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The distribution of repressive histone modifications on silenced *FMR1* alleles provides clues to the mechanism of gene silencing in fragile X syndrome

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Fragile X syndrome (FXS) is the most common heritable cause of intellectual disability and the most common known cause of autism. Most cases of FXS result from the expansion of a CGG·CCG repeat in the 5′ UTR of the *FMR1* gene that leads to gene silencing. It has previously been shown that silenced alleles are associated with histone H3 dimethylated at lysine 9 (H3K9Me2) and H3 trimethylated at lysine 27 (H3K27Me3), modified histones typical of developmentally repressed genes. We show here that these alleles are also associated with elevated levels of histone H3 trimethylated at lysine 9 (H3K9Me3) and histone H4 trimethylated at lysine 20 (H4K20Me3). All four of these modified histones are present on exon 1 of silenced alleles at levels comparable to that seen on pericentric heterochromatin. The two groups of histone modifications show a different distribution on fragile X alleles: H3K9Me2 and H3K27Me3 have a broad distribution, whereas H3K9Me3 and H4K20Me3 have a more focal distribution with the highest level of these marks being present in the vicinity of the repeat. This suggests that the trigger for gene silencing may be local to the repeat itself and perhaps involves a mechanism similar to that involved in the formation of pericentric heterochromatin.

INTRODUCTION

Fragile X syndrome (FXS; MIM no.: 300624) is an X-linked developmental disorder whose symptoms include intellectual disability, autistic-like symptoms, attention deficits, hyperactivity, insomnia, anxiety, depression, aggressive behavior, seizures and hypersensitivity to sensory stimuli (1). FXS results from mutations in the Fragile X Mental Retardation 1 (*FMR1*) gene. This gene encodes a translational regulator, FMRP, that is ubiquitously expressed at high levels early in embryonic development (2). The most common FXS mutation is an expansion of a CGG·CCG repeat tract in the 5′-untranslated region of the Fragile X mental retardation 1 (*FMR1*) gene from a premutation (PM) allele with 55–200 repeats to an allele having >200 repeats (3–5). These alleles, referred to as full mutation (FM) alleles, are transcriptionally silenced with a consequent deficiency of FMRP (6–8).

Although indirect evidence suggesting that FX alleles are transcriptionally silent and associated with heterochromatin preceded the identification of the *FMR1* gene in 1991, and the promoter and exon 1 of FX alleles have since been shown to have extensive CpG methylation and to be associated with certain marks of silent chromatin, the silencing mechanism remains the subject of much debate (3,9–25). Transcripts with long CGG tracts are known to form substrates for the RNA interference (RNAi) pathway (23). In addition, below the silencing threshold, an increase in the repeat number is associated with an increase in the amount of both the sense and antisense transcripts produced from the *FMR1* gene (21,26). Increases in repeat number also affect splicing and lead to the use of transcription start sites that are located further 5′ on the gene, in some cases as much as 10 kb away (21,26,27). Thus, during early embryogenesis when FX alleles are still active (20), unique transcripts may be produced or the existing transcripts may be made in sufficient quantity to trigger silencing. This silencing could occur via an RNAi-dependent or RNAi-independent process (28). In addition, a variety of CGG·CCG-repeat-binding proteins are found in human cells (29,30). By analogy with sequence-specific DNA-binding proteins such as MEF2 (31) and Sp1 (32), such proteins may recruit enzymes responsible for establishing

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and reinforcing gene silencing. Possible models for FX gene silencing thus include RNA-dependent and RNA-independent mechanisms in which the histone modifications are either nucleated within the repeat or initiate elsewhere in the gene as a downstream consequence of repeat expansion (21,23,33,34).

Distinct histone 'signatures' have been identified for various silenced regions of the genome that may reflect differences in the way that transcriptional repression is accomplished at these loci (35–39). To date, only two repressive histone modifications, H3K9Me2 and H3K27Me3, have been studied with respect to the silenced *FMR1* gene in individuals with FXS (16,19,40,41). H3K9Me2 and H3K27Me3 are modifications that are typically seen in unique regions of the genome that are developmentally repressed (38,42,43). Histone modifications, such as H3K9Me3 and H4K20Me3, that characterize repetitive DNA sequences like Satellite 2 (*Sat2*), the long array of 1 kb+ imperfect repeats found in pericentric heterochromatin (36,42–44), have yet to be examined. To better understand FX gene silencing, we have extended our previous studies on the *FMR1* gene to examine the *FMR1* gene of normal and silenced alleles for the presence and distribution of such modified histones. We show that FX alleles are enriched for both H39Me3 and H4K20Me3. The distribution of these modifications suggest that FX gene silencing may arise from events intrinsic and local to the repeat and result in a repressive histone profile reminiscent to that seen on human pericentric heterochromatin.

