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Mechanisms of *Igf2/H19* Imprinting: DNA Methylation, Chromatin and Long-Distance Gene Regulation

Hiroyuki Sasaki,[1] Ko Ishihara, and Reiko Kato[2]

Division of Human Genetics, Department of Integrated Genetics, National Institute of Genetics & Department of Genetics, Graduate University for Advanced Studies, 1111 Yata, Mishima, Shizuoka 411-8540

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The mouse *Igf2* and *H19* genes lie 70-kb apart on chromosome 7 and are reciprocally imprinted. Two regulatory regions are important for their parental allele–specific expression: a differentially methylated region (DMR) upstream of *H19* and a set of tissue-specific enhancers downstream of *H19*. The enhancers specifically activate *Igf2* on the paternal chromosome and *H19* on the maternal chromosome. The interactions between the enhancers and the genes are regulated by the DMR, which works as a selector by exerting dual functions: a methylated DMR on the paternal chromosome inactivates adjacent *H19* and an unmethylated DMR on the maternal chromosome insulates *Igf2* from the enhancers. These processes appear to involve methyl-CpG–binding proteins, histone deacetylases and the formation of chromatin insulator complexes. The *Igf2/H19* region provides a unique model in which to study the roles of DNA methylation and chromatin structure in the regulation of chromosome domains.

Key words: chromatin, chromosome domain, DNA methylation, enhancer, genomic imprinting.

Genomic imprinting refers to a sex-specific gene marking process that occurs in the germline, whereby subsets of mammalian genes are expressed differently depending on their parental origin (*1*). The parental allele–specific expression of imprinted genes has a crucial influence on normal mammalian development and is relevant to a number of genetic diseases and cancers (*1*). Notably, paternally expressed genes tend to promote cell proliferation and maternally expressed genes tend to have the opposite effect, although there are exceptions to this rule (*2*). Evidence from germline mutations in mice further indicates that some imprinted genes affect animal behavior (*3*, *4*).

The mouse insulin-like growth factor II (*Igf2*) and *H19* genes lie within a large imprinted region on distal chromosome 7 (*5*). The two genes are separated by only 70 kb and imprinted in opposite directions: only the paternal allele of *Igf2* is expressed and only the maternal allele of *H19* is expressed (*6*, *7*) (Fig. 1A). The protein product of *Igf2* is a fetal growth factor, but the function of *H19*, whose final product is an RNA, is still unclear. In this article, we will review the experimental evidence for the involvement of DNA methylation and chromatin structure in *Igf2/H19* imprinting. We will also review the findings obtained by germline deletion experiments in mice, which have revealed the long-distance mechanisms regulating the reciprocal imprinting of the two genes. Based on these findings, a chromatin model for *Igf2/H19* imprinting will be proposed.

Epigenetic modifications at *Igf2* and *H19*

Three important features are noted for the mechanisms of genomic imprinting: (i) heritability, (ii) reversibility, and (iii) the ability to affect gene expression. Parental marks or imprints established during gametogenesis must be maintained and propagated through fertilization and subsequent cell division during development. Conversely, imprints should be reversible or switchable upon each passage through the germline. Mechanisms that meet all these criteria include epigenetic modifications such as DNA methylation, histone acetylation and changes in higher-order chromatin structure (*1*, *8*).

The first indication that DNA methylation (methylation of cytosine at CpG dinucleotides) is involved in imprinting comes from studies on imprinted transgenes (*8*). In most cases, the foreign DNA was methylated when maternally derived but unmethylated when paternally derived (*8*). However, a study on an endogenous gene, *Igf2*, revealed that its promoter region, including a typical CpG island, was unmethylated at both parental alleles (although partial differences were seen in non-regulatory regions) (*9*). Similarly, DNase I sensitivity assays detected no difference in chromatin between the paternal and maternal *Igf2* alleles (*9*). In contrast, studies on *H19* showed the inactive paternal allele to be heavily methylated whereas the active maternal allele was unmethylated (*10*, *11*) (Fig. 1A). Furthermore, the chromatin of the methylated paternal allele was more resistant to nucleases than the maternal allele (*10*, *11*) (Fig. 1A). Subsequent studies revealed that the differences in methylation found in a 2-kb region (differentially methylated region, DMR) located 2-kb upstream from

[1] To whom correspondence should be addressed. Tel: +81-559-81-6795, Fax: +81-559-81-6800, E-mail: hisasaki@lab.nig.ac.jp

[2] Present address: Division of Molecular Genetics, Institute of Life Science, Kurume University, Aikawa-machi, Kurume 839-0861.

