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*H19*ICR mediated transcriptional silencing does not require target promoter methylation

Claudia Gebert [1], Qi Rong [1], Sangkyun Jeong [2], James Iben, Karl Pfeifer*

Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA

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ABSTRACT

Transcription of the reciprocally imprinted genes *Insulin-like growth factor 2* (*Igf2*) and *H19* is orchestrated by the 2.4-kb *H19* Imprinting Control Region (*H19*ICR) located upstream of *H19*. Three known functions are associated with the *H19*ICR: (1) it is a germline differentially methylated region, (2) it is a transcriptional insulator, and (3) it is a transcriptional silencer. The molecular mechanisms of the DMR and insulator functions have been well characterized but the basis for the ICR's silencer function is less well understood. In order to study the role the *H19*ICR intrinsically plays in gene silencing, we transferred the 2.4-kb *H19*ICR to a heterologous non-imprinted location on chromosome 5, upstream of the *alpha fetoprotein* (*Afp*) promoter. Independent of its orientation, the 2.4-kb *H19*ICR silences transcription from the paternal *Afp* promoter. Thus silencing is a function intrinsic to this DNA element. Further, ICR mediated silencing is a developmental process that, unexpectedly, does not occur through DNA methylation at the target promoter.

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1. Introduction

Genomic imprinting is an epigenetic mechanism that regulates transcription of about 100 genes in mammals [1]. Imprinted genes display parent-of-origin specific gene expression and only one of the two parental alleles is expressed while the other becomes silenced. Imprinted genes are usually located in clusters scattered throughout the genome. One such imprinted cluster is the *Igf2*/*H19* locus on mouse chromosome 7 (Fig. 1A). Loss of imprinting defects at this locus are associated with Beckwith Wiedemann and Russell-Silver syndromes and with many cancers [2].

Igf2 and *H19* are 80-kb apart and share enhancers that are located downstream of the *H19* gene (Fig. 1A) [3,4]. Transcription of both genes is regulated in *cis* by a 2.4-kb DNA element, the *H19* imprinting control region (*H19*ICR) [5–7]. The ICR is located between the two genes, just upstream of the *H19* promoter. Deletion and mutational studies at the endogenous locus have demonstrated three functions for the region. First, the ICR is the only germ line differentially methylated region (gDMR) at the locus and thus is responsible for establishing all the differences in DNA methylation that distinguish maternal and paternal chromosomes in later development [8–10]. Second, when non-methylated, as on the maternal chromosome, the ICR binds CTCF protein and forms a transcriptional insulator that blocks activation of the maternal *Igf2* promoter by the shared downstream enhancers [11]. Third, a paternally inherited, methylated ICR is required for the developmentally regulated silencing of the adjacent paternal *H19* promoter [6,12].

To test whether these three functions are intrinsic to the 2.4-kb *H19*ICR we generated insertion mutation mouse models that carry the ICR at the non-imprinted *alpha-fetoprotein* (Afp) location on mouse chromosome 5 (Fig. 1B) [13]. The *Afp* gene is highly expressed in fetal liver but rapidly repressed after birth. Mice with one functioning copy of *Afp* are healthy and fertile.

We first looked at the DNA methylation patterns of maternally and paternally inherited ICR insertions and saw that the *65* CpGs within the *H19*ICR became methylated but only on paternal chromosomes. That is, there is no detectable methylation upon maternal inheritance but upon paternal inheritance, the ICR is fully protected from digestion by methylation sensitive enzymes. Thus the 2.4-kb *H19*ICR has intrinsic gDMR activity [13].

We next looked at transcriptional insulation. When inserted at the *Afp* location and maternally inherited, the ICR is not methylated, binds the protein CTCF, and therefore insulates the maternal *Afp*

* Corresponding author.
E-mail address: pfeiferk@mail.nih.gov (K. Pfeifer).
[1] These authors equally contributed to this work.
[2] Current address: Medical Research Division, Korea Institute of Oriental Medicine, Daejeon 305-811, South Korea.

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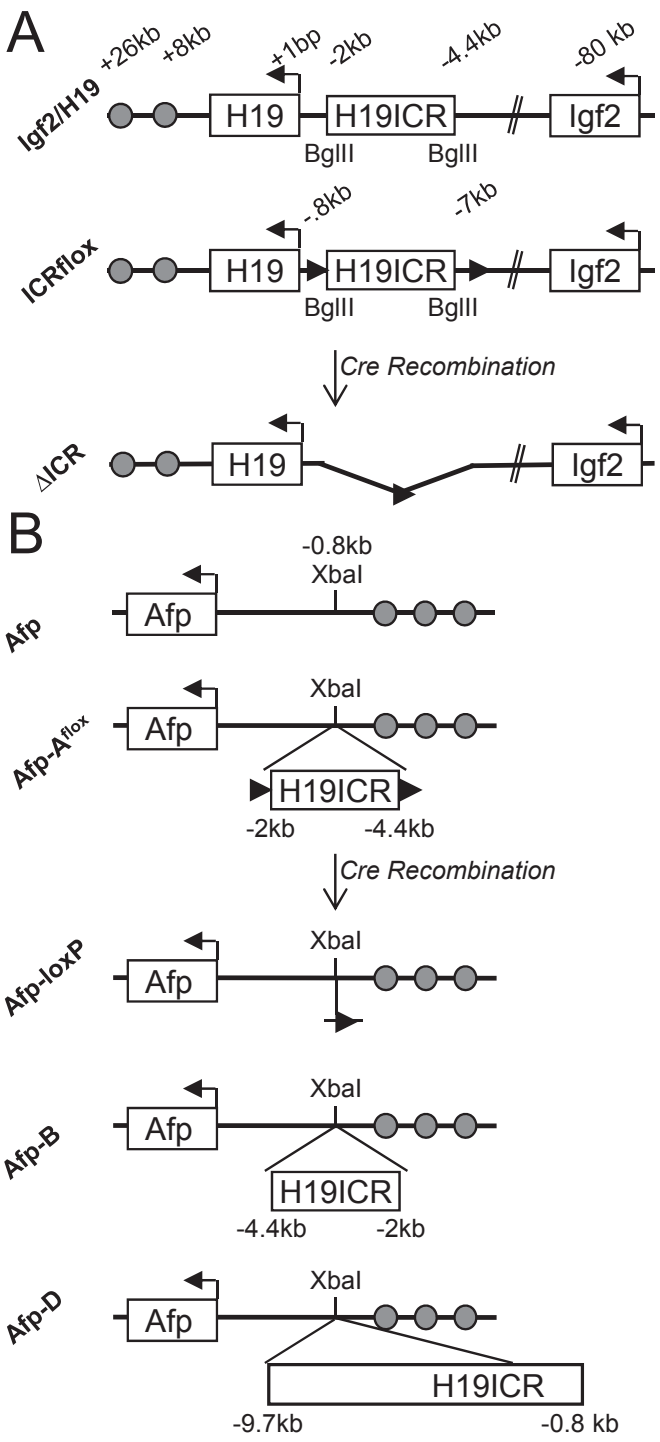

