Igf2 imprinting does not require its own DNA methylation or *H19* RNA

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Three models have been proposed to explain the imprinting of the mouse *Igf2* gene on the maternal chromosome. We ruled out the importance of DNA methylation at *Igf2* by showing that silencing of *Igf2* accompanying the loss of DNA methylation could be overcome by a mutation at the neighboring *H19* gene that activates *Igf2*. By replacing the *H19* structural gene with a protein-coding gene, we have ruled out a role for *H19* RNA in the imprinting of *Igf2*. This replacement resulted in sporadic activation of the *H19* promoter on the paternal chromosome without affecting the level of expression of *Igf2*, a finding that is inconsistent with strict promoter competition between the genes. We conclude that a transcriptional model involving access to a common set of enhancers shared between *Igf2* and *H19* is the most likely explanation for *Igf2* imprinting.

[Key Words: Genomic imprinting; *H19*; *Igf2*; DNA methylation; promoter competition; chromatin]

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The mammalian genome harbors a small number of genes whose expression is affected by the parental origin of the chromosome, a process known as genomic imprinting (Barlow 1995; Bartolomei and Tilghman 1997). The number of imprinted genes that have been identified now numbers >20, and comparisons among these genes have begun to reveal some common features. Foremost among these is allele-specific DNA methylation, which has been implicated in the mechanism by which a cell discriminates between the two parental alleles of an imprinted gene and determines which will be transcribed (Li et al. 1993). Furthermore, imprinted genes are frequently linked within clusters that contain both maternally and paternally expressed genes (Zemel et al. 1992; Mutirangura et al. 1993; Rougeulle et al. 1997; Vu and Hoffmann 1997; Wutz et al. 1997). Finally, an unusual feature of these clusters is that they contain at least one imprinted gene that encodes an untranslated RNA (Brannan et al. 1990; Kay et al. 1993; Wevrick et al. 1994; Wutz et al. 1997).

All of these properties are evident within an ~800-kb cluster of genes that resides on the distal end of mouse chromosome 7 (Caspary et al. 1998). This cluster contains the paternally expressed *insulin-like growth factor 2* (*Igf2*) and *insulin-2* (*Ins2*) genes, which are flanked on the telomeric side by the maternally expressed *p57^{Kip2}*, *Mash2*, and *K_vlqt1* genes and on the centromeric side by the maternally expressed *H19* gene. Of these genes, the one whose imprinting mechanism is best understood is *H19*, which encodes the first of the untranslated RNAs

to be shown to be imprinted (Brannan et al. 1990; Bartolomei et al. 1991). The silence of *H19* on the paternal chromosome is thought to be the result of DNA methylation within the gene and its 5' flank that is established during spermatogenesis and maintained throughout embryogenesis (Bartolomei et al. 1993; Brandeis et al. 1993; Ferguson-Smith et al. 1993; Li et al. 1993).

The absence of any DNA methylation on the silent *Igf2* gene forced us to consider alternative models to explain its imprinting. In fact, it is the expressed paternal allele of *Igf2* that is specifically methylated in two locations: within the gene itself and in its 5' flank (Sasaki et al. 1992; Feil et al. 1994). Furthermore, this correlation between methylation and expression of *Igf2* extends to the choroid plexus, a tissue that expresses both alleles of *Igf2* and exhibits biallelic methylation (DeChiara et al. 1991; Feil et al. 1994; Svensson et al. 1995), and to mutations at *H19* that activate *Igf2* (Forne et al. 1997). These results, taken together with the loss of *Igf2* expression in embryos deficient in DNA methyltransferase (Dnmt), led to the proposal that methylation was acting as a positive signal for transcription, possibly by inhibiting the binding of a repressor (Walter et al. 1996).