Abbreviations: DMR, differentially methylated region; *Dnmt*, DNA (cytosine-5) methyltransferase; *Igf2*, insulin-like growth factor II, *Igf2r/Mpr*, insulin-like growth factor II receptor/mannose-6-phosphate receptor; LCR, locus control region; *L23mrp*, L23 (mitochondrial)-related protein.

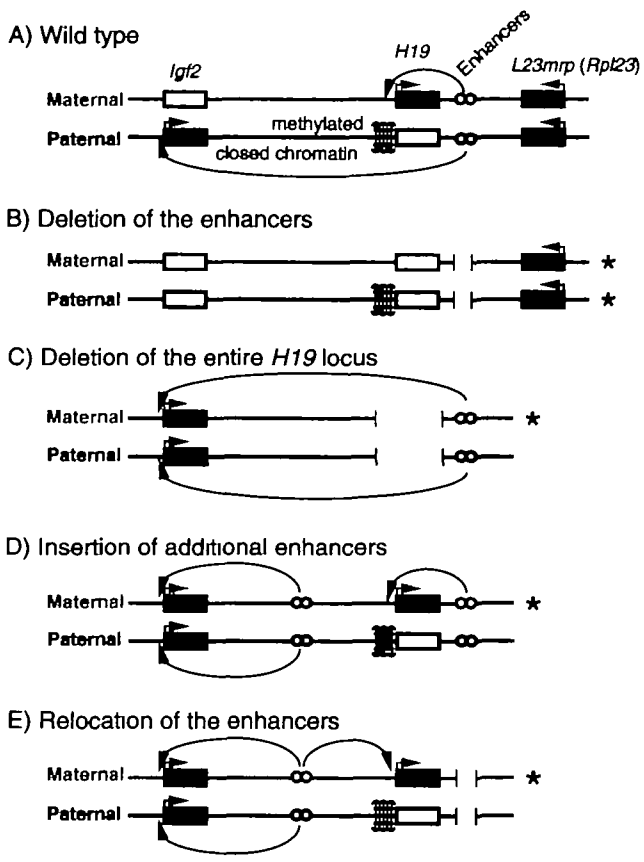

Fig 1. **Germline mutation experiments reveal roles for the downstream enhancers in *Igf2/H19* imprinting.** The genes are depicted as boxes and the two endoderm-specific enhancers as circles. Short arrows above the boxes show that the genes are transcriptionally active. Filled lollypops show a region with methylated DNA and closed chromatin structure. Interactions between the enhancers and the promoters, deduced from the results of the experiments (see text), are also indicated. Asterisks indicate the affected parental chromosome.

the transcription start site were already present in the gametes (methylated in sperm and unmethylated in oocytes), fulfilling the criteria for a parental imprint (*12*). In contrast, methylation differences around the transcription start site were established only after implantation (*13*).

Unequivocal evidence for the involvement of CpG methylation in imprinting comes from studies on mice carrying a mutation in a DNA methylase gene, *Dnmt* [for DNA (cytosine-5) methyltransferase, now called *Dnmt1*] (*14*). Mouse embryos homozygous for the mutated *Dnmt1* allele exhibit genome-wide hypomethylation and loss of the allele-specific expressions of *Igf2*, *H19*, and the maternally expressed gene encoding insulin-like growth factor II receptor/mannose-6-phosphate receptor (*Igf2r/Mpr*) (Table I) (*14*). The results indicate that CpG methylation is required for either the maintenance of imprints or for the establishment of imprinted allele-specific expression. However, this study did not address the question of whether the germline parental imprints are methylations or not because the parents of these embryos possessed at least one functional *Dnmt1* allele (heterozygotes) and thus were functionally normal.

