ARTICLE

Differential epigenetic modifications in the *FMR1* gene of the fragile X syndrome after reactivating pharmacological treatments

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The fragile X syndrome is caused by a >200 CGG repeat expansion within the *FMR1* gene promoter, with consequent DNA hypermethylation and inactivation of its expression. To further clarify the mechanisms that suppress the activity of the mutant gene and the conditions that may permit its reactivation, we investigated the acetylation and methylation status of three different regions of the *FMR1* gene (promoter, exon 1 and exon 16) of three fragile X cell lines, using a chromatin immunoprecipitation (ChIP) assay with antibodies against acetylated-H3/H4 histones and against dimethylated lysine residues K4 and K9 of histone H3 (H3-K4 and H3-K9). We then coupled the ChIP assay with real-time PCR, obtaining absolute quantification of immunoprecipitated chromatin. Basal levels of histone acetylation and H3-K4 methylation were much higher in transcriptionally active wild-type controls than in inactive fragile X cell lines. Treatment of fragile X cell lines with the DNA demethylating drug 5-aza-2-deoxycytidine (5-azadC), known to reactivate the *FMR1* gene, induced a decrease of H3-K9 methylation, an increase of H3 and H4 acetylation and an increase of H3-K4 methylation. Treatment with acetyl-L-carnitine (ALC), a compound that reduces the *in vitro* expression of the FRAXA fragile site without affecting DNA methylation, caused an increase of H3 and H4 acetylation. However, H3-K4 methylation remained extremely low, in accordance with the observation that ALC alone does not reactivate the *FMR1* gene. Our experiments indicate that H3-K4 methylation and DNA demethylation are the main epigenetic switches activating the expression of the *FMR1* gene, with histone acetylation playing an ancillary role.

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Introduction

The fragile X syndrome (OMIM +309550) is the most common inherited form of mental retardation. It is caused by the expansion of a polymorphic CGG trinucleotide repeat in the promoter of the *FMR1* gene, consisting of more than 200 repeats (full mutation) instead of 6–54 CGG repeats, as in normal alleles.^{1,2} In affected patients, cytosines of the CGG expansion and of the upstream CpG island are abnormally methylated and lead to

transcriptional inactivation of the gene^{3,4} and absence of the FMR1 protein (FMRP). FMRP is an mRNA-binding protein, whose absence was recently shown to cause a translational dysregulation of several mRNAs normally associated with FMRP, allowing the identification of candidate genes relevant to this phenotype.^{5,6} Rare individuals of normal intelligence have been described, who express FMRP and harbor a completely or partially unmethylated full mutation, clearly indicating that DNA methylation is the key factor in silencing *FMR1*.^{7,8}

We previously demonstrated that treatment of fragile X lymphoblastoid cell lines with the DNA methylation inhibitor 5-aza-2-deoxycytidine (5-azadC) leads to the transcriptional reactivation of the mutant *FMR1* gene.⁹ We then studied single-cell methylation by analyzing the promoter region of the gene with the bisulfite-sequencing technique and found that a large proportion of cells (70–90%) became unmethylated after 5-azadC treatment.¹⁰ On the other hand, the amount of *FMR1* mRNA, measured by quantitative fluorescent RT-PCR, indicated that transcriptional reactivation efficiency was only 15–20% in comparison to a normal cell line,¹⁰ possibly due to a residual methylation of the CGG repeat. Our results confirmed the role of DNA methylation in the transcriptional silencing of the *FMR1* gene.

Research over the past few years focused on the direct and indirect mechanisms by which DNA methylation mediates gene silencing. DNA methylation can directly inhibit binding of transcription factors to gene promoters, while indirect mechanisms are mediated by local chromatin changes. Methylated CpG residues can then be recognized by methyl binding proteins, such as MeCP2 and MBD1-4, which recruit histone deacetylases (HDACs).^{11,12} HDACs remove acetyl groups from lysines of histone H3 and H4 amino-terminal tails, eventually leading to a more condensed chromatin structure (heterochromatin).¹³ In normal controls, the *FMR1* gene is associated with acetylated histones H3 and H4, while acetylation is reduced in fragile X cells.¹⁴ Increased acetylation of *FMR1*-associated histone H3 and H4 was induced by treatment with 5-azadC and with the HDAC inhibitor trichostatin A.¹⁴ We then reported that histone hyperacetylating drugs (HDAC inhibitors), such as sodium butyrate (BA) and 4-phenylbutyrate (4-PBA), synergistically potentiate the *FMR1* gene reactivation induced by 5-azadC.¹⁵ This observation confirms that CpG cytosine methylation and histone deacetylation cooperate in silencing chromatin domains.