An alternative to this hypothesis was based on the very similar patterns of expression of *Igf2* and *H19* during development. Bartolomei and Tilghman (1992) proposed that their imprinting is linked through a competition for a common set of transcriptional enhancers. In this scenario, the silence of the maternal *Igf2* gene is a consequence of its failure to compete successfully with the unmethylated *H19* gene in *cis*. This model gained considerable support when it was demonstrated that both genes require for their expression two endoderm-

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specific enhancers that lie 3' of the *H19* gene (Leighton et al. 1995b). Furthermore, the insertion of an extra set of enhancers between the two genes results in the activation of the unmethylated maternal *Igf2* gene (Webber et al. 1998). Finally, two targeted mutations that delete the competitor *H19* gene and either 10 kb (*H19 Δ 13*) or 40 bp (*H19 Δ 3*) of its 5' flank also result in complete or partial activation, respectively, of the maternal *Igf2* gene 90 kb away (Leighton et al. 1995a; Ripoché et al. 1997).

In this report we perform an epistasis test to assess the relative importance of DNA methylation, versus the absence of the *H19* region, to *Igf2* expression. We conclude that the *Igf2* gene does not require DNA methylation for its expression in the embryo. Furthermore, we generate a targeted replacement of the *H19* structural gene to definitively show that the RNA itself plays no role in the imprinting of *Igf2*.

Results

Igf2 is expressed in the absence of DNA methylation in *H19* mutant animals

To assess the relative importance of methylation in *Igf2* imprinting, we performed an epistasis test to ask whether the silencing of *Igf2* by loss of DNA methylation in *Dnmt $^{-/-}$* embryos could be overcome by the *H19 Δ 13* mutation that activates maternal expression of *Igf2*. We utilized mice bearing the null *Dnmt S* allele; homozygous mutant embryos do not survive past e10, and exhibit a marked reduction in both genome-wide and *Igf2*-specific methylation (Li et al. 1992, 1993; Tucker et al. 1996). *Dnmt $^{-/-}$* embryos that had also inherited the *H19 Δ 13* mutation on either the maternal or paternal chromosome were harvested at e9.5. DNA was prepared from yolk sacs for genotyping, and the levels of *Igf2* RNA were determined by RNase protection in pooled embryos of the same genotype. As the results in Figure 1 illustrate, *Igf2* expression is unaffected by the loss of DNA methylation when the *H19 Δ 13* mutation is present. The levels of *Igf2* expression in *Dnmt $^{-/-}$; *H19 Δ 13/+* embryos were similar to those obtained in*

Figure 1. Expression of *Igf2* RNA in *Dnmt $^{-/-}$; *H19 Δ 13/+* double mutant embryos. Embryos were generated by crossing *Dnmt $^{+/-}$* heterozygotes to *Dnmt $^{-/-}$; *H19 Δ 13/+* double heterozygotes. Embryos were pooled according to genotype and the levels of *Igf2* and rpl32 mRNAs were determined by RNase protection. The level of *Igf2* RNA is expressed as a percent of that in wild-type embryos, after normalization to rpl32 RNA by PhosphorImager densitometry.**

Dnmt $^{+/-}$ littermates, on the basis of comparisons with rpl32 RNA, which served as an internal control. The levels were independent of the parental origin of the *H19 Δ 13* mutation, as would be predicted if the loss of methylation had erased all differences between the parental chromosomes. From this observation, we conclude that the expression of *Igf2* at this stage in development does not require DNA methylation, but does require the methylation or deletion of the *H19* gene region.

Igf2 imprinting does not require *H19* RNA in cis

Although the original enhancer competition model predicted a strictly transcriptional role for the *H19* gene in *Igf2* imprinting, a function for the RNA product of the gene has been suggested, as both the *H19 Δ 13* and *H19 Δ 3* mutations that disrupted *Igf2* imprinting removed both the promoter and the structural *H19* gene (Leighton et al. 1995a; Ripoché et al. 1997). Moreover, the presence of untranslated RNAs in multiple imprinted regions, together with the compelling evidence that expression of an untranslated RNA encoded by the *Xist* gene is required for X chromosome inactivation (Penny et al. 1996; Marahrens et al. 1997), raised expectations that there might be some function for these RNAs in imprinting. Functional evidence for the importance of sequences within the *H19* structural gene came from the experiments of Pfeifer et al. (1996) who observed that when the *H19* structural gene was replaced with the luciferase gene within the context of a 14-kb transgene, imprinting of the transgene was lost.

