CpG methylation regulates the *Igf2/H19* insulator

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The differentially methylated 5′-flank of the mouse *H19* gene unidirectionally regulates the communication between enhancer elements and gene promoters and presumably represses maternal *Igf2* expression in vivo [1–6]. The specific activation of the paternally inherited *Igf2* allele has been proposed to involve methylation-mediated inactivation of the *H19* insulator function during male germline development [1–4, 6]. Here, we addressed the role of methylation by inserting a methylated fragment of the *H19*-imprinting control region (ICR) into a nonmethylated episomal *H19* minigene construct, followed by the transfection of ligation mixture into Hep3B cells. Individual clones were expanded and analyzed for genotype, methylation status, chromatin conformation, and insulator function. The results show that the methylated status of the *H19* ICR could be propagated for several passages without spreading into the episomal vector. Moreover, the nuclease hypersensitive sites, which are typical for the maternally inherited *H19* ICR allele [1], were absent on the methylated ICR, underscoring the suggestion that the methylation mark dictates parent of origin-specific chromatin conformations [1] that involve CTCF [2]. Finally, the insulator function was strongly attenuated in stably maintained episomes. Collectively, these results provide the first experimental support that the *H19* insulator function is regulated by CpG methylation.

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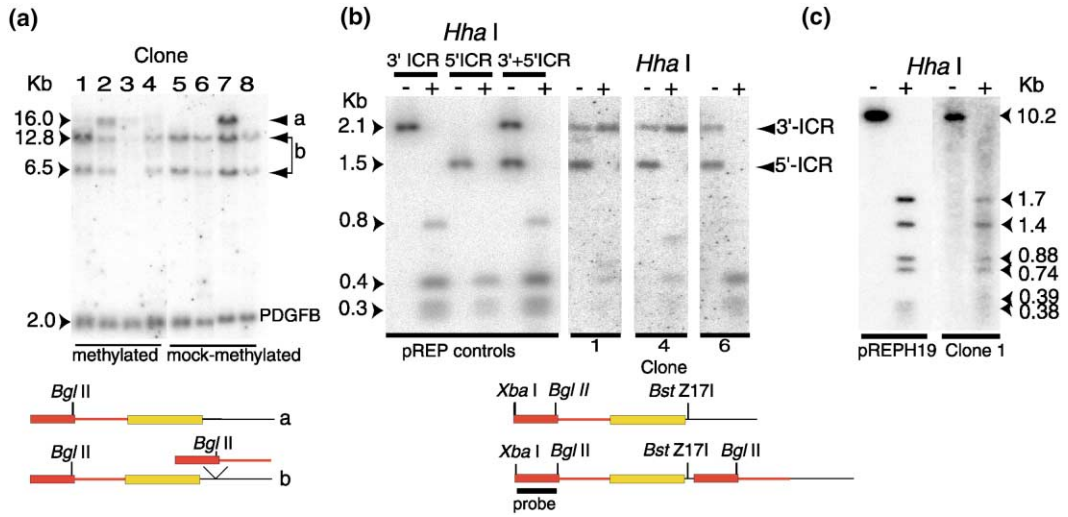
Results and discussion

The insulator assay used in this report is based on the episomal propagation of the *H19* ICR (which results in a proper chromatin conformation) and the specific positioning of the insulator segment between a strong enhancer and reporter gene [1, 2]. To test the effect of DNA methylation on the insulating properties of the *H19* ICR, an in vitro-methylated (by *Sss I* methyltransferase) [7] or mock-methylated ICR segment was positioned between the reporter gene (in this instance, the mouse *H19* gene) and the SV40 enhancer in the episomal pREPH19 vector, as has been described [1, 2]. This construct retained the position of a second *H19* ICR in its natural 5′-position (see Figure 1). The 5′-ICR was not deleted, as it provided an internal control with respect to methylation analyses (see below). The construction of these vectors involved a series of complicated steps, including the ligation of an in vitro-methylated insulator segment into a nonmethylated episome. The screening for properly ligated constructs was carried out by transfection of the crude ligation mixture into Hep3B cells. These cells display a negligable de novo methylation preference for the ICR and the rest of the episome during the course of the experiments. Moreover, they maintain the *H19* insulator function in stably transfected cells with high fidelity [1].

