An enhancer deletion affects both *H19* and *Igf2* expression

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The distal end of mouse Chromosome 7 contains four tightly linked genes whose expression is dependent on their parental inheritance. *Mash-2* and *H19* are expressed exclusively from the maternal chromosome, whereas *Insulin-2* (*Ins-2*) and *Insulin-like growth factor 2* (*Igf2*) are paternally expressed. The identical expression during development of the 3'-most genes in the cluster, *Igf2* and *H19*, led to the proposal that their imprinting was mechanistically linked through a common set of transcriptional regulatory elements. To test this hypothesis, a targeted deletion of two endoderm-specific enhancers that lie 3' of *H19* was generated by homologous recombination in embryonic stem cells. Inheritance of the enhancer deletion through the maternal lineage led to a loss of *H19* gene expression in cells of endodermal origin, including cells in the liver, gut, kidney, and lung. Paternal inheritance led to a very similar loss in the expression of *Igf2* RNA in the same tissues. These results establish that *H19* and *Igf2* utilize the same endoderm enhancers, but on different parental chromosomes. Mice inheriting the enhancer deletion from fathers were 80% of normal size, reflecting a partial loss-of-function of *Igf2*. The reduction was uniformly observed in a number of internal organs, indicating that insulin-like growth factor II (IGFII), the product of *Igf2*, acts systemically in mice to affect prenatal growth. A modest decline in *Ins-2* RNA was observed in the yolk sac. In contrast *Mash-2*, which is expressed in spongiotrophoblast cells of the placenta, was unaffected by the enhancer deletion.

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Genomic imprinting is a process by which the two parental alleles of an autosomal gene are differentially expressed (Solter 1988; Efstratiadis 1994). The majority of imprinted genes that have been identified in mice and humans exist in clusters of reciprocally imprinted genes, suggesting the possibility of a common imprinting mechanism involving relatively large chromosomal domains. One such cluster lies on the distal end of the mouse Chromosome 7 (Zemel et al. 1992; Guillemot et al. 1995). It contains at least four imprinted genes spanning ~350 kb of DNA: *Mash-2*, a helix-loop-helix transcription factor, and *H19*, a noncoding RNA, lie at the extremes of the cluster and are maternally expressed (Bartolomei et al. 1991; Guillemot et al. 1995). Two growth-factor genes, *Insulin-2* (*Ins-2*) and *Insulin-like growth factor 2* (*Igf2*) lie between them and are paternally expressed (DeChiara et al. 1991; Giddings et al. 1994) (Fig. 1A).

A plausible explanation for the imprinting of the 3'-most genes, *H19* and *Igf2*, was first suggested by their essentially identical patterns of expression during development (Bartolomei and Tilghman 1992; Zemel et al. 1992; Bartolomei et al. 1993). The model proposed that the genes compete for a common set of regulatory elements, such as enhancers. On the paternal chromosome, sperm-specific methylation of the *H19* promoter and

gene prevent its expression (Bartolomei et al. 1993; Ferguson-Smith et al. 1993; Li et al. 1993; Tremblay et al. 1995) and thereby permit paternal-specific expression of the *Igf2* gene. On the undermethylated maternal chromosome, transcription of *H19* is favored by virtue of its proximity to the regulatory elements and/or the greater strength of its promoter relative to *Igf2*. This model implies that the *H19* gene is autonomously imprinted, whereas the imprinting of *Igf2* requires its linkage to the *H19* gene.