Histone amino-terminal tails can be modified through the attachment (or removal) of acetyl-, phosphate- or methyl-groups.¹⁶ Recently, attention has been focused on the methylation of histone H3 at lysines 4 and 9 (H3-K4 and H3-K9), which play an important role in transcriptional regulation. In particular, H3-K4 methylation correlates with activation of gene expression (high in

euchromatin) and H3-K9 methylation with transcriptional inactivation (high in heterochromatin).^{17,18} Coffee *et al*¹⁹ reported that exon 2 of the *FMR1* gene has decreased H3-K4 and increased H3-K9 methylation, in accordance with its silent status, and that 5-azadC treatment induces an increase of H3-K4 methylation, but only a transient decrease of H3-K9 methylation.

Here we present a detailed analysis of the epigenetic modifications of the *FMR1* gene in normal and fragile X cell lines, using a chromatin immunoprecipitation (ChIP) protocol coupled with real-time PCR, which allows better quantitation of epigenetically modified histones. In contrast to previous reports,^{14,19} we tested three different regions of the *FMR1* gene (promoter region, exon 1 next to the CGG repeat tract and exon 16) and found significant differences before and after 5-azadC treatment. All ChIP experiments were accompanied by measurement of *FMR1* mRNA levels by quantitative RT-PCR, so that a correlation between epigenetic modifications and transcriptional activity of the gene could be established. We also report on the epigenetic effect of acetyl-L-carnitine (ALC), a drug which reduces the cytogenetic expression of the fragile site FRAXA,²⁰ possibly through direct histone acetylation.

Materials and methods

Cell cultures and drugs treatments

Lymphoblastoid cell lines were established by Epstein–Barr virus (EBV) transformation from peripheral blood lymphocytes of male fragile X patients and two normal control males. The three fragile X cell lines used in these experiments were: E3 carrying an *FMR1* allele with 250 CGG repeats, S1 with 450 CGGs and E6 with over 550 CGG repeats. Cells were grown in RPMI1640 medium with 10% fetal calf serum and penicillin/streptomycin at 37°C with 5% CO₂. Medium was changed every 48 h. Cells were treated daily with 1 µM 5-azadC (Sigma) for 7 days. Treatment with 10 mM ALC (Sigma-Tau) was also for 7 days, with only two additions of the drug.

RNA extraction and reverse transcriptase-PCR

Total RNA from treated and untreated cell lines was extracted with the single-step acid phenol method, using Trizol (Invitrogen). RNA was reverse-transcribed by MoMLV-reverse transcriptase (Gibco-BRL). PCR was performed as previously described,⁹ employing 1 µl of cDNA with specific primers for *FMR1* and for the housekeeping gene hypoxanthine guanine phosphorybosyltransferase (*HPRT*) as internal control. PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, at 55°C for 1 min and at 72°C for 2 min and then a final step of extension at 72°C for 5 min.

Table 1 Histone acetylation and H3-K4/H3-K9 methylation in wild-type and fragile X cell lines before and after 5-azadC treatment