Following hygromycin selection for 3–4 weeks, a total of 23 out of 50 individual cell clones could be expanded. The first round of genotyping revealed that the episomal vector had integrated into the genome in 9 clones (data not shown), as determined by Southern blot hybridization analyses of total cellular DNA. The high proportion of stably integrated DNA is presumably due to an excess amount of open-ended plasmid fragments that did not ligate in vitro prior to transfection. Since the episome approach avoids position-dependent chromatin effects, which would be inherent to stably integrated plasmids, only cell clones that showed identical band patterns to the parent episomal vector were processed further. Of the remaining 14 clones that were episomally maintained, 7 clones displayed a rearranged plasmid, and 4 clones contained a mixed population of plasmids (with and without the insert) (Figure 1a and data not shown). The remaining 3 clones (2 from methylated, and one from mock-methylated ligation mixtures) were judged to be correct, as determined by multiple Southern analyses using the whole plasmid as a probe.

The three cell clones with the correct plasmid genotype, i.e., episomes with an ICR fragment inserted in the

Figure 1



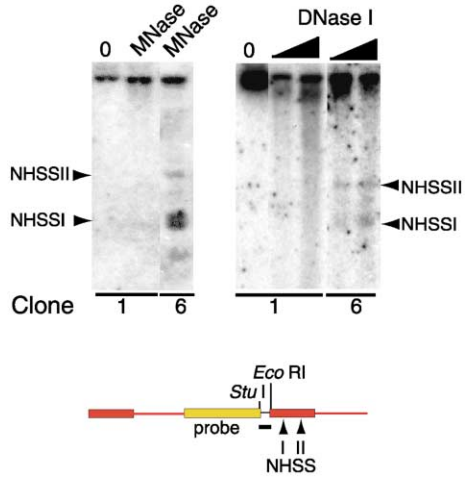
The isolation and identification of Hep3B cell clones maintaining a preimposed methylation pattern in the episomally maintained ICR. **(a)** A subset of Hep3B clones demonstrating the genotyping assay, using BglII. Note that some clones have episomal vectors both with and without the *H19* ICR in the 3′-position. The entire pREPH19 vector was used as a probe. The visualization of the *PDGF-B* fragment [1, 2] allowed an estimation of copy numbers of episomes. **(b)** Methylation analysis of the Hep3B clones. Extracted DNA was digested with XbaI, BglII, and BstZ171, to discriminate between 5′- and 3′-ICRs, and HhaI, to assess the methylation status. The control episomes had the 5′-ICR and 3′-ICR specifically deleted. **(c)** The absence of methylation spreading of the entire plasmid of clone 1. Extracted DNA was digested with XbaI, BglII, BstZ171, and HhaI to assess the methylation status. The resulting Southern blot was hybridized to a radioactively labeled probe that was derived from the entire pREP4 (Invitrogen) plasmid.

3′-position (Figure 1b) from both the methylated and mock-methylated group, were subjected to methylation analysis using methylation-sensitive restriction enzymes. The 5′-ICR, which was part of the *H19* minigene [1, 2] and could be discriminated from the 3′-ICR by using restriction enzymes, served as an internal control. Figure 1c shows that the in vitro methylation of the 3′-ICR could be propagated in Hep3B cells without spreading into neighboring plasmid sequences in clones 1 and 4 (data not shown). We observed, however, that the 5′-ICR appeared to be moderately methylated in clones 1 and 4 (Figure 1b). Although this suggested some degree of methylation spreading from the 3′-ICR to the 5′-ICR during the process of clone propagation, the overall difference in the methylation status enabled us to examine the effects of preimposed local methylation patterns on both chromatin structure and insulator function.

In addition to others, we have shown earlier that the target sites for the CTCF protein within the *H19* ICR are essential for the insulator function [1–4, 6]. The CTCF-*H19* ICR interaction is methylation-sensitive and generates diagnostic nuclease hypersensitive sites (NHSSs) [1, 2], which are abrogated in point-mutated CTCF target sites ([1], M. Kanduri et al., submitted). Consequently, we examined the chromatin conformation in cells maintaining the in vitro-generated methylation pattern of the *H19* ICR. Figure 2 shows that limited MNase and DNase I digestion of nuclei extracted from clone 1 (with the methylated *H19* ICR in the 3′-position) does not generate any detectable NHSSs, in contrast to clone 6 (with the unmethylated *H19* ICR of the 3′-position). This data shows that in vitro-generated methylation patterns in the 3′-ICR attenuate protein-DNA interactions within the CTCF binding sites in transfected cells, which is reminiscent of the chromatin conformation of the paternal ICR allele [1, 2].