RESULTS

A localized region of elevated H3K9Me3 and H4K20Me3 occurs on FX alleles coincident with the repeat

We examined three regions of both active and silenced *FMR1* alleles for association with H3K9Me3 and H4K20Me3. Since *FMR1* is located on the X chromosome, we used cells from males to avoid complications arising from the presence of two X chromosomes in females. Two of the regions we tested were located within the body of the *FMR1* gene. Because of the technical difficulties associated with the PCR of long CGG·CCG-repeat tracts and the vast difference in the efficiency of amplification of short (active) and long (silenced) alleles, we used primer pairs from the region immediately downstream of the repeats in exon 1 to examine chromatin changes in the repeat region. We also used a primer pair in intron 1, ∼1 kb downstream of exon 1 to examine chromatin changes downstream of the repeat (Fig. 1A). A region 5′ of the *FMR1* promoter and ∼1 kb upstream of the major transcription start site of the *FMR1* gene, referred to as the upstream region, was also examined. Exon 1 of *GAPDH*, a robustly expressed housekeeping gene was used as a negative control. *Sat2* was used as a positive control.

Normal cells had levels of H3K9Me3 and H4K20Me3 that were not significantly above *GAPDH* in any of the three regions tested. In contrast, exon 1 in FXS cells showed levels of these histones that were comparable to *Sat2* and significantly higher than *GAPDH* (Fig. 1B and C). The level of both modified histones then dropped off significantly in both the 5′ and 3′ directions. This would be consistent with the idea that the events that nucleate the deposition of these repressive histone marks initiate within or very close to the repeat.

A zone of developmentally regulated heterochromatin is located 5′ of both active and silenced FMR1 alleles

To assess whether the repressive histone marks previously shown to be associated with FX alleles, H3K9Me2 and H3K27Me3, have a similar distribution, we analyzed the association of these histones with the same three regions of the *FMR1* locus in cells from unaffected individuals and those with FXS. As with H3K9Me3 and H4K20Me3, these two modified histones were present at elevated levels on FX alleles. However, in contrast to what was seen with the first pair of modified histones, H3K9Me2 and H3K27Me3 showed a similar elevated distribution on all three regions tested in FX cells (Fig. 2A and B) with the levels of these marks being comparable to that seen on *Sat2*.

H3K9Me2 and H3K27Me3 were also present at similar levels on the upstream region of normal alleles (Fig. 2A and B). However, significantly lower levels of these histones were found on exon 1 or intron 1 of such alleles (Fig. 2). While this work was in progress, DNA methylation of the upstream region of normal alleles was reported (33). Previous work had suggested that the *FMR1* gene was embedded in a much larger region of altered chromatin (45). Our work, together with the other recently published study, demonstrates that this region of altered chromatin actually abuts the 5′ end of the *FMR1* gene. Furthermore, our data suggest that this upstream heterochromatin zone (UHZ) has elevated levels of H3K27Me3 and H3K9Me2, but not H3K9Me3 or H4K20Me3.

Thus, our data suggest that the complement of histone modifications present on the UHZ of normal and FX alleles is different from that seen within the body of FX alleles. The UHZ histone profile is reminiscent of normal, developmentally repressed single copy genes, whereas the profile of all four repressive marks on exon 1 of FXS alleles was indistinguishable from that seen on *Sat2*. Thus, it may be that the mechanism of FX gene silencing is different from the mechanism responsible for establishing the UHZ.

Since the histone modification 'signature' of the UHZ is typical of developmentally repressed genes, we examined the deposition of H3K27Me3 on this region in normal human embryonic stem cells (hESCs) before and after differentiation. We found that in undifferentiated ESCs, H3K27Me3 levels were low (Fig. 2C). However, on differentiation of the hES cells into embryoid bodies (EBs), an increase in the levels of H3K27Me3 occurred. This increase was confined to the UHZ as it is in lymphoblastoid cells from unaffected individuals (Fig. 2C). A similar situation was seen in mouse (data not shown) Thus, the deposition of H3K27Me3 on the UHZ is an early step in normal human and murine embryonic development.