To examine the role of *H19* RNA in the silencing of *Igf2*, a targeting strategy was devised to delete all but the first three nucleotides of the *H19* transcript and replace them with a gene coding for the protein luciferase (*luc*) (Fig. 2). The neomycin resistance (*neo R*) selectable marker was flanked by loxP sites to facilitate removal by Cre-mediated homologous recombination, which would leave the targeted locus modified only within the coding region of the *H19* gene (Sauer and Henderson 1989). Correctly targeted events and accurate excision of *neo R* were verified with probes that lie outside of the targeting vector (Fig. 2B). RNase protection analysis confirmed the absence of maternal *H19* RNA (data not shown).

The expression of the *luc* gene in neonatal liver was examined by an RNase protection assay in progeny of *+/*H19 luc** mothers (Fig. 3A). Regardless of whether the mutation was on the grandmaternal or grandpaternal chromosome, the gene was expressed at equivalent levels, suggesting that resetting from a male to a female epigenetic state was occurring. Similar results were obtained in skeletal muscle (data not shown). To ask whether the maternally inherited *H19 luc* locus was unmethylated, the 5' flank and the *luc* gene were examined by digestion of genomic DNA from neonatal liver with methylation-sensitive restriction enzymes (Fig. 3B). A region encompassing from -2 to -4 kb within the 5' flank of the gene, which is thought to contain the primary paternal gametic mark but is always unmethylated

Figure 2. Strategy for replacing *H19* RNA with luciferase RNA. (A) Structure of the endogenous *H19* locus, along with the positions and sizes of restriction fragments and radiolabeled probes (shaded boxes) used in B, are illustrated at the top. The targeting vector contains the luciferase coding region (LUC) inserted at +3 bp of the *H19* structural gene, followed by the *neo^R* gene linked to the *tk* gene by an internal ribosomal entry site (*neo^R/tk*) driven by the *phosphoglycerokinase* promoter. The *neo/tk* gene is flanked by *loxP* sites from bacteriophage P1 (▶). The regions of homology between the targeting vector and the endogenous locus are indicated by broken lines. The vector also includes the *diphtheria toxin A* (DTA) chain gene for negative selection (McCarrick et al. 1993). The structure of the correctly targeted locus, before and after excision of *neo/tk* with Cre recombinase, are illustrated, along with the restriction fragments used to diagnose them. The position of probe C is indicated by the shaded box. (B) DNAs prepared from E14.1 wild-type cells (+/+) and a correctly targeted line before (+/Luc-Neo) and after (+/Luc) excision of *Neo/tk* were analyzed by digestion with restriction enzymes and hybridized to probes A–C, as indicated. The size marker was λ DNA digested with *HindIII*. (P) *PstI*; (Bg) *BglII*; (R) *EcoRI*; (H) *HindIII*.

on the maternal chromosome (Tremblay et al. 1995, 1997), is equally unmethylated in maternal *H19^{luc}* mice. The lack of methylation is also evident within the promoter and the *luc* gene itself (data not shown). These results are consistent with the findings of Ripoché et al (1997), who showed that a *neo^R* gene inserted in place of the *H19* promoter and structural gene was appropriately unmethylated and expressed on the maternal chromosome.

Although there is no requirement for maternal *H19* RNA in the embryo, the RNA could be required in *trans* for setting the maternal imprint in primordial germ cells or in early germ-cell development, in which the gene has

been shown to be expressed from both chromosomes (Szabo and Mann 1995). To test this, we examined transmission of a paternally inherited *H19^{luc}* allele from both *H19^{luc}* homozygous and *H19^{Δ13}/H19^{luc}* females. The females were crossed to wild-type males and +/*H19^{Δ13}* males so that the expression and methylation of the