	Wild-type 1	Wild-type 2	E3	E3+5azadC	S1	S1+5azadC	E6	E6+5azadC
<i>Promoter</i>								
H3 acetylation	63.433 ± 45.254	81.345 ± 45.991	1.099 ± 0.804	8.562 ± 7.484	1.125 ± 0.272	8.934 ± 4.484	0.553 ± 0.210	4.026 ± 2.323
H4 acetylation	28.672 ± 11.011	32.508 ± 21.021	1.650 ± 0.339	3.020 ± 0.588	0.959 ± 0.858	7.363 ± 6.532	4.842 ± 4.608	14.372 ± 13.350
H3-K4 methylation	58.023 ± 23.347	38.210 ± 9.032	5.655 ± 5.326	45.169 ± 8.157	1.665 ± 0.349	3.440 ± 1.315	2.614 ± 0.543	27.103 ± 14.365
H3-K9 methylation	0.287 ± 0.069	0.903 ± 0.580	7.034 ± 4.108	7.373 ± 4.112	3.342 ± 2.814	0.941 ± 1.164	5.886 ± 4.594	4.469 ± 1.187
<i>Exon 1</i>								
H3 acetylation	469.079 ± 111.573	784.391 ± 513.503	3.948 ± 1.765	40.339 ± 38.744	2.605 ± 0.656	27.419 ± 22.948	6.365 ± 6.366	11.501 ± 12.081
H4 acetylation	321.215 ± 116.079	92.690 ± 73.999	2.359 ± 0.388	6.303 ± 3.986	1.439 ± 0.667	15.100 ± 10.491	26.848 ± 35.228	34.214 ± 45.917
H3-K4 methylation	93.379 ± 78.901	181.553 ± 145.491	6.524 ± 2.875	45.680 ± 43.288	6.703 ± 4.794	21.897 ± 5.496	7.624 ± 1.482	49.913 ± 10.521
H3-K9 methylation	1.273 ± 0.906	4.384 ± 4.263	13.671 ± 8.697	14.099 ± 4.281	51.235 ± 39.692	49.364 ± 23.810	25.615 ± 2.813	15.487 ± 2.264
<i>Exon 16</i>								
H3 acetylation	0.881 ± 1.023	0.848 ± 0.495	1.482 ± 1.265	1.542 ± 1.601	0.664 ± 0.530	1.775 ± 1.830	1.240 ± 1.363	1.691 ± 2.235
H4 acetylation	0.800 ± 0.502	0.543 ± 0.354	1.455 ± 1.402	0.988 ± 0.947	0.620 ± 0.535	2.625 ± 5.446	2.396 ± 1.847	1.211 ± 0.809
H3-K4 methylation	1.188 ± 0.792	1.226 ± 0.984	0.500 ± 0.332	0.835 ± 0.008	0.720 ± 0.376	1.537 ± 1.489	0.216 ± 0.40	0.885 ± 0.046
H3-K9 methylation	1.095 ± 0.269	1.766 ± 1.947	1.804 ± 0.908	1.552 ± 0.541	1.330 ± 0.545	2.399 ± 2.195	4.406 ± 1.217	3.505 ± 0.492

All values correspond to the mean amount of immunoprecipitated DNA (nanograms) ± standard deviation.

Quantitative RT-PCR analysis

For a quantitative estimate of the relative *FMRI* mRNA levels, we adapted the technique described by Tassone *et al.*²¹ using an ABI 7700 Sequence Detector with dual-labeled TaqMan probes. The *FMRI* amplicon is a 89-bp product spanning the junction between exon 13 and 14 of the gene (positions 1432–1520 of GenBank sequence NM_002024). The following primers and TaqMan probe were employed: forward 5'-GGA ACA AAG GAC AGC ATC GC-3', reverse 5'-CTC TCC AAA CGC AAC TGG TCT-3'; TaqMan probe 5'-(FAM)-AAT GCC ACT GTT CTT TTG GAT TAT CAC CTG AA-(TAMRA)-3'. The relative amount of *FMRI* mRNA was assessed by comparison with the human *HPRT* mRNA detected with the Pre-Developed TaqMan Assay Reagent ABI 4310890E (huHPRT endogenous control). Final reaction volume was 25 µl in the TaqMan Universal PCR Master Mix (ABI 4304437) with 900 nm of each primer and 100 nm dual-labeled probe for *FMRI*. Cycle parameters were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C denaturation and 1 min at 60°C annealing/extension. Relative *FMRI* levels were calculated as follows: $2^{-[\Delta C_t(\text{fragile X}) - \Delta C_t(\text{control})]}$ = $2^{-\Delta \Delta C_t}$, where ΔC_t equals $C_t(\text{FMRI}) - C_t(\text{HPRT})$ as discussed by Tassone *et al.*²¹

ChIP assay

We employed the ChIP Assay Kit and antibodies from Upstate Biotechnology and followed the manufacturer's protocol. Histone acetylation was studied by ChIP with antibodies directed against histone H4 acetylated at lysines 5, 8, 12 and 16, and histone H3 acetylated at lysines 9 and 14. Histone methylation analysis was performed using two different antibodies against histone H3 dimethylated at lysine 9 or at lysine 4. After ChIP, DNA was extracted with a standard procedure (phenol/chloroform/isoamyl alcohol) (25:24:1) and digested with *XhoI*, prior to quantitative fluorescent PCR analysis, in order to separate the CGG repeats, which could interfere with amplification efficiency.