The *FMR1* locus produces a complex mixture of long and short RNAs

A number of different RNA species have been implicated in gene silencing (46). One species consists of long non-coding transcripts that act in *cis* or *trans* to target silencing complexes

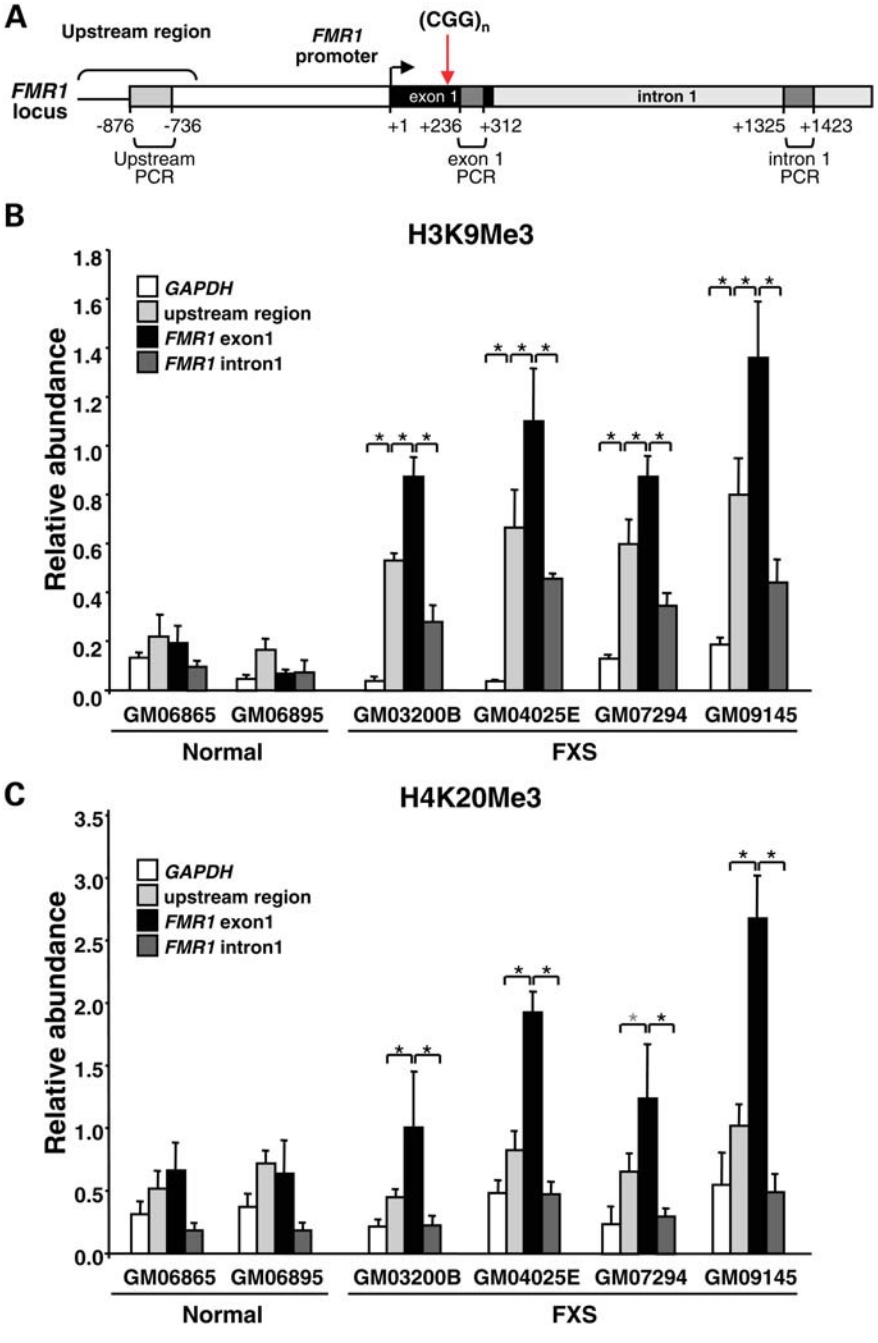

Figure 1. The distribution of H3K9Me3 and H4K20Me3 on normal and FX alleles. (**A**) Diagrammatic representation of the *FMR1* 5′ end showing location of the amplified regions used in these experiments. All the numbering is relative to the 3′ most transcription start site (Site I). (**B**) and (**C**) ChIP analysis of the abundance of H3K9Me3 (B) and H4K20Me3 (C) in normal and FX cells. The *Y*-axis in each case shows the abundance relative to *Sat2* which is set to 1. The data in each panel are an average of at least three independent ChIP experiments. The black asterisks indicate that regions that are significantly different ($P < 0.04$). The gray asterisks indicate differences that approach statistical significance ($P = 0.06$).

