Relationship between DNA methylation, histone H4 acetylation and gene expression in the mouse imprinted *Igf2-H19* domain

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Abstract DNA methylation and histone H4 acetylation play a role in gene regulation by modulating the structure of the chromatin. Recently, these two epigenetic modifications have dynamically and physically been linked. Evidence suggests that both modifications are involved in regulating imprinted genes – a subset of genes whose expression depends on their parental origin. Using immunoprecipitation assays, we investigate the relationship between DNA methylation, histone H4 acetylation and gene expression in the well-characterised imprinted *Igf2-H19* domain on mouse chromosome 7. A systematic regional analysis of the acetylation status of the domain shows that parental-specific differences in acetylation of the core histone H4 are present in the promoter regions of both *Igf2* and *H19* genes, with the expressed alleles being more acetylated than the silent alleles. A correlation between DNA methylation, histone hypoacetylation and gene repression is evident only at the promoter region of the *H19* gene. Treatment with trichostatin A, a specific inhibitor of histone deacetylase, reduces the expression of the active maternal *H19* allele and this can be correlated with regional changes in acetylation within the upstream regulatory domain. The data suggest that histone H4 acetylation and DNA methylation have distinct functions on the maternal and paternal *Igf2-H19* domains.

Key words: Imprinting; Histone acetylation; DNA methylation; Chromatin; *Igf2*/*H19*

1. Introduction

DNA methylation and histone acetylation alter the chromatin structure to repress or activate transcription. Recent studies have suggested that DNA methylation and histone deacetylation may operate along a common mechanistic pathway to repress transcription[1–3]. In mammals, these two epigenetic modifications appear to play a role in genomic imprinting, a mechanism of gene regulation whereby the two alleles of a gene are expressed differentially according to their parental origin [4–6].

Igf2 and *H19* are closely adjacent, reciprocally imprinted genes, localised on human chromosome 11 and mouse chromosome 7. *Igf2* encodes a foetal growth factor and is normally expressed from the paternal allele, while *H19* transcription is exclusively from the maternal allele, producing a non-coding RNA of unknown function. The strongest evidence for the involvement of CpG methylation in *Igf2* and *H19* regulation comes from studies in mice deficient for a DNA methyltransferase gene, *Dnmt-1*. Indeed, these mutant embryos could not express *Igf2* but expressed *H19* from both parental alleles [7]. In addition, sites of regional and parental-specific methylation have been mapped [6] (Fig. 1), with the paternal *H19* allele being heavily methylated compared to the active maternal *H19* allele. The involvement of chromatin structure is evident from the identification of allele-specific differences in nuclease accessibility [8,9] (Fig. 1) and replication timing in imprinted domains [10,11]. Recent work indicates that histone acetylation, one of the epigenetic modifications able to affect the chromatin structure, is involved in *Igf2* and *H19* regulation. Parental-specific acetylation has been identified in the coding region of the *H19* gene and treatment with specific inhibitor histone deacetylase induced changes in the expression of *Igf2* and *H19* [12–14]. However, the precise epigenetic changes responsible for this were not determined.

Molecular and genetic analyses have shown that two regions play key roles in allele-specific expression of *Igf2* and *H19*: a differentially methylated region (DMR) upstream of *H19* [15–17] and a set of tissues-specific enhancers downstream of *H19* [18,19]. The enhancers specifically contribute to the activation of *Igf2* on the paternal chromosome and to the activation of *H19* on the maternal chromosome. The interactions between the enhancers and the genes are regulated by the DMR. When this region is methylated, the *H19* gene is inactivated. Reciprocally, when this region is unmethylated, the *Igf2* gene is insulated from the enhancers [4–6]. Recently, Felsenfeld and Tilghman have proposed a model in which the unmethylated status of DMR allows the binding of the enhancer-blocking activity protein CTCF [20–22]. The presence of this protein may insulate the *Igf2* gene from the enhancer. Importantly, this factor has been shown to be associated with a histone deacetylase activity[23]. Hence, this domain provides a unique model to study the roles of DNA methylation and histone acetylation in the regulation of gene expression.