Quantification of immunoprecipitated DNA (IP-DNA) by real-time PCR

The levels of acetylation of histone H3 and H4 and of methylation of lysines H3-K9 and H3-K4 of both *FMRI* and *HPRT* were measured by fluorescent PCR by ABI 7700 Sequence Detector with dual-labeled TaqMan probes.

Primers and probes employed for PCR analysis were designed to amplify three different portions of the *FMRI* gene: the promoter region (positions 13376–13448 of GenBank L29074), exon 1 near the CGG repeat tract (positions 13940–14012 of GenBank L29074) and the last amplicon overlapping exon 16 (positions 47305–47386 of GenBank L29074). For *HPRT*, the amplified amplicon was

that overlapping exon 6 (positions 34946–35022 of GenBank sequence M26434). The sequences of primers and probes were as follows: 5'-AACTgggATAACCGgATgCAT-3' forward, 5'-ggCCAgAACgCCCATTTC-3' reverse and 5'-(FAM)TgATTTCgCgCCACTgAgTgCAC (TAMRA)-3' probe for the promoter region; 5'-CgCTAgCAgggCTgAAGAgA-3' forward, 5'-CTTgTAgAAAgCgCCATTggA-3' reverse and 5'-(FAM)AtggAggAgCTggTggTggAAGTgC (TAMRA)-3' probe for exon 1; 5'-AATATTCTCATAggAAACgACgATCA-3' forward, 5'-CTgTTgTTCTTCCTTTAgCCTCTC TT-3' reverse and 5'-(FAM)TCCCGAACAgATAATCgTCCACgTAATCC (TAMRA)-3' probe for exon 16; 5'-TgACACTggCAAAACAATgCA-3' forward, 5'-CCTTgCgACCTTgACC ATCT-3' reverse and 5'-(VIC)TTTgC TTTCTTggTCAggCagTATAATCCA(TAMRA)-3' probe for *HPRT* exon 6. Cycle parameters were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, for denaturation and 1 min at 60°C for annealing/extension. A standard curve for the three *FMR1* and for the single *HPRT* amplicon was constructed with five different DNA dilutions of known concentration (X -axis = $\log[X]$) and the corresponding C_t values (Y -axis). The unknown amount of acetylated and methylated IP-DNA of *FMR1* and *HPRT* (X -axis = $\log[X]$) was calculated from C_t values, through the standard curve plot. Normalized *FMR1* levels were estimated dividing the amount of *FMR1* IP-DNA by the amount of *HPRT*. The acetylation and methylation levels of the treated cell lines has been compared to the levels of untreated and/or control male cell lines.

Statistical analysis

All variables were analyzed by means of descriptive statistics (mean, median, standard deviation and standard error of mean) and the τ -test was used to compare the means of the continuous variables in the untreated and treated group. We tested the null hypothesis that 5-azadC and ALC treatments did not induce any increase of the H3–H4 acetylation or changes in K4 and K9 methylation in the three regions of the *FMR1* gene analyzed in the three cell lines. The F -test was employed to explain the total variation, evaluating the ratio between the values before (value = 1) and after treatments. If τ -test or F -ratio were near 1, the null hypothesis was valid; on the contrary, if τ -test or F -ratio were > 1 , the null hypothesis was rejected.

All tests were two-tailed, and the level of significance was 0.05. The collection and analysis of data were performed using the Statistical Package for Social Sciences Release 6.0 (SPSS; Chicago, IL, USA).

Results

Table 1 summarizes the results of ChIP experiments performed on two wild-type and three fragile X cell lines before and after 5-azadC treatment, while Figure 1 illustrates in graphical form only the results of one fragile X cell line (E6) as example. The data in Table 1 clearly demonstrate that histone acetylation and H3-K4 methylation levels in the promoter and exon 1 region were significantly higher in wild-type controls, while H3-K9

Figure 1 ChIP analysis after 1 μ M 5-azadC treatment for 7 days of histone H3 and H4 acetylation (a) and of histone H3-K4 and H3-K9 methylation (b) in the E6 fragile X cell line. Each box-plot depicts the amount (ng) of IP-DNA in three different regions of the *FMR1* gene, corresponding to the promoter, exon 1 and exon 16. The plus and minus signs beneath each box-plot indicate whether treatment had been administered (+) or not (–).