Figure 3. Maternal inheritance of *H19^{luc}*. (A) Expression of *H19^{luc}* and rpl32 were measured by RNase protection in day 4 and 5 neonatal livers of progeny of +/*H19^{luc}*, *H19^{luc}/H19^{luc}*, and *H19^{Δ13}/H19^{luc}* mothers and *M. castaneus* (+) fathers. (B) Genomic DNA from wild type (+/+) and progeny of *H19^{luc}/+* females and *M. castaneus* males was digested with *SacI* alone (–) or *SacI* together with *HpaII*, *HhaI*, or *MspI*, as indicated. The DNA was detected with a 4-kb *EcoRI* hybridization probe (shaded rectangle) derived from the 5' end of the *H19* gene. The positions of the *SacI* (S) sites, including the polymorphic site in *M. castaneus* (S*) are indicated, along with the start of *H19* transcription (arrow). (M) Maternal allele; (P) paternal allele. The size markers are indicated at right. (C) *Igf2* RNA was assayed by an allele-specific RNase protection assay in a subset of the animals described in A as well as in *M. castaneus* neonatal liver (C, lane 1) and C57BL/6J liver (D, lane 2). The maternal genotypes are +/*H19^{luc}* (lane 4), *H19^{luc}/H19^{luc}* (lane 5), and *H19^{Δ13}/H19^{luc}* (lane 6).

H19^{luc} gene could be assessed in either the presence or absence of the paternally provided *H19* gene. No differences in the expression of *H19^{luc}* RNA were observed in any of these genetic combinations, providing strong evidence that there is no requirement for *H19* RNA in the maternal germline for resetting or maintaining the unmethylated, transcriptionally active status of the *H19^{luc}* allele (Fig. 3A and data not shown).

Having verified that replacement of *H19* with *luc* resulted in normal maternal expression from the *H19* promoter, we next examined the impact of this mutation on *Igf2* imprinting. Heterozygous, homozygous, and compound heterozygous female mice bearing the *H19^{luc}* allele (*H19^{luc}/+*, *H19^{luc}/H19^{luc}*, and *H19^{luc}/H19^{Δ13}*) were crossed to *Mus castaneus* males and *Igf2* expression was examined in livers and skeletal muscle from neonatal progeny by allele-specific RNase protection (Fig. 3C; data not shown). No activation of the maternal *Igf2* gene was observed under any circumstance, establishing that imprinting of the *Igf2* gene does not require the presence of the *H19* RNA in *cis*. Thus, unlike the previous two mutations at *H19*, both of which removed the promoter and structural gene, the loss of the structural gene alone has no consequence for *Igf2* imprinting.

Sporadic methylation and silencing of *H19^{luc}* on the paternal chromosome

When the *H19^{luc}* mutation was inherited from fathers, the level of expression of *luc* RNA in the progeny was surprisingly variable, suggesting that there had been sporadic relaxation of the imprint (Fig. 4). The levels varied from 5% to 55% of that measured in age-matched progeny inheriting *H19^{luc}* maternally, on the basis of comparisons with the levels of rpl32 RNA for normalization. The variability could not be ascribed to the presence or absence of wild-type *H19* during spermatogenesis in the father, as progeny of both *H19^{luc}* homozygotes and *H19^{luc}/H19^{Δ13}* males displayed the same degree of variability in *luc* imprinting as those from *H19^{luc}* heterozygotes. Furthermore, the variability did not depend on the presence of *H19* RNA during embryogenesis, as it occurred in both *+/H19^{luc}* and *H19^{Δ13}/H19^{luc}* progeny (Fig 4).

To examine the basis for the variability, the levels of

luc methylation in progeny showing relaxation of the imprint after paternal inheritance were examined. To simplify the analysis, we used tissues from *H19^{Δ13}/H19^{luc}* animals in which the *H19* gene and its 5' flank were deleted on the maternal chromosome. In progeny exhibiting the lowest levels of *luc* expression, the methylation pattern was indistinguishable from that observed on a wild-type paternal *H19* gene throughout the 5' flank; that is, both the 4-kb *SacI* fragment encompassing the gametic imprint as well as the smaller promoter fragment resisted digestion with *HhaI*. Moreover, this methylation extended into the *luc* gene itself, reminiscent of the methylation of the *H19* structural gene (data not shown). In contrast, in animals in which the expression of *luc* was detected, there was an inverse correlation between the expression level and the degree of methylation of the promoter, but not the 5' gametic imprint region, which remained fully methylated (Fig. 5). Thus, the replacement of the *H19* structural gene with the *luc* gene results in some degree of undermethylation of the promoter and sporadic expression of *luc*. This result suggests that sequences within the *H19* structural gene contribute to the maintenance of its silent state on the paternal chromosome. CpG density alone, however, cannot account for this, as the *luc* gene, which is almost identical in length to *H19*, has almost twice the number of CpG dinucleotides. On the other hand, the *luc* gene's overall G+C content is 43%, which is closer to the genome average than *H19*, which is 56%.