To gain further understanding of the relationship between DNA methylation, histone acetylation and *Igf2-H19* gene expression, we used a chromatin immunoprecipitation (CHIP) assay to assess the acetylation status associated with normal and perturbed imprinted activity [24]. By using primary embryonic fibroblasts derived from normal embryos and embryos containing maternal duplication/paternal deficiency of the imprinted region on distal chromosome 7 (MatDi7), we evaluated the levels of H4 acetylation across this domain on both paternal and maternal alleles. These levels were corre-

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lated with the previously reported methylation status of the corresponding regions. In addition, to assess the functional role of histone H4 acetylation in the *Igf2* and *H19* imprinting, we analysed the effects of an inhibitor of histone deacetylase both on *Igf2* and *H19* expression and on the levels of histone H4 acetylation in this domain. Our study reveals that sites of allele-specific acetylation are sensitive to trichostatin A (TSA) treatment and may be involved in the regulation of *Igf2* and *H19* at least on the maternal chromosome.

2. Materials and methods

2.1. Cell culture and drug treatment

Cultures of cells derived from normal and MatDi7 embryos have been generated as described previously [8,25]. Cells were plated in 150 mm dishes at 2×10^6 cells per plate and cultured in DMEM (Gibco/BRL) containing 10% (v/v) heat-inactivated foetal calf serum. After 24 h, 100 ng/ml TSA (Sigma) was added to exponentially growing cells. Cells were harvested for CHIP assay or for isolation of nucleic acids after 3 h.

2.2. Antibodies

The preparation and characterisation of polyclonal antisera against acetylated H4 have been described previously [26]. For the experiments described in this paper, antisera R232/8 (H4Ac8), R252/16 (H4Ac16), R101/12 (H4Ac12) and R41/5 (H4Ac5) were used [24].

2.3. CHIP

Immunoprecipitations were performed using affinity-purified antisera exactly as described previously [27,28]. DNA was isolated from antibody-bound (i.e. acetylated) and unbound (i.e. non-acetylated) chromatin fractions and equal amounts (based on [^{3}H]thymidine counts) applied to nylon filters by slot-blotting. DNA was hybridised with ^{32}P-labelled probes corresponding to key areas in the imprinted domain. Quantitation was performed using a phosphorimager (Molecular Dynamics) as described previously [27,28].

2.4. Probes

Probes used to scan the *Igf2-H19* region were as follows (Fig. 1): (1) subclone of 5 kb *Bam*HI fragment 3 kb upstream of *Igf2*; (2) 1 kb *Hin*cII exon 2 fragment; (3) 0.9 kb *Kpn–Bam*HI fragment which covers a part of *Igf2*-DMR2 region (intron 5–exon 6) [29]; (4) 0.9 kb intergenic *Eco*RI/*Pst*I subclone of A4 [30]; (5) 527 bp PCR fragment encompassing HS1 [9]; (6) 940 bp PCR fragment which covers the silencer element; (7) 544 bp PCR fragment which covers the G-repeat; (8) 0.8 kb fragment of which the 3′ end is an *Eco*RI site at the *H19* transcription start site; (9) 1 kb *Xba*I fragment containing the first downstream enhancer.

3. Results

3.1. Histone acetylation level is very variable in the Igf2-H19 domain

To evaluate the levels of histone H4 acetylation across the *Igf2-H19* regions, CHIP assays were performed with a panel of polyclonal antisera generated against the four different acetylated lysine residues of histone H4, H4Ac8, H4Ac16, H4Ac12 and H4Ac5 [24]. Chromatin was prepared by nuclease digestion from normal embryonic fibroblasts. Nine regions were analysed (Fig. 1A). Regions 1, 3, 5, 6 and 8 have previously been shown to be differentially methylated [8,31–33] and regions 1–6 and 9 contain DNase I hypersensitive sites [30]. Results for the nine regions tested and the two controls, β-actin and α-heterochromatin, are shown in Table 1. Levels of acetylation are presented as the ratio of probes hybridised to the bound (B, acetylated) and unbound (UB, non-acety-