Several lines of evidence have lent support to this model. First, transgenes carrying the *H19* gene region, including the majority of the paternal-specific 5' methylation domain and two 3' endoderm-specific enhancers, display appropriate tissue-specific expression and imprinting at heterologous chromosomal locations, demonstrating the autonomy of *H19*'s imprinting (Bartolomei et al. 1993). In contrast, a survey of 26 kb DNA within the *Igf2* locus failed to identify any enhancer elements in a muscle cell line (Kou and Rotwein 1993). Furthermore, a transgene that contained the 5' end of the *Igf2* gene fused to β -galactosidase was not appropriately expressed in mice and imprinted in only a minority of the lines examined (Lee et al. 1993). This suggested that the 5' end of the *Igf2* gene was not sufficient for either its tissue-specific or allele-specific expression. Second, a targeted

Figure 1. Targeting of the *H19* enhancer region in ES cells. (A) The locations of *Mash-2*, *Ins-2*, *Igf2*, and *H19* on mouse chromosome 7 (Zemel et al. 1992; Guillemot et al. 1995). The distance between *Mash-2* and the other genes is ~200–300 kb. (●) The *H19* enhancers. (B) The structure of the targeting vector that was used to replace the *H19* enhancers (●) and a DNase I hypersensitive site (vertical arrow) is shown on the middle line. The transcriptional orientations of the PGKneoBpA (*neo*) and herpes simplex virus thymidine kinase genes (*tk*) are indicated by the horizontal arrows. The broken lines join the regions of homology shared by the endogenous locus and the targeting vector. The positions of the restriction enzyme sites used to diagnose a targeting event, and the sizes of the fragments produced, are indicated above the endogenous and targeted loci. The positions of probes A and B are indicated by the hatched boxes. Restriction endonuclease recognition sites are abbreviated as follows: (R) *EcoRI*; (S) *SalI*; (B) *BamHI*; (Bg) *BglII*; (X) *XbaI*; (Xm) *XmnI*. (C) Genomic DNAs derived from wild-type (+/+), heterozygous (+/-), and homozygous (-/-) progeny were digested with *EcoRI* or *XmnI* and hybridized to probe A or B, respectively.

deletion of the *H19* gene on the maternal chromosome resulted in the loss of imprinting of *Igf2*, indicating that the sequences required for *Igf2* imprinting reside close to the *H19* gene (Leighton et al. 1995).

The enhancer competition model rests on the premise that *H19* and *Igf2* utilize the same enhancers. Only two enhancers have been identified in the vicinity of the *Igf2* and *H19* genes, at +9 and +11 kb relative to the start of transcription of the *H19* gene (Yoo-Warren et al. 1988) (Fig. 1A). In both tissue-culture cells and transgenic mice, these enhancers direct expression of *H19*-based constructs in endodermal cell types in liver, gut, choroid plexus, and yolk sac (Brunkow and Tilghman 1991; J.R. Saam, unpubl.). In support of the notion that both the maternal and paternal copies of these enhancers are active, they were shown to be in equally open chromatin conformation (Bartolomei et al. 1993; Ferguson-Smith et al. 1993). To ask whether they are required for expression of both *H19* and *Igf2*, a line of mice was generated in which the enhancers were deleted, and the consequence to the expression of *H19* and *Igf2* was assessed.

Results

Deletion of the *H19* enhancers

A gene targeting approach was used to delete the endo-

derm-specific enhancers in the *H19* locus in mouse embryonic stem (ES) cells. A 6.2-kb region of DNA was replaced with a *neo^r* cassette conferring resistance to the drug G418 (Fig. 1B). The deleted region contains the two enhancers as well as a DNase I hypersensitive site that was identified in nuclei from neonatal liver (Brunkow and Tilghman 1991). The significance of this hypersensitive site is unclear, as its inclusion in transgenic constructs did not affect the expression patterns of the *H19* gene.

The *neo^r* ES cells harboring the correct homologous recombination event were identified by Southern blot analysis with probes A and B, which lie outside the DNA in the targeting vector (Fig. 1C). These cells were injected into host blastocysts, and the resulting chimeric mice were bred to generate progeny carrying the enhancer deletion in the germ line (Stewart 1993).