to the target gene independent of the RNAi machinery. The second species are small RNAs produced from longer sense–antisense pairs by the RNAi machinery. We have used a combination of northern blotting and RNase protection to examine the RNAs produced by the *FMR1* locus. Our northern blotting data on total RNA suggests that a variety of long antisense transcripts are made including very high levels of a ~5 kb transcript that hybridizes strongly with the UHZ probe (Fig. 3A). This is presumably not the previously described *FMR4* gene which initiates in a similar location, since the *FMR4* transcript is only 2.5 kb (47). It is also not one of the splice isoforms generated from the antisense transcript, *ASFMR1*, since it hybridizes only weakly with sequences from the promoter region and exon 1. Unlike the previously described antisense transcripts (21,47), these RNAs are seen at similar levels in carriers of normal alleles having <54

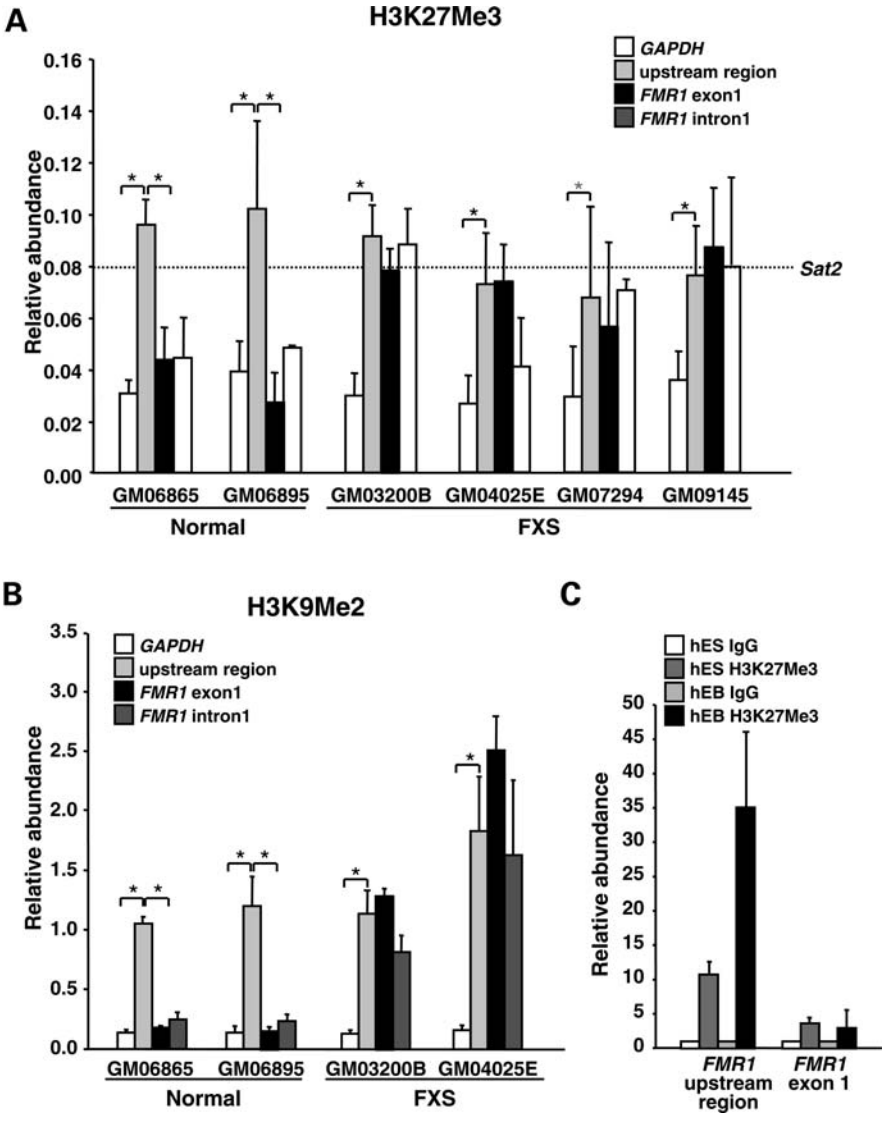

Figure 2. The distribution of H3K27Me3 and H3K9Me2 on the *FMR1* locus. ChIP analysis of the abundance of H3K27Me3 (**A**) and H3K9Me2 (**B**) on normal and FX alleles. The *Y*-axis shows the abundance relative to either *MYOD* (A), a positive control for H3K27Me3 enrichment or *Sat2* (B). The data shown are an average of at least three independent experiments. In (A), the dotted line shows the average abundance of H3K27Me3 on *Sat2*. The black asterisks indicate those regions that are significantly different ($P < 0.04$). The gray asterisks indicate regions whose differences approach statistical significance ($P = 0.06$). (**C**) ChIP analysis of H3K27Me3 on the upstream region and exon 1 of the *FMR1* gene in normal hESCs and hEBs. The *Y*-axis shows the abundance of H3K27Me3 relative to IgG.

