovered and by day 16 was at or above the level seen in untreated cells (fig. 6). These results demonstrate that the heterochromatic mark of methylation of histone H3 at lysine 9 at *FMR1* did not track with DNA demethylation, as did the euchromatic marks of H3 methylation at lysine 4 and histone acetylation, suggesting that this histone modification is related in a more complex way to DNA methylation.

Discussion

Elsewhere, we showed that treatment of fragile X cells with TSA resulted in acetylation of histone H4 at *FMR1* but had little, if any, effect on histone H3 acetylation and failed to reactivate transcription (Coffee et al. 1999). Loss of DNA methylation after azadC treatment resulted in an increase in both histone H3 and H4 acetylation and transcriptional reactivation. This suggested that histone H3 deacetylation, but not histone H4 deacetylation, is linked to DNA methylation and transcriptional silencing (Coffee et al. 1999). Here, we extend this initial observation by showing that acetylation of histone H4 at naturally occurring fragile X *FMR1* alleles can vary between 10% and 50% of normal levels. The extent of histone H4 deacetylation correlated with increasing CGG repeat length. One possible basis for this observation could be an increasing abundance of HDACs at the locus, resulting from a larger number of binding sites for methylcytosine-binding proteins in the longer CGG repeat alleles. Histone H3 was more profoundly and uniformly deacetylated across the spectrum of CGG repeat sizes, suggesting that loss of this modification is more important in transcriptional silencing. A similar linkage of DNA methylation to histone H3 deacetylation, but not histone H4 deacetylation, was found at the imprinted *Snrpn* and *U2af1-rs1* genes in mice (Gregory et al. 2001).

The spectrum of histone H4 acetylation found at *FMR1* in fragile X cells suggested that there is a gradation in the possible heterochromatin-like states that *FMR1* can populate. Supporting this notion is the observation shown here, and also by others (Chiurazzi et al. 1999), that *FMR1* transcription is reactivated earlier by azadC treatment in fragile X cells with shorter CGG repeat expansions. The earlier azadC reactivation of *FMR1* that possesses partially acetylated histones is reminiscent of the synergistic reactivation achieved by dual treatment with HDAC inhibitors and a subthreshold dose of azadC (Chiurazzi et al. 1999). This relatively

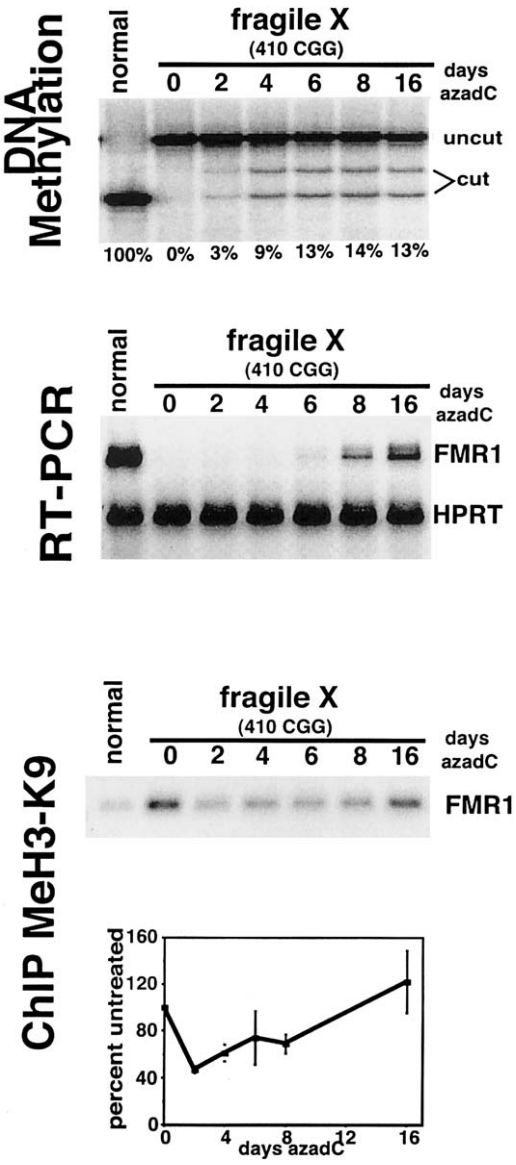

Figure 6 DNA methylation, RT-PCR, and ChIP analysis of *FMR1* during the course of azadC treatment. The fragile X cell line carrying 410 CGG repeats was treated with 1 μM azadC for 16 d. Equal numbers of cells were harvested at the indicated times for DNA, RNA, and chromatin analysis. DNA demethylation is expressed as the sum of intensities of the digested bands divided by the sum of the intensities of the digested and undigested bands. Histone methylation at lysine 9 during the course of azadC treatment is plotted as percent of untreated for three independent experiments.

large restoration of *FMR1* mRNA is not accompanied by a proportional increase in FMRP accumulation. This is consistent with prior work indicating that expansion-containing mRNA is refractory to efficient translation (Feng et al. 1995).

Partial or even full acetylation of histone H4, however, did not increase the accessibility of *FMR1* chromatin to nucleases, indicating that H4 acetylation is not sufficient to relax chromatin condensation at *FMR1*. However, the observation that histone H4 acetylation—either at the naturally occurring 230–CGG repeat allele or induced by HDAC inhibitor treatment of cells with large repeat tracts—augments azadC-mediated transcriptional reactivation suggests that the extent of histone H4 acetylation correlates with the preparation of this locus to become transcriptionally active. Whether histone H4 acetylation is necessary for transcription of *FMR1* remains to be determined.