Table 2 Histone acetylation and H3-K4/H3-K9 methylation in wild type and fragile X cell lines before and after ALC treatment

	Wild-type 1	Wild-type 2	E3	E3+ALC	S1	S1+ALC	E6	E6+ALC
<i>Promoter</i>								
H3 acetylation	63.433 ± 45.254	81.345 ± 45.991	0.694 ± 1.154	1.297 ± 1.145	0.331 ± 0.257	2.681 ± 2.762	0.799 ± 0.333	1.981 ± 1.076
H4 acetylation	28.672 ± 11.011	32.508 ± 21.021	0.478 ± 0.102	1.605 ± 1.263	0.330 ± 0.386	1.193 ± 0.960	2.565 ± 1.038	3.999 ± 1.963
H3-K4 methylation	58.023 ± 23.347	38.210 ± 9.032	2.296 ± 1.488	2.777 ± 0.993	1.127 ± 0.413	1.196 ± 0.264	3.536 ± 0.647	5.863 ± 2.029
H3-K9 methylation	0.287 ± 0.069	0.903 ± 0.580	3.057 ± 0.912	2.117 ± 1.142	1.109 ± 0.308	1.649 ± 1.641	7.386 ± 0.822	2.360 ± 0.328
<i>Exon 1</i>								
H3 acetylation	469.079 ± 111.573	784.391 ± 513.503	10.876 ± 10.837	36.129 ± 33.782	1.258 ± 0.840	3.741 ± 1.991	4.604 ± 5.103	11.870 ± 6.816
H4 acetylation	321.215 ± 116.079	92.690 ± 73.999	1.555 ± 0.885	5.414 ± 6.930	0.997 ± 1.222	1.991 ± 1.705	17.042 ± 17.990	22.063 ± 30.514
H3-K4 methylation	93.379 ± 78.901	181.553 ± 145.491	3.005 ± 1.334	3.285 ± 0.997	1.521 ± 0.455	1.603 ± 0.897	8.237 ± 1.048	13.087 ± 0.458
H3-K9 methylation	1.273 ± 0.906	4.384 ± 4.263	2.938 ± 1.716	2.388 ± 1.800	4.921 ± 3.501	4.905 ± 3.008	40.268 ± 7.644	12.385 ± 1.797
<i>Exon 16</i>								
H3 acetylation	0.881 ± 1.023	0.848 ± 0.495	0.189 ± 0.127	0.687 ± 0.096	0.347 ± 0.235	1.024 ± 0.452	0.669 ± 0.353	1.163 ± 0.406
H4 acetylation	0.800 ± 0.502	0.543 ± 0.354	0.436 ± 0.110	1.399 ± 1.986	0.345 ± 0.356	0.526 ± 0.262	1.134 ± 0.749	1.413 ± 0.872
H3-K4 methylation	1.188 ± 0.792	1.226 ± 0.984	2.667 ± 0.928	1.327 ± 0.270	0.543 ± 0.294	0.630 ± 0.308	2.953 ± 0.116	3.306 ± 0.430
H3-K9 methylation	1.095 ± 0.269	1.766 ± 1.947	2.942 ± 0.747	4.261 ± 1.938	0.424 ± 0.181	0.119 ± 0.071	6.093 ± 1.145	3.578 ± 0.170

All values correspond to the mean amount of immunoprecipitated DNA (nanograms) ± standard deviation.

methylation was significantly higher in fragile X cell lines. Treatment with 5-azadC of the three fragile X cell lines induced an increase in histone acetylation. This increase was limited to the promoter and exon 1 regions, while no significant difference was observed in the region corresponding to exon 16 (Figure 1a). Likewise, H3-K4 methylation increased significantly in all three fragile X cell lines, again more pronouncedly around the promoter and exon 1 region, but not in exon 16 (Figure 1b). These epigenetic changes correlated with the transcriptional reactivation of the gene, which amounted to 8–15% of wild-type values, as measured by quantitative fluorescent RT-PCR, in all three fragile X cell lines (data not shown). In accordance with previous results,¹⁹ we observed a significant reduction in H3-K9 methylation levels in fragile X cell line E6 (Figure 1b). However, H3-K9 methylation did not change after 5-azadC treatment in fragile X cell lines S1 and E3 (Table 1).