TABLE I. **Targeted disruption of *Dnmt1* affects the allelic expression patterns of imprinted genes.**

Gene	Allele	Expression	
		Wild-type	Mutant[a]
Igf2	Paternal	+	−
	Maternal	−	−
H19	Paternal	−	+
	Maternal	+	+
Igf2r/Mpr	Paternal	−	−
	Maternal	+	−

[a]The data on mutant mice are according to Li *et al.* (*14*)

Another epigenetic mechanism closely associated with imprinting is the timing of DNA replication. In non-imprinted gene regions, homologous chromosomes replicate synchronously during S phase. In contrast, imprinted gene regions, including the *Igf2/H19* region, replicate asynchronously (*15*, *16*), suggesting the presence of allelic differences in the higher-order chromatin structure. However, a direct link between asynchronous replication and parental allele-specific expression is still lacking.

Original enhancer competition model

The identification of allele-specific epigenetic modifications at *H19* and the lack of clear epigenetic differences at *Igf2* together derive a model that explains the reciprocal imprinting of the two genes. This model, called the "enhancer competition model" (*9*, *18*) (Fig. 1A), is also based on their similar patterns of expression in many endoderm- and mesoderm-derived tissues. A presupposition then is that the two genes share a common set of tissue-specific enhancers. At least two endoderm-specific enhancers have been identified in the *H19* region (*17*). Thus, in this model, the enhancers specifically activate *H19* on the maternal chromosome, perhaps due to proximity or high affinity, leaving the maternal *Igf2* allele silent or only weakly expressed. On the paternal chromosome, the enhancers cannot interact with *H19* because the 5′ region of *H19* is heavily methylated and assumes a closed chromatin structure. The enhancers then engage with *Igf2*, which is 80 kb away. Consistent with this model, the promoters of *Igf2* are by themselves incapable of driving strong reporter gene expression in transgenic mice and the transgene expression is dependent on the presence of the enhancers (*19*).

The downstream enhancers

Direct evidence that the endoderm enhancers downstream of *H19* are involved in *Igf2/H19* imprinting comes from a series of germline deletion experiments by Tilghman and colleagues. First, they deleted the two endoderm enhancers and observed that neither *H19* nor *Igf2* was expressed on the chromosome with the deletion (*20*) (Fig. 1B). This indicated that *H19* and *Igf2* utilize the same endoderm enhancers, but on different parental chromosomes. Interestingly, the mutation did not affect the expression of *L23mrp* (or *Rpl23*), a biallelically expressed gene located only 40 kb downstream of *H19*, suggesting that *L23mrp* is insulated from the enhancers (*21*) (Fig. 1B). Second, a 13-kb deletion containing both the *H19* transcription unit and DMR activated the normally silent *Igf2* allele on the maternal chromosome (*22*) (Fig. 1C), suggesting that a function of the *H19* region is to imprint *Igf2*. Third, when an extra set of enhancers was inserted midway between *Igf2* and *H19*,

the normally silent maternal *Igf2* allele was activated in endodermal tissues without changing the level of *H19* expression (*23*) (Fig. 1D). This is consistent with a relief from competition. Lastly, when the enhancers were relocated equidistant from *Igf2* and *H19*, the normally silent maternal *Igf2* allele was activated and expression of the maternal *H19* allele was reduced (*23*) (Fig. 1E). These findings indicate that the endoderm enhancers are essential for the imprinted expression of both *Igf2* and *H19* and that the locations of the enhancers are important.

Although these endoderm-specific enhancers were the only known enhancers of the region, both *Igf2* and *H19* are expressed and imprinted in many mesoderm-derived tissues. To look for additional enhancers of the region, we sequenced a 40-kb region containing *H19* in both human and mouse and identified a total of ten evolutionarily conserved regions (200–500 bp in size, sequence identity 65–85%), two of which were coincident with the regions of the endoderm enhancers (*24*). Subsequent studies in transgenic mice revealed that at least five of the remaining eight regions function as enhancers in specific mesoderm and ectodermal tissues (*24*). Thus it appears that a large cluster of tissue-specific enhancers, resembling the locus control region (LCR) of the β-globin gene cluster (*25*), is involved in the reciprocal imprinting of *Igf2/H19*.