Complementary patterns of histone H3 methylation at lysines 4 and 9 are emerging as a common theme in the difference between transcriptionally active and inactive loci. For example, at the chicken β-globin locus, transcriptionally active genes are associated with histone H3 methylated at lysine 4, and transcriptionally repressed genes are associated with methylation of histone H3 at lysine 9 (Litt et al. 2001). Similarly, there is a complementary pattern of histone H3 methylation at lysines 4 and 9 at the Prader-Willi imprinting centers in humans (Xin et al. 2001). Here we show that there is also a complementary pattern of histone H3 methylation at lysine 4 and 9, associated with *FMR1* in normal and fragile X cells, that correlates with gene expression. In normal cells, histone H3 associated with *FMR1* is methylated at lysine 4 but not at lysine 9. In fragile X cells, histone H3 associated with *FMR1* is undermethylated at lysine 4 and hypermethylated at lysine 9.

Methylation of histone H3 at lysine 9 at expanded *FMR1* alleles suggests that a histone methyltransferase, such as SUV39H1 (Rea et al. 2000), is being targeted to the locus. The chromodomain of the heterochromatin protein HP1 has been shown to have an affinity for histone H3 tails methylated at lysine 9, suggesting a model in which methylation of histone H3 at lysine 9 targets HP1 to a locus resulting in heterochromatin formation and transcriptional repression (Bannister et al. 2001; Lachner et al. 2001). Indeed, methylation of histone H3 at lysine 9, directed by Rb, targets HP1 to the cyclin E promoter, correlating with the repression of its transcription (Nielsen et al. 2001). This model may also apply to *FMR1* in fragile X syndrome. Maintenance of methylation of histone H3 at lysine 9 after TSA treatment would explain why *FMR1* remains in a condensed state, despite an increase of histone H4 acetylation to normal levels, since a chromodomain-containing protein, such as HP1, could remain targeted to the gene.

We also show that, after removal of azadC, there is a coordinated remethylation of *FMR1* DNA, renewed deacetylation of histones, and transcriptional silencing. This result demonstrates that these changes are not stable without the continuous presence of azadC. It also reinforces the observation that these changes are tightly coupled to the methylation state of the DNA. We emphasize that, although suggestive, the data do not prove that these chromatin changes are causally involved in transcription.

Like the marks of histone H3 and H4 acetylation, methylation of histone H3 at lysine 4 increased after azadC treatment, indicating that this modification is also coupled to DNA methylation of *FMR1*. In contrast to these changes, methylation of H3 at lysine 9 was transient, decreasing to ~50% of the starting level but recovering to the original level by day 16. *FMR1* transcripts continued to accumulate throughout the course of azadC treatment, indicating that the chromatin changes due to drug treatment may override the repressive effects of methylation of histone H3 at lysine 9, possibly by inhibiting binding of proteins, such as HP1. Since there is an increase in acetylation but no net change in methylation of H3 lysine 9 after 16 d, the increase in acetylation must be due to acetylation of other accessible lysine 9s of different H3 tails that reside in the same nucleosome, on neighboring nucleosomes, or even on different chromosomes in the population of cells.

The continued association of partially demethylated *FMR1* DNA with histone H3 methylated at lysine 9, coupled with the observation that *FMR1* DNA becomes remethylated after withdrawal of azadC, suggests that undermethylated *FMR1* DNA associated with histone H3 methylated at lysine 9 could be a target for DNA methyltransferase. This is consistent with a recent model in which methylation of histone H3 at lysine 9 is a determinant of DNA methylation (Tamaru and Selker 2001). In our working model, histone H3 associated with expanded, unmethylated CGG repeat tracts would become a target for a histone methyltransferase such as SUV39H1. The association of *FMR1* DNA containing expanded CGG tracts with histone H3 methylated at lysine 9 would then result in the targeting of a DNA methyltransferase to *FMR1* by association with a chromodomain-containing protein. There is precedent in model organisms for the association of a DNA methyltransferase activity with a chromodomain. For example, in *Arabidopsis thaliana,* DNA methylation and histone methylation are linked by a set of chromomethylase proteins, which contain both a chromodomain and the catalytic domain of a DNA methyltransferase (Lindroth et al. 2001). It also has been shown recently that, in *N. crassa,* DNA methylation is dependent upon histone H3 methylation at lysine 9 and that disruption

of histone methylation leads to DNA demethylation (Tamaru and Selker 2001). In our working model, after establishment of DNA methylation, HDACs and other components of the transcriptional repression machinery would be targeted to *FMR1* by association with methyl DNA-binding proteins, such as MeCP2, which condenses the chromatin at the locus, repressing transcription of the gene. HDAC inhibitors are able to partially reverse this process by inhibiting H4 deacetylation at the locus, but, because histone H3 is methylated at lysine 9, acetylation of this key amino acid would be blocked, preventing the transition from a transcriptionally silent condensed chromatin state back to a transcriptionally active open chromatin state. This model suggests the possibility that disruption of histone H3 methylation at lysine 9 at *FMR1* in fragile X cells could also lead to DNA demethylation, resulting in chromatin opening and transcriptional reactivation of the gene.

Acknowledgments

We thank J. Boss, P. Vertino, and P. Wade for helpful discussions. This work is supported by a postdoctoral research grant from the FRAXA Research Foundation and by National Institutes of Health grant HD35576.

Electronic-Database Information

The accession number and URL for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *FMR1* [MIM 309550])

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