Maternal inheritance of the *H19* enhancer deletion

To examine the consequences of the enhancer deletion on the expression of the *H19* gene, heterozygous females were mated to wild-type males, and RNA was prepared from tissues of neonatal progeny. The levels of *H19* and *Igf2* RNAs were determined by an RNase protection assay (Fig. 2A). The heterozygous progeny (-/+) exhibited

A. Maternal inheritance

B. Paternal inheritance

Figure 2. Effect of the *H19* enhancer deletion on the expression of *H19* and *Igf2*. (A) Total RNA was isolated from the tissues indicated from one wild-type (+/+) and two maternal heterozygous (-/+) littermates. Each RNA was hybridized in the same tube to radiolabeled *H19* and *Igf2* RNA probes, with the following amounts of RNA and ages: p3 gut (10 μ g); p7 liver (10 μ g), kidney (15 μ g), lung (20 μ g), skeletal muscle (1 μ g), and brain (30 μ g); E13.5 yolk sac (3 μ g) and placenta (1 μ g). p0 is the day of birth. The migration of the *H19* and *Igf2* protected fragments are indicated by arrows (right). (Lanes M) Radiolabeled pBR322 digested with *Msp*I; (lanes pH19 and pIgf2) the migration of the full-length probes; (lanes H19 and Igf2) protected fragments from neonatal liver RNA hybridized with the *H19* probe or *Igf2* probe separately. (B) Total RNA was isolated from wild type (+/+) and paternal heterozygotes (-/+), and treated as in A.

the most dramatic decline in *H19* RNA in liver, which is primarily composed of endodermal cells. In gut, kidney, and lung, where both endodermal and mesodermal cells are present, a substantial decline in *H19* RNA was also observed. In the rest of the tissues sampled, including extraembryonic tissues such as yolk sac and placenta, as well as muscle and brain, there was either no decrease or a very modest (<50%) decline in *H19* RNA.

The absence of an effect of the enhancer deletion was anticipated in skeletal muscle because both transgene and transfection studies had suggested that no regulatory elements required for muscle expression were present in the 6.2-kb deletion (Brunkow and Tilghman 1991). In brain, however, the absence of an effect was unexpected, as transgenes under the control of the *H19* enhancers had displayed brain-specific expression (K. Pfeifer and J.R. Saam, unpubl.). This suggests that additional functionally redundant regulatory elements act in these tissues.

The levels of *Igf2* RNA were unaffected by the maternal deletion of the enhancers, consistent with the fact that this allele of *Igf2* is normally silent. Therefore, *Igf2* RNA was used as a control to assess the extent of the decline of *H19* RNA as determined by densitometry and

comparison of the levels of *H19* and *Igf2* transcripts. In maternal heterozygotes, liver maintained ~10% of the wild-type level of *H19* RNA, gut and kidney ~25%, and lung 30%. An allele-specific assay of RNA obtained from the progeny of female heterozygotes crossed to males harboring the *Mus castaneus* allele of the *H19* gene demonstrated that the decline in *H19* expression in liver and gut originated from the maternal allele (Fig. 3A).

Paternal inheritance of the enhancer deletion

To assess the effect of the enhancer deletion on the *Igf2* gene, male heterozygotes were crossed to wild-type females, and *Igf2* and *H19* RNAs were measured in tissues isolated from the heterozygous progeny. As shown in Figure 2B, the levels of *Igf2* RNA declined in exactly the same manner as *H19* RNA in the maternal heterozygotes. That is, the decrease was most pronounced in liver, but was also evident in gut, kidney, and lung. Thus the 3' endoderm enhancers are required equally for expression of both genes but on different parental chromosomes. An allele-specific RNase protection assay re-

Figure 3. Allele specificity of *H19* and *Igf2* expression. (A) Female heterozygotes were crossed to male B6(CAST-*H19*) (Tremblay et al. 1995), and RNA was prepared from neonatal liver, gut, and muscle from wild type (+/+), and maternal heterozygous (+/-) littermates. The RNAs were analyzed by use of an allele-specific RNase protection assay. Arrows indicate the migration of maternal (Mat) and paternal (Pat) transcripts. (Lane Cas) The migration of protected fragment obtained from homozygous neonatal B6(CAST-*H19*) liver RNA. (P) Full-length probe. (B) B6(CAST-*H19*) females were crossed to males carrying the *H19* enhancer mutation, and the progeny were analyzed for *Igf2* RNA expression with an allele-specific assay as in A.

vealed that the residual expression of *Igf2* in liver and gut derives from the paternal allele (Fig. 3B).