The sporadic expression of *luc* on the paternal chromosome provides an explanation for the failure of *H19-luc* fusion transgenes to be imprinted (Pfeifer et al. 1996). One difference between the *luc* transgene and the germline replacement is the maintenance of DNA methylation in the 5' flank in the latter. It may be that the 14-kb transgenes contained only a subset of the epigenetic information required for maintenance of DNA methylation, a conclusion that is consistent with the recent observation that single copy 130-kb YAC transgenes of the locus are correctly imprinted (Ainscough et al. 1997).

H19^{luc} expression has no effect on *Igf2* expression

It was of considerable interest to ask whether transcription of *luc* on the paternal chromosome affected *Igf2*

Figure 4. Expression of *luc* RNA upon paternal inheritance. Males of the genotype indicated (paternal genotype) were crossed to wild-type (+/+) females or *+/H19^{Δ13}* females and the levels of *luc* RNA and rpl32 RNA was determined by RNase protection assay in neonatal livers of the progeny. The levels of *luc* RNA, as determined by PhosphorImager densitometry and normalization to rpl32 RNA levels, are expressed as a percent of the level upon maternal inheritance (*H19^{luc}/+*).

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Figure 5. Effect of paternal *H19^{luc}* inheritance on DNA methylation. (A) Genomic DNA from livers of wild-type (+/+), +/*H19^{luc}* heterozygotes, and *H19^{Δ13}/H19^{luc}* homozygotes were digested with *SacI* and *XbaI* alone (–, first two lanes) or *SacI*, *XbaI*, and *HhaI* (+), separated on 1% agarose gels, and hybridized to a 4-kb *EcoRI* fragment that detects the 3.8-kb *SacI* fragment in the 5' flank of the + and *H19^{luc}* alleles (top) or a 600-bp *XbaI*–*EcoRI* fragment from the 5' end of the *H19* gene that detects a 1.2-kb *SacI* fragment encompassing the + promoter and a 0.7-kb *XbaI* fragment encompassing the *H19^{luc}* promoter (bottom). The level of expression of paternal *luc* RNA in the four compound heterozygotes is indicated as a percent of maternal *luc* RNA expression. (B) A map of the *H19* 5' region, with the start of transcription indicated by the arrow, is drawn below, along with the positions of the *SacI* and *XbaI* fragments assayed (solid rectangles). (S) *SacI*; (X) *XbaI*. (|) *HhaI*; (*) *HhaI* site assayed in the bottom of A. The probes used in A are indicated by hatched boxes.

expression. If a strict competition for transcription governs its expression, we would anticipate a decrease, analogous to the effect of mutations in the *β-globin* gene promoter on *γ-globin* gene expression in chickens, in which promoter competition has been clearly demonstrated (Choi and Engel 1988; Foley and Engel 1992). However, there was no correlation between the levels of *Igf2* RNA (normalized to rpl32 RNA levels) and the expression of paternal *luc* RNA (Fig. 6A). Moreover, the birth weights of the animals were unaffected by paternal *luc* expression (Fig. 6B). These data argue against a strict competition between the *H19* and *Igf2* promoters in maintaining imprinted *Igf2* expression.

Discussion

The maintenance of *Igf2* imprinting in mice that have substituted *luc* for the *H19* coding region rules out a role

for *H19* RNA itself in imprinting. Two previous mutations at the locus, both of which removed both the *H19* gene and its promoter, had resulted in the inappropriate expression of the maternal *Igf2* gene (Leighton et al. 1995a; Ripoché et al. 1997). These findings, coupled with the demonstration by Marahrens et al (1997), that the *Xist* gene itself is required for X chromosome inactivation, made the *H19* RNA an attractive candidate for playing a role in imprinting. However, this is clearly not the case. Instead, the important epigenetic mark for *Igf2* imprinting must lie in the vicinity of the promoter and the 5' flank of the *H19* gene.