Function of the differentially methylated region (DMR)

Although it was shown that a 13-kb *H19* region is essential for *Igf2* imprinting (*22*) (Fig. 1C), the most important part was yet to be determined. For example, the RNA-coding region could play a critical role since *Xist*, a transcript expressed specifically from inactive X chromosomes, is thought to initiate chromosome inactivation by functioning as an RNA (*26*, *27*). Replacement of the *H19* coding region by a luciferase gene, however, caused only a slight derepression of the transgene on the paternal chromosome, with no apparent effect on *Igf2* expression or imprinting (*28*) (Fig 2B). This suggested that the *H19* RNA is basically irrelevant. Removal of the *H19* transcription unit including the promoter and the transcription start site caused only minimal activation of the maternal *Igf2* allele (*29*) (Fig. 2C). This indicated that, in contrast to the prediction based on strict enhancer competition, transcriptional activity of the *H19* promoter is not required to silence the maternal *Igf2* allele. Furthermore, the results suggested that a 10-kb region containing the DMR blocks the enhancer-*Igf2* interaction on the maternal chromosome but not on the paternal chromosome.

Direct evidence for the crucial function of the DMR was obtained by producing mice harboring a 1.6-kb deletion of the DMR. *H19* was activated and *Igf2* expression was reduced when the deletion was paternally inherited; *H19* expression was reduced and *Igf2* was activated upon maternal inheritance of the deletion (*30*) (Fig. 2D). These findings indicate that the methylated DMR on the paternal chromosome is required to silence the adjacent *H19* and that the unmethylated DMR on the maternal chromosome is required to block the interactions between *Igf2* and the enhancers. Thus, the DMR serves as an inactivation center when methylated and as a chromatin insulator or boundary element (*31*) when unmethylated. Interestingly, this insulator activity of the unmethylated DMR may be orientation-dependent since the DMR did not block enhancer–promoter interactions when the enhancers were placed on the 5′ side (*23*) (Fig. 1E).

We recently identified an evolutionarily conserved sequence element that appears repeatedly within the human and mouse DMRs (*24*). The highly conserved 15-bp core region of the consensus sequence is GC-rich and contains four methylatable CpG sites. Gel shift assays showed that these sequences form complexes with specific nuclear factors from various sources and, furthermore, that complex formation by one of these factors is inhibited by target site methylation (*24*). It is conceivable that the binding of these factors to the unmethylated DMR is one of the crucial steps in the regulation of *Igf2/H19* imprinting.

A chromatin model

Taking all the above findings into account, we propose a revised model to explain the mechanisms of *Igf2/H19* imprinting (Fig. 3). There are two important points to note. First, the LCR-like region downstream of *H19* contains some enhancers with overlapping tissue specificities (*24*); activation complexes formed on these enhancers might assemble further to form a larger holocomplex. The holocomplex and the promoter of either *Igf2* or *H19* are brought together to form a transcription complex, perhaps by chromatin looping Second, the selection of which gene is to be activated is not determined by strict enhancer competition: it is determined by the function of the DMR. The methylated DMR on the paternal chromosome inactivates the adjacent *H19*, perhaps by spreading methylation during development. This silencing appears to involve the binding of methyl-CpG–binding proteins and the deacetylation of histones. Methyl-CpG–binding proteins recruit histone deacetylase complexes (*32–35*) and, furthermore, an inhibi-

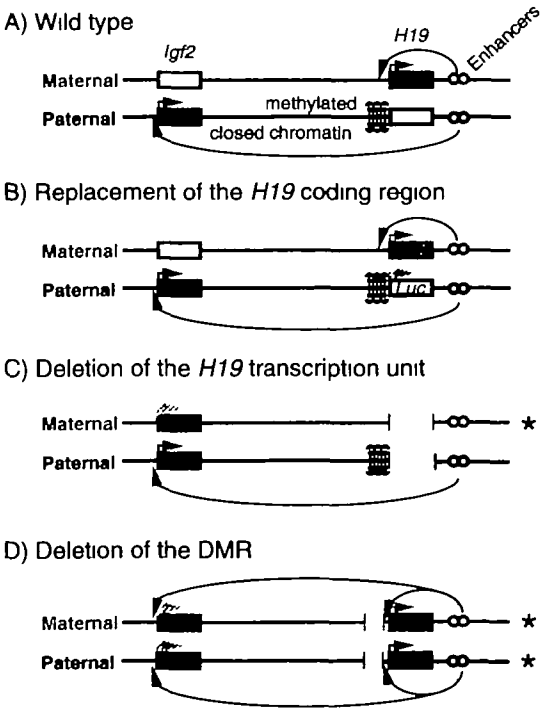

Fig 2 Germline mutation experiments reveal multiple functions of the DMR in *Igf2/H19* imprinting. See Fig 1 for legend.