The increased levels of H3/H4 acetylation after 5-azadC treatment, though significant, did not reach the corresponding levels of wild-type controls. In fact, histone acetylation in the promoter and exon 1 region of fragile X cell lines rose at most to 10% (H3) and 30% (H4) of wild-type levels after 5-azadC treatment (Table 1). Histone acetylation in exon 16 was extremely low in both wild-type and fragile X cell lines and was not affected by treatment. The 5-azadC treatment also induced a significant increase of H3-K4 methylation, approximately 10–50% of levels found in wild-type controls, for the promoter and exon 1 region, respectively. Once again, H3-K4 methylation in exon 16 was low and unchanged by treatment (Table 1).

ChIP results obtained before and after treatment with ALC are summarized in Table 2, while the experiments performed on the E6 cell line are illustrated in Figure 2. All three fragile X cell lines showed an increase, although modest, of H3 and H4 acetylation levels in the promoter and exon 1 region. Treatment with ALC did not modify histone acetylation status in exon 16, similar to what was observed after 5-azadC treatment (Figure 2a). As with 5-azadC treatment, the increased levels of histone acetylation induced by ALC remained lower than those of wild-type controls. However, in contrast to 5-azadC, treatment with ALC (10 mM for 7 days) did not induce reactivation of the *FMR1* gene, as mRNA levels were measured by quantitative fluorescence RT-PCR and remained undetectable in all three fragile X cell lines (data not shown). Failure of *FMR1* reactivation correlates with the extremely low levels of H3-K4 methylation, which did not increase at all after ALC treatment in any of the fragile X cell lines (Figure 2b). Interestingly, levels of H3-K9 methylation in the exon 1 region did decrease in E6 (Figure 2b) but not in the other two fragile X cell lines (E3 and S1). Table 3 summarizes the epigenetic changes that we observed in the promoter and exon 1 region of the *FMR1* gene in the tested cell lines before and after treatments.

Figure 2 ChIP analysis after 10 mM ALC treatment for 7 days of histone H3 and H4 acetylation (**a**) and of histone H3-K4 and H3-K9 methylation (**b**) in the E6 fragile X cell line. Each box-plot depicts the amount (ng) of IP-DNA in three different regions of the *FMR1* gene, corresponding to the promoter, exon 1 and exon 16. The plus and minus signs beneath each box-plot indicate whether treatment had been administered (+) or not (–).

Table 3 Summary of the observed epigenetic changes in a methylated full mutation before and after 5-azadC and ALC treatment, compared to a wild-type cell line

	Wild-type	Full	Full+5azadC	Full+ALC
DNA methylation	–	+	–	+ ^a
Histone H3 acetylation	+	–	+	+
Histone H4 acetylation	+	–	+	+
H3-K4 methylation	+	–	+	+/-
H3-K9 methylation	–	+	+/-	+/-
<i>FMR1</i> mRNA	+	–	+	–

^aData reported by Pascale *et al.*²⁴

Discussion

We have previously shown that transcription and translation of a methylated full mutation can be partially restored by treating fragile X cells with the DNA demethylating agent 5-azadC.^{9,10} We also showed that compounds known to increase histone acetylation can act synergistically with 5-azadC.¹⁵ To better understand the mechanisms of the *FMR1* gene reactivation, we undertook a systematic study of its epigenetic status. In particular, we tested acetylation and methylation of histones H3 and H4, in three different regions of the gene, that is, promoter, exon 1 and exon 16. As expected, we found that the levels of histone H3 and H4 acetylation are much higher in wild-type (*FMR1*-active) cells, as compared to fragile X (*FMR1*-inactive) cells. Similar results had been obtained by Coffee *et al.*,¹⁴ although these authors only investigated the epigenetic status of *FMR1* intron 1, approximately 200 bp downstream of exon 1

region. By analyzing multiple regions of the gene, we found that transcriptional reactivation of three fragile X cell lines with 5-azadC was paralleled by increased acetylation of both H3 and H4, exclusively in the promoter region and in exon 1. The exon 16 region remained substantially unmodified, possibly because its acetylation level is very low already before treatment, as in wild-type cells, indicating that the epigenetic modifications relevant to the transcription of the *FMR1* gene are concentrated in the 5' region. We did not observe a clear correlation between the extent of the tested epigenetic modifications and the size of the CGG expansion, among the three fragile X cell lines after 5-azadC treatment. In future studies, it might be interesting to test other tracts of the gene in order to characterize the extent of the active chromatin domain in the normal and reactivated *FMR1* gene.