The fact that the *H19^{luc}* mice display no phenotype whatsoever leaves unexplained the evolutionarily conserved primary sequence and secondary structure of the RNA (Brannan et al. 1990; Pfeifer and Tilghman 1994). It could be that the RNA has a function unrelated to imprinting that is either nonessential or redundant. Alternatively, it could be that the sole function of the gene is to be transcribed at high rates to imprint *Igf2* and that the conservation of its product reflects the necessity of packaging and sequestering a very abundant RNA. If so, this would be an entirely novel function for a gene.

From the outset, the epigenetic configuration of the *Igf2* and *H19* genes suggested that the genes were not being regulated identically. The finding of extensive methylation of the paternally inherited *H19* gene suggested a straightforward explanation for its silence, given the long-standing correlation between gene silencing and DNA methylation. Furthermore, the differential hypersensitivity of the maternal and paternal *H19* promoters to nuclease digestion reinforced the idea that the two alleles were in different chromatin conformations (Bartolomei et al. 1993; Brandeis et al. 1993; Ferguson-Smith et al. 1993). In contrast, *Igf2* is methylated exclusively on the expressed paternal chromosome, and the two parental promoters are in equally hypersensitive states (Sasaki et al. 1992). Despite the correlation between *Igf2* methylation and expression observed in vivo, our findings demonstrate that this methylation is not required for *Igf2* expression, but is likely the consequence of its expression.

The initial model proposed by Bartolomei and Tilghman (1992) suggested that the transcriptional status of *H19* was mediating the imprinting of *Igf2*. The possibility of pairing a transcription unit that is the target of epigenetic information (i.e., *H19*) with an oppositely imprinted response gene (*Igf2*) has now been raised for another imprinted gene, *Igf2r*. Like *Igf2*, its methylated epigenetic mark occurs on the expressed maternal chromosome, within a large intron of the gene (Stoger et al. 1993). Wutz et al (1997) have now shown that this CpG island is the promoter for a paternally expressed antisense transcript that is required for *Igf2r* imprinting. Barlow (1997) has suggested that the mechanism underlying *Igf2r* imprinting is promoter competition with the antisense RNA coding gene, like the one we have described at *Igf2* and *H19*.

More recent observations suggest that imprinting control may be more complex than strict promoter compe-

Figure 6. Effect of paternal *luc* expression on *Igf2* expression. (A) Expression of *Igf2* and rpl32 RNAs in paternal *H19^{luc}* heterozygotes was determined by an RNase protection assay (Webber et al. 1998). The level of *luc* RNA in each animal is indicated as a percent of maternal *luc* RNA, and the level of *Igf2* RNA is expressed as a percent of that in wild-type embryos after normalization to rpl32 RNA by PhosphorImager densitometry. (B) The weights of +/*H19^{luc}* neonatal offspring (expressed as percent of mean +/+ littermate weight) were plotted against paternal *luc* RNA expression (expressed as percent of maternal *luc* RNA levels).

tion. First, in an effort to understand the basis for the competition between *Igf2* and *H19*, we transposed the 3' *H19* enhancers to a position midway between the genes (Webber et al. 1998). To our surprise, the *Igf2* gene was expressed to the exclusion of *H19*, essentially reversing the direction of the imprint on the maternal chromosome. This argues that the position of a gene and its enhancers relative to the epigenetic mark may play a crucial role in the nature and direction of the imprint.