Fig. 1. *Mouse models used in this study.* (A) *Igf2/H19* wild type and mutant alleles. The relative locations of the *H19* and *Igf2* genes and the *H19ICR* are depicted along with the shared endodermal and mesodermal enhancers (filled circles at +8 and +26 kb, respectively). All coordinates are relative to the *H19* start site. The *ICRflox* allele [6] carries *loxP* insertions at −0.8 and −7-kb. Thus *cre*-mediated recombination results in deletion of the entire *H19ICR* but leaves the *H19* promoter in tact. (B) *Afp* wild type and insertion alleles. The relative locations of the *Afp* gene and its three enhancer elements (located between −1 and −7.6-kb) are depicted along with the *XbaI* site where *H19*ICR sequences were inserted. These coordinates are relative to the *Afp* transcriptional start site. *Afp*−A^{flox} has the *H19*ICR carried on a 2.4-kb *BglII* fragment inserted at the *XbaI* site. *Cre*-mediated recombination of *Afp*−*A*^{flox} removes the *H19*ICR but a residual 194-bp including a single loxP site remains. *Afp*−B differs from *Afp*−A in the orientation of the inserted ICR. *Afp*−D carries the *H19*ICR inserted in the same orientation as *Afp*−B but has an additional 4.8-kb of sequences that separate the *H19ICR* from the *Afp* promoter. For *Afp*−A^{flox}, *Afp*−B, and *Afp*−D, the numbers below the ICR delineate the endogenous coordinates of the *H19*ICR fragment.

promoter from interacting with its enhancers so that maternal *Afp* expression is not detected [14]. In fact, multiple studies in transgenic mice and cell culture models support the idea that insulator function is entirely intrinsic to the *H19*ICR [11].

Our understanding of the methylated ICR as a transcriptional silencer is more limited. At the endogenous *Igf2/H19* locus, silencing of the *H19* promoter is a developmental process. Expression of the *H19* gene is bi-allelic in early embryos. Repression of the paternal *H19* allele in the epiblast correlates with the "spread" of DNA methylation from the ICR to the adjacent CpG-rich *H19* promoter/exon 1 region [10,15]. Sometime after the *H19* promoter has become methylated, its silencing is permanent and independent of the continued presence of the paternal *H19*ICR [6,9,12]. For these reasons, it has been assumed that DNA methylation spreading is the mechanism that silences the paternal *H19* gene.

In this study, we test whether gene silencing is an intrinsic property of the 2.4-kb *H19*ICR and whether the spread of DNA methylation is essential for the ICR's function as a silencer. Unlike the *H19* promoter, the *Afp* promoter does not harbor any CpG-rich motifs but only a few scattered CpG dinucleotides [16]. However, when upstream of the *Afp* gene, the *H19*ICR successfully silenced the paternal *Afp* allele without changing *Afp* promoter methylation. DNA methylation is therefore likely not the mechanism that is used by the ICR to silence nearby promoters. However, we present developmental analyses that are consistent with the idea that DNA methylation contributes to the stability of gene silencing.

2. Materials and methods

2.1. Mice

Animal research was approved by the NICHD Animal Care and Use Committee and done according to NIH guidelines. Key mouse lines are depicted in Fig. 1. ICR^{flox} and ΔICR are from Srivastava [6]. *Afp*−A, *Afp*−B and *Afp*−D are from Park [13]. *Afp*−A^{flox} was generated for this study and is similar to *Afp*−A except that the 2.4-kb *Bgl*II fragment carrying the *H19*ICR is flanked with loxP sites. To generate *Afp*−loxP animals, *Afp*−A^{flox} males were crossed to females transgenic for *EIIa-Cre* (Jackson Labs 003724). *Albumin*-cre [17] and *Sox2*-cre [18] mice were backcrossed into FVB to introduce a single nucleotide polymorphism that distinguishes FVB and SvJ129 alleles of *Afp*.

2.2. Parent-of-origin specific gene expression at Afp and H19

Total RNA was extracted from liver, converted into cDNA, and maternal and paternal specific transcription of *Afp* and *H19* was quantitated using DNA melting curve analyses [19]. Data were analyzed using the unpaired *t*-test.

2.3. Bisulfite sequencing

Methods to quantitate DNA methylation are detailed in the legend to Supplemental Fig. 1.

3. Results

3.1. The 2.4-kb H19ICR silences the exogenous Afp promoter

When paternally inherited, ICR insertions at the *Afp* locus are