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

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Received 19 September 2000; revised 24 November 2000; accepted 28 November 2000
First published online 20 December 2000

Edited by Takashi Gojobori

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, Dnmt1. 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 imprinting 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-
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 2×10⁶ 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 [3H]thymidine counts) applied to nylon filters by slot-blotting. DNA was hybridised with ³²P-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 BamHI fragment 3 kb upstream of Igf2; (2) 1 kb HincII exon 2 fragment; (3) 0.9 kb Kan–BamHI fragment which covers a part of Igf2-DMR2 region (intron 5–exon 6) [29]; (4) 0.9 kb intergenic EcoRI/PstI subclone of A4 [30]; (5) 527 bp PCR fragment encompassing HSI [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 EcoRI site at the H19 transcription start site; (9) 1 kb XbaI 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-acetyl-
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.

<table>
<thead>
<tr>
<th>Lys</th>
<th>Igf2-P2</th>
<th>Igf2-DMR2</th>
<th>Igf2-DMR2-H19</th>
<th>H19-DMR</th>
<th>H19-silencer H19</th>
<th>H19-G-repeat</th>
<th>H19-promoter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys8</td>
<td>1.86 (±0.19)</td>
<td>2.46 (±0.23)</td>
<td>0.3 (±0.1)</td>
<td>2.15 (±0.69)</td>
<td>1.07 (±0.02)</td>
<td>1.05 (±0.01)</td>
<td>1.01 (±0.01)</td>
</tr>
<tr>
<td>Lys32</td>
<td>1.27 (±0.00)</td>
<td>2.53 (±0.0)</td>
<td>ND</td>
<td>1.1 (±0.01)</td>
<td>1.07 (±0.0)</td>
<td>1.15 (±0.0)</td>
<td>3.66 (±0.35)</td>
</tr>
<tr>
<td>Lys36</td>
<td>2.94 (±0.00)</td>
<td>2.69 (±0.00)</td>
<td>ND</td>
<td>1.14 (±0.0)</td>
<td>1.07 (±0.01)</td>
<td>1.05 (±0.01)</td>
<td>2.40 (±0.0)</td>
</tr>
</tbody>
</table>

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

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![Fig. 2. Effect of TSA treatment on Igf2 and H19 expression.](image-url)
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 acetylation across the H19-Igf2 domain. Histogram showing H4 acetylation levels at lysines 16 and 18 expressed as a ratio of values obtained with chromatin from normal untreated cells or cells grown for 3 h in the presence of TSA.

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.

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 localized 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 hypomethylation 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 chromosmes [13,40].

Acknowledgements: We are grateful to Adele Murrell for the DMR2 probe and to Fay Shamanski, Jayne Lavender and Tamzin Randall for assistance during the course of this analysis. This work was supported by the BBSRC, the CRC and the Isaac Newton Trust. V.G. was an EMBO Research Fellow.

References