repeats, as well as carriers of PM and FM alleles. Since the RNAs are the same size in all of these individuals, these transcripts likely do not contain the repeats. Failure to detect the previously described *ASFMR1* and *FMR4* antisense transcripts by northern blotting would be consistent with previous reports and presumably reflects the much lower levels of these transcripts (21). Since the RNAs we describe are found at equivalent levels in both normal and FX cells, it seems unlikely that they are involved in FX gene silencing, although it may be that they contribute to the formation of the zone of heterochromatin in which the *FMR1* gene is embedded in both normal and FXS cells.

In addition to these long RNAs, small RNAs, ~20 nt in length, that are derived from the *FMR1* locus were also detected using probes from the UHZ, promoter and exon 1. Similar levels of these RNAs were seen in normal cells as well as in patient cells with or without gene reactivation with 5-azadeoxycytidine (5-aza), a DNA methyltransferase inhibitor (Fig. 3B and data not shown). These RNAs correspond to the antisense strand of the *FMR1* locus and are slightly smaller than the small RNAs produced from the DMPK locus in both normal cells and cells from patients with myotonic dystrophy type I (DM1; MIM no.:160900), a CTG·CAG-repeat expansion disorder (Fig. 3B) (48). Moreover, in contrast to what is seen in DM1, where the small antisense RNAs are limited to the region that includes the repeat and that becomes heterochromatinized, these small RNAs extend far beyond the repeat and are not associated with heterochromatin in normal cells, despite being present at levels similar to the small DMPK RNAs.

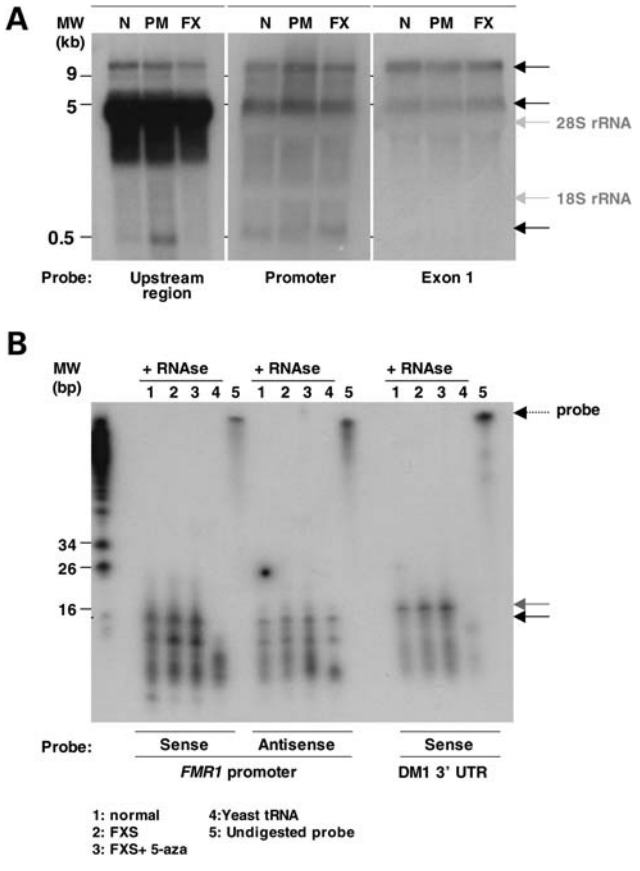

Figure 3. Transcripts produced from the *FMR1* locus. (**A**) Northern analysis of total RNA from normal (N), PM and FX cells using the UHZ, promoter and exon 1 PCR products indicated in Figure 1A as probes. The black arrows on the right-hand side of the autoradiographs mark the positions of the three major products seen with all three probes. (**B**) RNase protection assays to detect small RNAs produced from the *FMR1* promoter in normal alleles and in both silenced FM alleles and FM alleles reactivated by treatment with 5-aza. The molecular weight marker is *Msp*I-digested pBR322. The gray arrow indicates the small antisense RNA produced from the DMPK allele. The black arrow indicates the small antisense RNA produced from the *FMR1* allele.