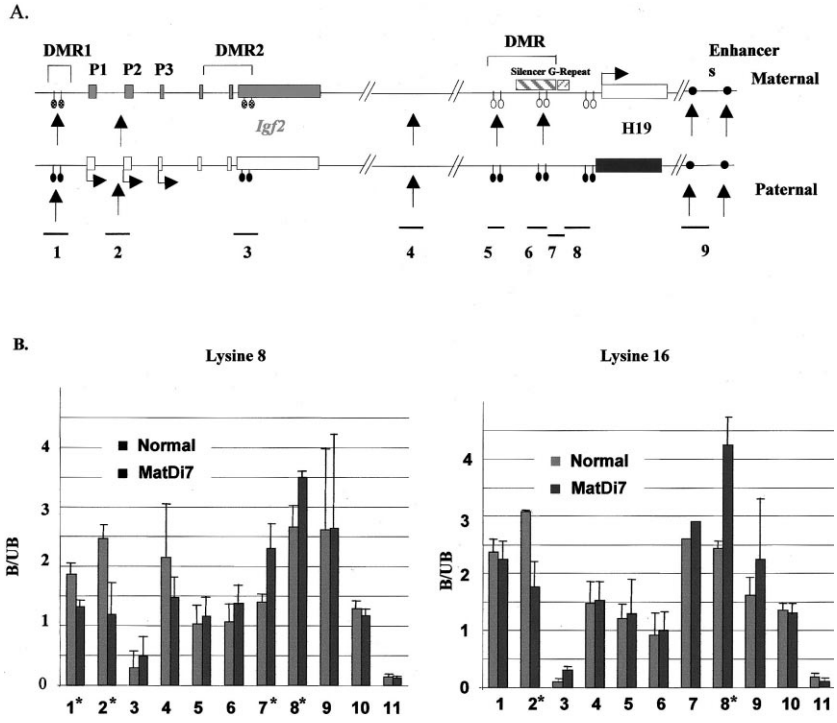

Fig. 1. A: Schematic of the 100 kb region encompassing *Igf2* and *H19* analysed in this study, including previously determined areas of DNA methylation and nuclease hypersensitivity on the two parental chromosomes [8,9,30]. Vertical arrows indicate sites or regions of nuclease hypersensitivity. Horizontal arrows show transcriptional initiation sites. □ Active; ■ inactive. Probes used are indicated by horizontal lines and numbered. B: Levels of H4 acetylation at lysines 8 and 16 across the *Igf2/H19* domain in embryonic fibroblasts from normal mice and mice with two copies of MatDi7. Acetylation levels are expressed as the ratio of hybridisation to DNA from the antibody-bound (B, acetylated) and unbound (UB, non-acetylated) fractions for each of the probes indicated in A. Values shown are the average of several independent experiments and error bars are ±S.E.M. * indicates statistically significant values.

Table 1
Acetylation ratios for regions in the *Igf2-H19* imprinted domain in normal embryonic fibroblasts

H4AcLys	*Igf2*-DMR1	*Igf2*-P2	*Igf2*-DMR2	Intergenic	*H19*-DMR-HS1	*H19*-silencer HS2	*H19*-G-repeat	*H19* promoter	Downstream enhancers	β-Actin	Heterochromatin
Lys8	1.86 (±0.19)	2.46 (±0.23)	0.3 (±0.1)	2.15 (±0.9)	1.03 (±0.31)	1.07 (±0.2)	1.4 (±0.14)	2.67 (±0.43)	2.62 (±0.98)	1.3 (±0.1)	0.15 (±0.05)
Lys12	1.27 (±0.1)	1.29 (±0.70)	ND	1.36 (±0.04)	1.09 (±0.01)	ND	ND	3.66 (±1.35)	1.01 (±0.01)	1.3 (±0.1)	0.15 (±0.03)
Lys16	2.55 (±0.05)	3.09 (±0.01)	0.1 (±0.1)	1.47 (±0.38)	1.2 (±0.26)	1.07 (±0.1)	2.6 (±0.1)	2.43 (±0.1)	1.62 (±0.14)	1.25 (±0.1)	0.175 (±0.05)

Numbers shown are average ratios of acetylated/unacetylated chromatin (±S.D.) for 2–4 independent CHIPs.

lated) fractions measured by slot-blotting followed by quantitative phosphorimaging. The levels of acetylation at β-actin (B/UB ratio = 1.25) and centric heterochromatin (B/UB ratio = 0.2) for the three antibodies included in Table 1 are as anticipated from previous results [27]. Antibodies to H4Ac5, the site that is acetylated least frequently in mature chromatin, precipitated too little chromatin to allow complete analysis, though the results that were obtained (not shown) were consistent with those presented in Table 1.

Levels of H4 acetylation in the imprinted domain from normal (N) cells reveal regional differences along the locus. The *Igf2* locus is characterised by a very high level of acetylation at the P2 promoter region (probe 2). The upstream region (probe 1) exhibits a intermediate level and the downstream DMR exhibits a very low acetylation level comparable to that observed for heterochromatic regions. The intergenic strongly hypersensitive regions (probe 4) [9,30] show an intermediate level. At the *H19* locus, we observed increased acetylation at the G-repeat region (probe 7) and even higher levels at the promoter region (probe 8). In general, the same overall pattern of acetylation was seen for all four H4 lysines, though acetylation of lysine 12 was markedly lower than that of 8 and 16 on the *Igf2* promoter and higher on the *H19* promoter (Table 1). It is interesting to note that the *Igf2* and *H19* promoters (probes 2 and 8) exhibit higher levels of acetylation than have previously been shown for other pol II transcribed genes [34]. These results suggest that regional differences are correlated with areas of known regulatory function and implicates histone acetylation in regional control.