Second, in this report we observed significant levels of expression of the paternal *luc* gene without a concomitant change in the expression of *Igf2*, a finding that directly contradicts strict promoter competition. It is possible that the substitution of the *luc* gene for *H19* reduced the transcription rate from the *H19* promoter, and thus, it is a poor competitor for transcription with *Igf2*. However, if this were the case, according to strict promoter competition, one might have expected an increase in maternal *Igf2* expression. Rather, it appears that the reciprocal expression of *H19* and *Igf2* is unlike that between the adult and embryonic chicken β -globin genes, in which an increase in expression of one gene is always accompanied by a decrease in the other (Foley and Engel 1992). Our data show that the *H19* promoter can be active on the paternal chromosome without decreasing *Igf2* expression. On the basis of both of these studies, we propose that it is the methylation of the epigenetic mark upstream of *H19* that is essential for *Igf2* expression, and would further suggest that the function of methylating this mark may be to inactivate a chromatin boundary that isolates *Igf2* from the downstream enhancers. On the maternal chromosome, where the epigenetic boundary is unmethylated, and therefore active, *Igf2* is insulated from its transcriptional regulatory elements.

Material and methods

Mutant genotyping

Dmmt genotyping was accomplished by a PCR reaction that detected both the wild-type locus with forward *Dmmt* 1114 primer (5'-CCTTCAGTGTGTACTGCAGTCG-3') and reverse *Dmmt* 1169 primer (5'-AATGAGACCGGTGTCGACAG-3') and the targeted locus with forward *Dmmt* 1114 primer and reverse *Pgk3* primer (5'-CTTGTGTAGCGCCAAGTGC-3'). Genomic DNA (100 ng) was amplified for 35 cycles at 90°C for 30 sec, 53°C for 30 sec, 72°C for 30 sec in 20 μ l.

H19^{Δ13} is a replacement from an *EcoRI* site at -10 kb to a *SalI* site at +3 kb at the *H19* locus with the *Pgkneo* cassette (Leighton et al. 1995a). Genotyping was performed by use of the forward primer in the region 5' of the *H19^{Δ13}* deletion (5'-CAGTGTGGGAAACAGCCT-3') and *Pgk3* for the reverse primer. *luc* genotyping by PCR utilized the forward primer LH195' (5'-GC-CATGTACTGATTGGTTGA-3') and the reverse primer Luc3' (5'-TTGCTCTCCAGCGGTTCCAT-3'). Conditions for amplification were identical to those used for *Dmmt* genotyping.

RNA analysis

RNAs were prepared from pools of from four to five embryos in the *Dmmt*;*H19^{Δ13}* crosses with the RNeasy kit from Ambion. Ten micrograms of each RNA were hybridized to radiolabeled RNA antisense probes specific for rpl32 (Webber et al. 1998), *Igf2* (Leighton et al. 1995a), and *H19* (Brunkow and Tilghman 1991) by use of the Ambion RPAII kit. Protected fragments were resolved by electrophoresis on 7.5% denaturing acrylamide gels run in 1× TBE.

The *luc* riboprobe was generated by ligating a 540-bp *XbaI*-*EcoRI* fragment from pGL2 into BS KSII⁻, linearizing with *BsrFI* and transcribing with T3 polymerase. Ten micrograms of total neonatal liver RNA was used in each assay.

Generation of *H19^{luc}* mutant mice

H19 genomic clones were isolated from a 129Sv/J bacteriophage

genomic library (Stratagene). A *Bgl*III–*Dra*III fragment of the *H19* 5' flank from –1.8 kb to +3 bp and a *Sall*–*Bgl*III 6.5-kb fragment of *H19* 3'-flanking sequence were cloned into Bluescript KSII[–]. The *luc* was isolated from the pGL2 vector (Promega) as a *Hind*III–*Sall* fragment and ligated to the 5' arm. The diphtheria toxin A-chain cassette was ligated downstream of the 3' homologous sequence. *neo/tk* (Wu et al. 1994) was ligated between two tandem loxP sites and inserted immediately 3' of *luc* in the final targeting vector. E14.1 ES cells (Kuhn et al. 1991) were transfected as described (Webber et al. 1998) and selected for 6–7 days with 200 µg/ml G418 (active) before picking *neo*^R clones. Cre excision and derivation of chimeric mice and germ-line off-spring were performed as described (Webber et al. 1998).

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***Igf2* imprinting does not require its own DNA methylation or *H19* RNA**

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