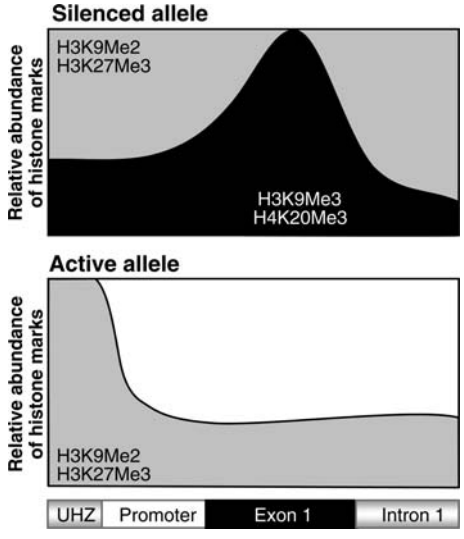

Figure 4. Diagrammatic representation of distribution of the two categories of repressive histone modifications associated with FX alleles. The distribution of H3K9Me2 and H3K27Me3 is shown in gray and the distribution of H3K9Me3 and H4K20Me3 is shown in black.

This leaves the possibility that small RNAs produced from the repeats themselves, either because of their higher molar amount or because of some as yet unknown biological property, are particularly efficient at inducing gene silencing when the repeat number exceeds 200. This possibility is appealing since the distribution of H3K9Me3 and H4K20Me3 suggests that nucleation of the heterochromatin occurs close to or within the repeat. We have been able to detect small RNAs corresponding to the CGG repeats in both normal cells and patient cells where the *FMR1* gene has been reactivated (data not shown). However, because CGG repeats are found elsewhere in the human genome, it is unclear whether these small repeat containing RNAs are derived from the *FMR1* region or not. Whatever their origins, we have found no evidence of any RNA species unique to patient cells even when silenced alleles were reactivated with 5-aza. Nor do we see altered levels of any of these RNAs. Thus, although it is tempting to think that transcripts from the *FMR1* locus play a role in FX gene silencing, we have been unable to find any clear evidence that they do. Our data thus raise the possibility that RNA-independent mechanisms may be more likely to be involved in FX gene silencing.

DISCUSSION

We have extended the work from a variety of laboratories, including our own, to more extensively characterize the *FMR1* gene and the region of the X chromosome in which it is embedded. We showed that the *FMR1* gene lies immediately downstream of a UHZ that is established during early embryonic development in both normal and FX alleles. The UHZ of active alleles has a histone profile similar to what is seen on developmentally repressed single copy regions of the genome being enriched primarily for H3K9Me2 and H3K27Me3. In contrast, the body of the silenced *FMR1* gene contains, in addition to the modified histones seen on the UHZ, elevated levels of H3K9Me3 and H4K20Me3, two histone modifications not previously known to be associated with FX alleles (Figs 1 and 2). The fact that the histone profiles differ as they do suggests that gene silencing in FXS is unlikely to simply reflect the spread of silencing marks from the UHZ into the body of the *FMR1* gene as has recently been suggested (33).

All four modified histones are present on exon 1 of FX alleles at levels comparable to that found on *Sat2*. However, whereas H3K9Me2 and H3K27Me3 are broadly distributed across the whole 5′ end of silenced alleles, H3K9Me3 and H4K20Me3 levels were highest on exon 1 which contains the CGG-repeat tract as represented diagrammatically in Fig. 4. The difference in the spread of the H3K9Me2/H3K27Me3 histones and H3K9Me3/H4K20Me3 histones seen on FX alleles may be related to the tendency of H3K27Me3-enriched heterochromatin to spread widely in the absence of effective chromatin boundaries, whereas

'focal heterochromatin' with a much more limited ability to spread is often seen on genes enriched for H4K20Me3 and H3K9Me3 (49–51).

On normal alleles, spreading of the repressive histone marks from the UHZ into the body of the active *FMR1* gene may be prevented by factors such as CTCF and USF1/USF2. These proteins bind to the promoter of active *FMR1* alleles (21,52) and have the potential to enforce a chromatin boundary (53,54). In FXS, the putative chromatin boundary at the 5′ end of the *FMR1* gene appears to have been lost, and the UHZ and the repeat-induced heterochromatin have merged, with limited spread of the H3K9Me3/H4K20Me3 marks from the body of the *FMR1* gene into the UHZ (Fig. 4). However, since the histone profile on exon 1 of *FMR1* is similar to *Sat2* with respect to all four repressive heterochromatin marks, it may be that the FX gene silencing mechanism is similar to the one responsible for silencing of *Sat2*. Thus, it is unnecessary to invoke a corresponding spread of histone modifications in the other direction to explain the histone profile in the body of the silenced *FMR1* gene. However, it is possible that there are histone modifications that remain to be identified that originate in the UHZ and spread into the body of the *FMR1* gene when the chromatin boundary is lost, thereby contributing to FX gene silencing.