Methylation of H3-K4, normally much lower in fragile X cells compared to wild type, increased consistently after 5-azadC treatment, in the promoter and exon 1 region. Again, the relationship with the levels of transcriptional reactivation was not linear, given that the maximum reactivation was observed in line E3, rather than in line E6, which had higher H3-K4 methylation levels.

H3-K9 residue was definitely more methylated in exon 1 of fragile X cell lines when compared to wild-type controls, representing a localized epigenetic mark, characteristic for heterochromatin.²² However, no significant reduction of H3-K9 methylation was induced by 5-azadC in the E3 and S1 fragile X cell line. However, a 40% decrease was observed in exon 1 of line E6. Our results suggest that increases in H3 and H4 acetylation and in H3-K4

methylation, in addition to DNA demethylation, are critical for the transition from a transcriptionally non-permissive to a transcriptionally permissive configuration of the *FMR1* gene chromatin, as depicted schematically in Figure 3. The model is only an approximation and does not explain why the transcriptional reactivation is never complete. This could be due to incomplete demethylation of the CGG tract,³ or else to the fact that only a proportion of cells respond to the reactivating treatment, as previously discussed.¹⁰ On the other hand, variations in the level of H3-K9 methylation seem to be less critical with respect to the transition from heterochromatin to euchromatin.

To further dissect the role of DNA and histone methylation from that of histone acetylation, we studied the effect of ALC, a compound that should increase histone acetylation. We had previously shown that ALC inhibits the cytogenetic expression of the fragile site FRAXA.²⁰ It also proved effective in ameliorating the hyperactive behavior, when administered to children with the fragile X syndrome.²³ However, Pascale *et al*²⁴ showed that long-term ALC treatment did not cause DNA demethylation of the mutant *FMR1* promoter, nor reactivation of the gene. We now show that treatment with ALC of three fragile X cell lines results in significant acetylation of *FMR1*-associated H3 and H4 histones, especially in the promoter and exon 1 region. Interestingly, H3-K9 methylation was also reduced by ALC in the promoter and exon 1 region of the E6 cell line, a finding correlating with the increased acetylation of histone H3. In fact, H3-K9 can undergo only one of two possible epigenetic modifications, either acetylation or methylation. However, all these changes were not sufficient to restore transcription and, in accordance with the lack of *FMR1* reactivation, ALC treatment did not induce a significant increase in the level of H3-K4 methylation.

These results confirm our previous assumption¹⁵ that DNA methylation, which persists after ALC treatment,²⁴ rather than histone acetylation, is the dominant epigenetic factor in the regulation of transcription of the *FMR1* gene.

In conclusion, it appears that the epigenetic status of a methylated full mutation tends to be changed into that of a wild-type by treatment with 5-azadC, while treatment with ALC, which does not cause *FMR1* reactivation, affects significantly histone acetylation, but not histone and DNA methylation. Only H3-K4 methylation, particularly around the promoter and exon 1 region, correlates with DNA hypomethylation and gene transcription. In fact, *FMR1* is actively transcribed in the full mutation cell lines treated with 5-azadC, that have a methylated H3-K4.

It has been suggested that methylated H3-K9 recruits histone methyltransferases (HMTs) associated with HDACs via the heterochromatic protein 1-HP1,^{25,26} resulting in histone deacetylation. However, our experiments indicate that H3-K9 methylation and partial histone deacetylation can coexist with H3-K4 methylation and active *FMR1* transcription. We reached the same conclusion also by characterizing an exceptional cell line that harbors an unmethylated full mutation. This cell line is transcriptionally active and has high H3-K4 methylation levels, in spite of low histone acetylation and high H3-K9 methylation.²⁷

Understanding the molecular mechanisms by which the *FMR1* gene is transcriptionally regulated will have significant bearing on the ultimate goal of finding an effective cure for the fragile X syndrome.

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Figure 3 A simplified model of chromatin remodeling after 5-azadC and ALC treatment of a methylated full mutation. MBD: methyl-DNA binding proteins; HD: histone deacetylases; Ac: acetyl groups on histone H3 and H4 tails.

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