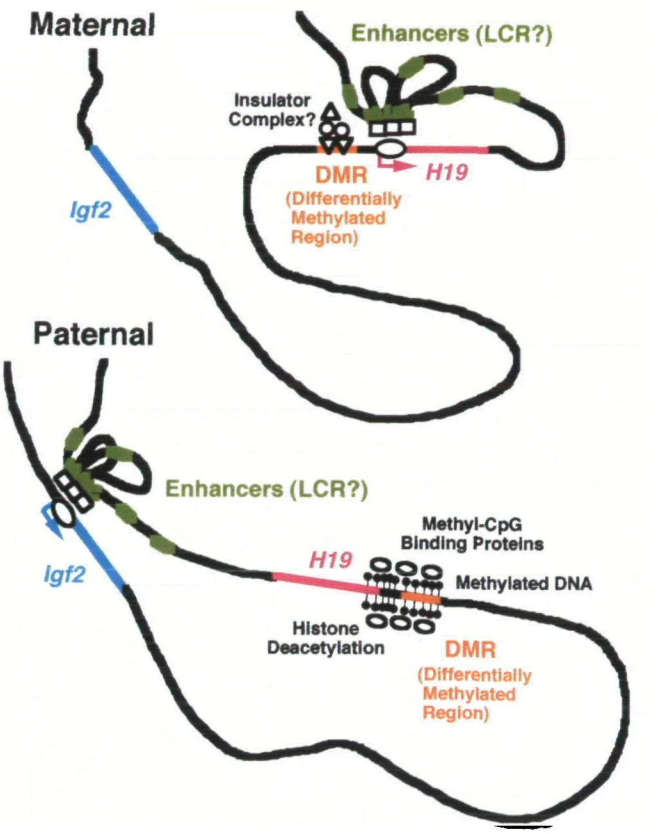

Fig 3 **A chromatin model of *Igf2/H19* imprinting.** The reciprocal, imprinted expression of *Igf2* and *H19* is dependent on the downstream LCR-like region, which interacts with the genes, perhaps through chromosome looping The DMR works as a selector or switch that determines which gene is to be activated by the putative LCR holocomplex A paternally derived methylated DMR inactivates adjacent *H19* by spreading its inactive chromatin state into the promoter region, leaving the enhancers accessible to *Igf2* (bottom). A maternally derived unmethylated DMR blocks the interaction between the enhancers and *Igf2*, perhaps by forming chromatin insulator complexes (top).

tor of histone deacetylase disrupts the imprinting of *H19* in some tissues (*36*) Thus the holocomplex cannot interact with the *H19* promoter and engages itself in the activation of *Igf2*. In constrast to this, both *Igf2* and *H19* are unmethylated and available for activation by the holocomplex on the maternal chromosome. However, the unmethylated DMR attracts sequence-specific nuclear factors and forms a chromatin insulator complex, which prevents the interaction between the holocomplex and *Igf2*. The formation of sequence-specific complexes is supported by the presence of maternal-specific DNase I hypersensitive sites (*37*, *38*). Since some of the evolutionarily conserved elements map at the same positions as the hypersensitive sites, the DNA-binding proteins, including the methylation-sensitive protein, are probably important for the formation of the insulator complex (*24*).

The above model provides a guide for future studies since most of the features described are testable. For example, whether the enhancer cluster meets the criteria for an LCR can be examined by producing transgenic mice. LCRs form a class of regulatory elements that confer high level, tissue-specific, site-of-integration-independent, copy number-dependent expression on linked transgenes (*25*). The precise function of the conserved elements within the DMR will be revealed by site-directed mutagenesis combined with gene knockout in mice. It will also be interesting to test whether the DMR functions as an inactivation center and/or an insulator in a heterologous system. Molecular cloning of cDNAs encoding the factors binding to the elements will shed light on the structure and function of the unique methylation-sensitive insulator complex. These studies should provide insight into the long-distance mechanisms regulating not only the imprinted domain but also other chromosome domains.

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