The fact that the body of the silenced gene shares a heterochromatin profile with *Sat2*, along with the fact that the highest levels of H3K9Me3 and H4K20Me3 are seen on exon 1, supports the idea that gene silencing in FXS is related to events directly triggered by the repeats perhaps in a manner analogous to the way that *Sat2* is silenced. This narrows the range of likely gene silencing mechanisms. The fact that no unique transcripts are seen in FX cells even when the *FMR1* gene is reactivated and that the levels of small RNAs corresponding to the repeat are similar in normal and FX cells raises the possibility that FX gene silencing occurs via an RNA-independent mechanism. Indeed, while *Sat2* was once thought to be silenced by an RNAi-based mechanism like the centromeric repeats of fission yeast (55), it is now known that the chromatin modifications on these repeats are unaffected in Dicer-null ES cells (56). This is consistent with an RNAi-independent mechanism (57). A similar lack of the effect of Dicer depletion has been reported for a transgene tandem array that normally undergoes silencing in mammalian cells (58). Interestingly, in the yeast mating-type region, heterochromatinization of the *cenH* DNA element, which has 96% homology to the yeast centromeric repeats, is regulated by both RNA-dependent and independent mechanisms. The RNA-independent mechanism is initiated by sequence-specific binding by members of the ATF/CREB family of transcription factors, Atf1 and Pcr1 (59,60). In the context of FX alleles, silencing may be initiated by one of the many CGG·CCG-repeat-binding proteins that have been described (29,30). It may be that these proteins recruit enzymes such as histone deacetylases and methylases that initiate gene silencing. Why 200 repeats would be required before silencing is seen is not clear. It may be that a critical level of binding of these proteins is only reached when the repeat number reaches 200 or that a factor that keeps the gene active also binds the repeat and is only titrated out when the repeat number reaches this threshold. The latter idea is appealing since it may also explain the hyperexpression of PM alleles. It is also possible that silencing is related instead to the length-dependent induction of the DNA damage response by FX repeats (61–64), since DNA damage leads to the recruitment of proteins involved in gene silencing such as SIRT1, EZH2, DNMT1 and DNMT3B (65,66).

Whatever the trigger for gene silencing, in addition to the shared histone profiles, both *Sat2* and FX gene silencing involve the protein deacetylase SIRT1 (67,68). SIRT1 is thought to act on pericentric heterochromatin via recruitment of the SUV39H1 histone methyltransferase rather than G9a, the methylase more commonly active on developmentally repressed regions of the genome (67,68). Since these methylases represent different potential druggable targets, understanding how far the similarities between *Sat2* and FX silencing extend may be useful in the development of strategies to reactivate FX alleles.

MATERIALS AND METHODS

Cell lines and reagents

Lymphoblastoid cell lines from normal (GM06865, GM06895) and fragile X patients (GM03200B, GM04025E, GM07924, GM09145) were obtained from Coriell Cell Repository (Camden, NJ, USA). Cells were grown in RPMI 1640 supplemented with 10% FBS and 1× antibiotic–antimycotic liquid consisting of penicillin, streptomycin and fungisone (Invitrogen, Carlsbad, CA, USA). To reactivate FX alleles, cells were treated with azadC as previously described (54). Chromatin immunoprecipitation assay kits and normal mouse and rabbit IgG were purchased from Upstate (Temecula, CA, USA). Antibodies against histone H3K9Me2 (ab1220), H3K9Me3 (ab8898), H3K27Me3 (ab6002) and H3K4Me2 (ab7766) were from Abcam (Cambridge, MA, USA). Antibodies against H4K20Me3 (39180) were from Active Motif (Carlsbad, CA, USA). Mouse embryonic stem cell chromatin immunoprecipated with antibodies to H3K27Me3 was a kind gift of Joseph Landry (NCI). H1 hESCs and differentiated EBs produced from these cells were a gift of Barbara Mallon (NIH Stem Cell Unit). The ESCs were propagated as described in http://stemcells.nih.gov/research/nihresearch/scunit/culture.asp#mef. The EBs were differentiated using DMEM supplemented with 10% FBS as described in http://stemcells.nih.gov/research/nihresearch/scunit/ebd.htm, except that the EBs were harvested after 4 days.