In situ hybridization analysis of the enhancer deletion

The tissue survey of the loss of *Igf2* and *H19* expression in Figure 2 was extended by use of *in situ* hybridization analysis of embryos. Embryos derived from crosses of either male or female heterozygotes to wild-type animals were harvested at embryonic day 13.5 (E13.5), sectioned, and hybridized to either *Igf2*- or *H19*-specific probes. Figure 4 (A,D) illustrates the overall similarity in the nor-

mal patterns of expression of *H19* and *Igf2* RNA at this stage of development (Lee et al. 1990; Poirier et al. 1991). The retention of high-level *Igf2* and *H19* RNA expression in paternal and maternal heterozygotes in mesodermal tissues such as the tongue, the somites, and the heart indicate that the enhancers for those tissues lie outside the deletion boundaries (see Fig. 4, B and C, for *Igf2* and *H19* RNA, respectively). More importantly, every tissue that exhibited a decline in *Igf2* RNA in the paternal heterozygotes also suffered a decline in *H19* RNA in the maternal heterozygotes.

If the two endoderm enhancers are both necessary and sufficient for all endoderm expression, one would predict that the residual expression of *H19* and *Igf2* RNAs in tissues such as gut, kidney, and lung would derive from expression in mesodermal cells. To test this, *H19* expression was examined in greater detail in those tissues (Fig. 5). In the fetal gut, wild-type animals express *H19* RNA at high levels in the gastric epithelial cells that line the lumen of the gut and at lower levels in the smooth muscle cells that surround them. In the maternal heterozygotes, the smooth muscle cell expression is maintained, whereas the endodermally derived epithelial cell expression is reduced (Fig. 5C). Likewise, in the fetal lung, the developing bronchioles are composed of bronchial epithelial cells surrounded by smooth muscle cells. It is evident in the maternal heterozygotes that *H19* expression is reduced in the epithelia but not the smooth muscle cells (Fig. 5A). Expression of *H19* in the kidney is primarily found in the mesonephric tubules, which lose their high level expression of *H19* RNA in maternal heterozygotes (Fig. 5B). In the fetal liver, the stippled pattern of hybridization in wild-type animals reflects the fact that at this stage in development, 50% of the cells are of hematopoietic origin, and these do not express *H19*. In the maternal heterozygotes, high-level *H19* expression becomes restricted to cells that surround the central vein and the visceral peritoneum. For each of these tissues, the extent of the decline in *H19* RNA, as measured by RNase protection, is an underestimate of the importance of the enhancers in specific endodermal cell types. Thus, the modest declines more often reflect tissue heterogeneity rather than redundancy in endoderm-specific *cis*-acting elements.

Effect of the enhancer deletion on *Ins-2* and *Mash-2*

The *Ins-2* gene, which lies 10 kb upstream of *Igf2*, is paternally expressed in the mouse yolk sac (Giddings et al. 1994). Like *Igf2*, its imprinting is disrupted by deletion of the *H19* gene region, indicating that it is also under the control of the *H19* gene region (Leighton et al. 1995). To ask whether its expression in the yolk sac is also dependent on the *H19* enhancers, a single-strand conformation polymorphism PCR assay was used to evaluate the levels of *Ins-2* RNA in wild-type and paternal heterozygous yolk sacs. As shown in Figure 6A, the levels of *Ins-2* RNA were reduced ~30% in the paternal heterozygotes, relative to wild-type littermates, equivalent in magnitude to the decline in *H19* and *Igf2* RNA.