To examine the effects of the methylation status of the inserted *H19* ICR on the enhancer-promoter communication (i.e., the activity of the mouse *H19* reporter gene), we performed RNase protection analysis of RNA extracted from each cell clone. The expression levels were normalized against copy numbers of the episomes, as has been accounted for earlier [1, 2]. Figure 3 shows that the reporter gene activity is 20- to 30-fold higher in clones 1 and 4 than in control cells with an unmethylated 3′-ICR, thereby linking methylation of the 3′-ICR with reduced or complete absence of insulator function. Figure 3 also shows that the unmethylated (or mock-methylated) *H19* ICR efficiently blocks the enhancer-promoter communication in the 3′-position, independent of the presence or absence of the 5′-ICR, ruling out any functionally important interaction between the unmethylated 5′- and 3′-ICRs that might complicate interpretations. Likewise, although the methylated 3′-ICR was only tested in the presence of a 5′-ICR, there is no precedent for a 5′-ICR to influence the activity of the 3′-ICR. It is possible, however, that

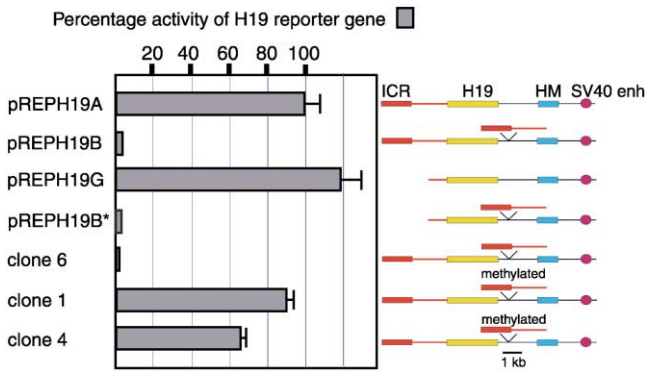
Figure 2



The methylated state of the *H19* ICR mimics parent of origin-dependent chromatin conformations. MNase and DNase I digestion of isolated nuclei was performed as has been described [1, 2]. The major nuclease hypersensitive sites (NHSSs) within the *H19* ICR are indicated in the figure. The extracted DNA was restricted by StuI; the 5′-site is positioned in the 3′-end of the murine *H19* reporter gene, whereas the downstream StuI site resides in the *EBNA* gene. This strategy specifically allowed visualization of only the 3′-ICR in the left-hand panel of the DNase 1 hypersensitivity analysis.

the varying degree of methylation spreading is correlated with different degrees of reporter gene activity in clones 1 and 4. We cannot exclude the possibility, therefore, that the methylation spreading inactivated the promoter function in a subpopulation of clone 4 cells.

Figure 3



CpG methylation regulates the *H19* insulator function. RNase protection analysis of *H19* reporter gene expression [1, 2]. Controls represent pooled, hygromycin-selected cells, which stably propagated the episomal vectors indicated in the figure. Reporter gene expression was normalized to the copy numbers of the episomes of the transfected cells. The mean deviation of a minimum of three different experiments is indicated for each vector construct, unless the differences were too small to allow visualization.

Our data documents that the methylation mark of the *H19* ICR functions as a switch to regulate the chromatin insulator function of this domain and, by extrapolation, that it controls the manifestation of the parent of origin-dependent expression of the mouse *Igf2* gene. It should be pointed out that this interpretation only applies for enhancers that are positioned downstream of the *H19* gene [8, 9]. This report also provides a plausible explanation as to why biallelic methylation of the *H19* ICR in human cancers may lead to a derepressed maternal *IGF2* allele, i.e., loss of imprinting [10]. Finally, the results open up the possibility that other chromatin insulators can be epigenetically regulated, thereby adding another layer of complexity to the organization of expression domains. For example, several known CTCF target sites behave as chromatin insulators in experimental assays, and some of these are regulated by CpG methylation (unpublished data). The functional link between the chromatin insulator protein CTCF with the methylation-sensitive enhancer-blocking function of the *H19* ICR, therefore, provides an opening to examine epigenetically regulated chromatin insulators on a genome-wide level.

Supplementary material

Supplementary material showing methodological details is available at http://images.cellpress.com/supmat/supmatin.htm.

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