Chromatin immunoprecipitation assays

The chromatin immunoprecipitation (ChIP) assay kit from Upstate was used according to the manufacturer's instructions. Real-time PCRs on the immunoprecipitated DNAs were carried out using the Power SYBR™ Green PCR master mix (Applied Biosystems) and the primer pairs shown in Table 1. The location of the *FMR1* amplicons is shown in Figure 1A. Regions of *GAPDH*, *Sat2* and *MYOD* were amplified using the primers recommended by Abcam, Inc. for use as negative and positive controls for ChIP. Their sequences are

Table 1. Primers used in this study

Primer name	Primer sequence
FMR1 primers	
Upstream-F	5′-ACAGTGGAATGTAAAGGGTTG-3′
Upstream-R	5′-GTGTTAAGCACTTGAGGTTCAT-3′
FMR1 exon1-F	5′-CGCTAGCAGGGCTGAAGAGAA-3′
FMR1 exon1-R	5′-GTACCTTGTAGAAAGCGCCATTGG AG-3′
FMR1 intron1-F2	5′-CTTGAAGGTGAATGAAGAATAGGTTG-3′
FMR1 intron1-R1	5′-TACAGCACCTACATACCAACAAACGC-3′
Control primers	
hsGAPDH exon1-F	5′-TCGACAGTCAGCCGCATCT-3′
hsGAPDH intron1-R	5′-CTAGCCTCCCGGGTTTCTCT-3′
hsSat2 repeat-F1	5′-ATCGAATGGAAATGAAAGGAGTCA-3′
hsSat2 repeat-R1	5′-GACCATTGGATGATTGCAGTCA-3′
hsMYO-D exon1-F	5′-CCGCCTGAGCAAAGTAAATGA-3′
hsMYO-D exon1-R	5′-GGCAACCGCTGGTTTGG-3′

listed in Table 1. For quantitation, the comparative threshold (Ct) method was used. The immunoprecipitated material was normalized to 5% of input and was expressed relative to the appropriate positive control. Student's *t*-test was used to calculate statistical significance.

Detection of antisense RNA by northern blot analysis

Total RNA was isolated using Trizol (Invitrogen) as per the manufacturer's instructions. Five micrograms of total RNA was mixed with equal volume of NorthernMax®-Gly sample loading buffer (Ambion, Austin, TX, USA) and incubated at 50°C for 30 min. The samples were then run on a 1% agarose gel in 1X NorthernMax®-Gly Gel Prep/Running buffer (Ambion). The gel was stained with ethidium bromide to check RNA integrity. Blotting to Hybond N^+ nylon membrane (Amersham) was carried out according to standard procedures. The blot was then rinsed with 5× SSC and crosslinked using the UVStratalinker (Stratagene, Santa Clara, CA, USA). For northern blot analysis, three templates were generated to make sense riboprobes for the detection of the antisense RNAs. The template for the 5′ flank region was prepared by linearizing a plasmid containing bases −510 to −950 of the *FMR1* gene. The template for the promoter region was prepared by linearizing a plasmid containing bases −19 to −121 of the *FMR1* gene. A PCR fragment containing T7 sequence linked to bases +137 to +412 of the *FMR1* gene was used as a template to generate sense probe in the exon 1/intron 1 region. Templates were transcribed using T7 polymerase plus (Ambion) and α-^{32}P-CTP (8000 Ci/mmol, MP Biomed, Solon, OH, USA) and the RNA purified according to standard procedures. After prehybridization in Ultrahyb buffer (Ambion), hybridization with the riboprobe was carried out in Ultrahyb buffer at 68°C overnight. The blot was then washed twice with 2× SSC/0.1% SDS for 5 min at 68°C followed by two 15 min washes with 0.1× SSC/0.1% SDS. The blot was then exposed to a Kodak Biomax XAR X-ray film.

RNase protection assays

RNase protection assays were performed using the mirVana™ miRNA detection kit (Ambion). Briefly, the riboprobes were transcribed as described above and purified by running on a 5% denaturing polyacrylamide gel. The gel was exposed to X-ray film and the band containing the full-length probe was cut and RNA was eluted in elution buffer provided in the kit overnight at 37°C. The template for the DM1 probe was prepared as described elsewhere (48). Five micrograms of total RNA was hybridized with 5×10^5 c.p.m. of the riboprobe at 50°C overnight. The hybridized probe/RNA was then digested with RNase A/T1 and precipitated. The protected fragments were detected by running the samples on a 15% (19:1, acrylamide:bis) polyacrylamide denaturing gel and exposing it to an X-ray film. Labeled pBR322 digested with *Msp*I was used for sizing of the protected fragments.

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Conflict of Interest statement. None declared.

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