Fig. 2. Effect of TSA treatment on *Igf2* and *H19* expression. A: Northern blots identifying *Igf2*, *H19* and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, control) mRNA extracted from normal (N) and MatDi7 embryonic fibroblasts cultivated in presence or absence of TSA (100 ng/ml). B: Histogram showing the *Igf2* and *H19* expression after TSA treatment determined by phosphorimager analysis of Northern blots. * indicates statistically significant values.

3.2. Presence of parental origin-specific differentially acetylated H4 at the promoter regions of the imprinted genes, Igf2 and H19

To evaluate the level of acetylation on each parental allele, we compared the level of histone H4 acetylation in primary embryonic fibroblasts derived from normal embryos with those from embryos containing maternal duplication/paternal deficiency of the imprinted region on distal chromosome 7, where *Igf2* and *H19* reside (Fig. 1). For all three antibodies, some areas of known regulatory function (probes 3, 5, 6 and 9) do not show statistically significant parental origin-specific differences (Fig. 1B).

Interestingly, levels of H4 acetylation at both lysines 8 and 16 on the *Igf2* promoter (probe 2) were 1.6–2.0-fold higher in normal than in MatDi7 cells, while at the *H19* promoter (probe 5), the MatDi7 cells were 1.3–1.7-fold higher than normal (Table 1). These differences are statistically significant. Thus, the two imprinted promoters show consistent parental origin-specific acetylation differences, with the active promoters, paternal *Igf2* and maternal *H19*, exhibiting hyperacetylation. Consistent differences of this magnitude were not seen in any of the other regions tested.

3.3. Effect of TSA treatment on Igf2 and H19 expression and on histone H4 acetylation levels across the H19-Igf2 domain

To investigate the role of histone acetylation in the regulation of these two imprinted genes, normal fibroblasts were treated for 3 h with an inhibitor of histone deacetylase, TSA. This treatment allows an overall increase in histone H4 acetylation in the absence of generalised toxic effects [35,36]. After 3 h TSA treatment, we did not observe any *Igf2* expression in the MatDi7 cells, suggesting that this treatment is not sufficient to induce reactivation of the silent maternal *Igf2* allele (Fig. 2). Strikingly, the H4 acetylation at the *Igf2* promoter region increased significantly at both lysine 8 and lysine 16 (Fig. 3). This increase can be attributed to the maternal allele since the same effect was observed in the MatDi7 cells which contain two maternal copies of the distal part of chromosome 7 (data not shown).

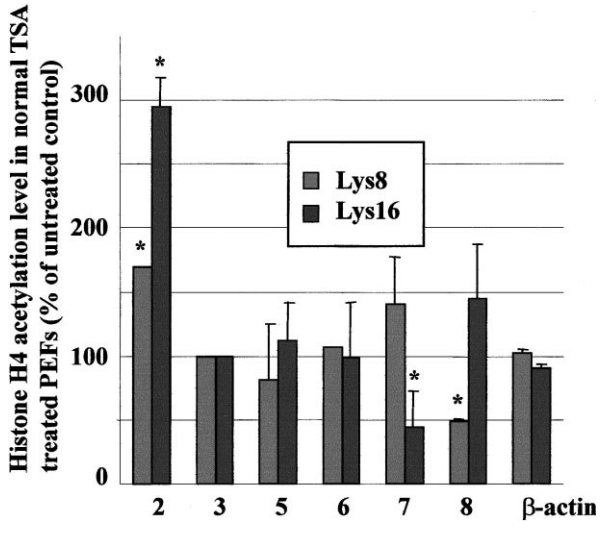

Fig. 3. Effect of TSA on histone H4 acetylation across the *Igf2-H19* domain. Histogram showing H4 acetylation levels at lysines 16 and 8 expressed as a ratio of values obtained with chromatin from normal untreated cells or cells grown for 3 h in the presence of TSA. * indicates statistically significant values.

Strikingly and in contrast to what it is generally expected for a specific histone deacetylase inhibitor, *H19* RNA levels decrease dramatically after TSA treatment. This *H19* down-regulation after TSA treatment was noted previously in a different experimental system [37]. This effect can be attributed to the expressed maternal allele since this down-regulation is also observed in the MatDi7 cells (Fig. 2). However, we also analysed the regional acetylation associated with this change in expression. Although no change is evident at regions 5 and 6 (which did not exhibit any regional allele-specific differences), we observe reproducible reciprocal changes in the upstream region (Fig. 3). Specifically, significantly decreased acetylation is observed on the *H19* promoter (probe 8) at lysine 8 and in the G-repeat region (probe 7) at the lysine 16. This result is consistent with the decrease in expression of *H19*.

4. Discussion

Our systematic regional analysis of the acetylation status across the *Igf2-H19* domain shows that parental origin-specific acetylation exists at the promoters of the two imprinted genes. This finding is inconsistent with data from Pedone et al., who, using CHIP followed by allele-specific PCR, were unable to detect allele-specific acetylation differences at *Igf2* [13]. This difference might be explained by the use of a different antibody (against tetra-acetylated H4) in their analysis. Our results indicate that allele-specific hyperacetylation is highly localised and is concordant with an unmethylated allele [38].

At the *H19* locus, regional acetylation levels are increased along a domain extending from the G-repeat region to the promoter. This whole region is highly methylated on the silent allele and unmethylated on the active allele. However, the DMR region, believed to be important in the regulation of *Igf2-H19* imprinting, is not differentially acetylated. Only the promoter is significantly differentially acetylated. These findings indicate that differential H4 acetylation is not always associated with differential methylation. Taken together, the data for *Igf2* and *H19* show that allele-specific acetylation differences can occur in the presence or absence of differential methylation. Regardless, allelic activity for both genes is associated with promoter hyperacetylation and hypomethylation. Furthermore the differences in acetylation levels between the inactive *Igf2* promoter and the inactive *H19* promoter suggest that DNA methylation and hypoacetylation may be mechanistically linked but such linkage is not required for gene silencing.

We show that TSA treatment perturbs acetylation levels in the differentially acetylated domains. Other regions are not significantly affected. The acetylation at the *P2-Igf2* promoter region on the maternal chromosome increased 2-fold after treatment. This was not associated with immediate reactivation of the maternal allele, although others have shown that after 24 h, reactivation can occur [13]. Unexpectedly and in contrast to the *Igf2* gene, *H19* expression was halved after TSA treatment. At the *H19* promoter, this was associated with a decrease in the level of Lys8 acetylation and at the G-repeat a reduction in Lys16 acetylation. Acetylation levels at other residues tested were not significantly changed. This

relationship between these two regulatory domains at *H19* suggests that lysine residues respond differently to TSA treatment, a finding also reported by Chen and Townes [35]. From this result, it is not clear whether a decrease in promoter Lys8 acetylation or a decrease in G-repeat Lys16 acetylation is causally related to the reduction in expression of *H19*. Neither change is associated with a change in the methylation state of the region (data not shown) indicating that acetylation differences alone can affect transcriptional levels at *H19*.

The upstream region of *H19* is an important regulatory region for controlling imprinting at the *Igf2-H19* locus. Maternally inherited deletions in this region cause reactivation of the silent *Igf2* allele, and a reduction in *H19* activity similar to those we report here. This region has been shown to contain an insulator element which, when unmethylated on the maternal chromosome, can bind the CTCF protein which promotes enhancer function at the *H19* promoter. On the methylated paternal allele, the CTCF factor does not bind and the enhancers are no longer insulated from the *Igf2* promoters, hence *Igf2* activity is facilitated on the paternal allele [20–22,39]. Interestingly, CTCF is associated with a deacetylase activity that is inhibited in the presence of TSA [23]. Based on these findings, it is possible that TSA affects the function of CTCF on the maternal allele and this is predicted to have a reciprocal relationship on the expression of *Igf2* and *H19*. The data are consistent with this; *H19* becoming down-regulated (this report) and *Igf2* up-regulated [12,13]. This finding is also independent of methylation changes that have been suggested to play a more important role in the regulation of *Igf2* activity and *H19* repression on the paternal allele. Hence the situation on the paternal allele is different. This is consistent with other data showing that TSA treatment is not sufficient to affect the activity of the *H19* and *Igf2* paternal alleles. Thus, although DNA methylation and histone acetylation both play a role in *Igf2-H19* imprinting, they most likely have distinct functions on the maternal and paternal chromosomes [13,40].

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