Figure 4. In situ hybridization analysis of *Igf2* and *H19* expression in embryos. Frozen sections of maternal heterozygous E13.5 embryos (A,C) and paternal heterozygous embryos (B,D) were hybridized to radiolabeled *Igf2* (A,B) or *H19* (C,D). (c) Choroid plexus; (t) tongue; (s) somites; (h) heart; (g) gut; (li) liver; (lu) lung. The sections were photographed at 8 $\times$ magnification by use of dark-field microscopy.

To control for the quality and amount of RNA, the same samples were assayed for *H19* RNA, which does not change in paternal heterozygotes (see Fig. 2B).

The *Mash-2* gene is maternally expressed in the spongiotrophoblasts of the placenta (Guillemot et al. 1995). The levels of *Mash-2* RNA were quantitated by an RNase protection assay with *Igf2* RNA as a control to ensure that the amounts of RNA were comparable between wild-type and maternal heterozygotes (see Fig. 2A). As shown in Figure 6B, *Mash-2* was unaffected by deletion of the *H19* enhancers on the maternal chromosome. This is consistent with the observation that the imprinting of *Mash-2* is not affected in mice carrying the *H19* gene deletion (T. Caspary, P.A. Leighton, unpubl.).

Effect of the *H19* enhancer deletion on growth

Insulin-like growth factor II (IGFII) is a critical growth

factor during the second half of embryogenesis in mice. Mice inheriting a null mutation in *Igf2* from fathers are 60% normal sized at birth, and the proportional dwarfism is maintained throughout their adult life (DeChiara et al. 1990, 1991). Unlike the *Igf2*-null mice, progeny derived from *H19* enhancer deletion fathers express lower levels of *Igf2* RNA only in endodermal tissues. To ask how this tissue-specific decline in IGFII affected overall growth, *H19* enhancer heterozygotes of both sexes were mated to wild-type mice, and the resulting progeny were weighed at birth and 3 weeks of age (Table 1). Mice inheriting the deletion from their fathers weighed between 70% and 73% of their wild-type littermates at birth, and 80% at 3 weeks of age. Thus, the paternal heterozygotes were intermediate in size between mice that were null for *Igf2* and wild-type animals. *H19* enhancer maternal heterozygotes, on the other hand, which exhibited a decrease in *H19* RNA

Figure 5. In situ hybridization analysis of *H19* expression in organs. Wild type (+/+) and maternal heterozygous embryos (-/+) were sectioned at E13.5, and hybridized to an *H19*-specific RNA probe. The organs indicated were photographed at high magnification. (m) Smooth muscle; (e) bronchial and gastric epithelia; (t) metanephric tubules; (CV) central vein. The sections were photographed at 200 $\times$ magnification by use of dark-field microscopy (bottom) and light-field microscopy (top).

only, were indistinguishable in size from their littermates at both stages of development.

A *trans*-gene that directed IGFII expression to the adult skin and uterus displayed selective somatic overgrowth in those tissues (Ward et al. 1994), but very little effect on overall growth. These observations suggest that IGFII acts locally, rather than systemically via the circulation. If this is the case, one would predict that the *H19* enhancer paternal heterozygotes would exhibit non-uniform growth retardation in the visceral organs. To test this, three affected organs (liver, lung, and kidney),

one expressing but unaffected organ (heart), and a non-expressing organ (spleen) from 3-week-old mice derived from both maternal and paternal heterozygotes were weighed. As the data in Table 2 indicate, the growth retardation in paternal heterozygotes was observed in spleen, a tissue that does not express IGFII, as well as in liver, kidney, and lung. The least affected tissue was heart, which showed a small decrease in size in both maternal and paternal heterozygotes. Thus, no evidence for gross disproportionate growth in the paternal heterozygotes was observed, suggesting that IGFII af-

Figure 6. The effect of the *H19* enhancer deletion on *Ins-2* and *Mash-2*. (A) Expression of *Ins-2* RNA in wild-type (+/+) and paternal heterozygous (+/-) E13.5 yolk sacs was determined by a 30-cycle PCR assay (Giddings et al. 1994). (panc) RNA isolated from adult pancreas. *H19* RNA served as a control for the amount of RNA (data not shown). The multiple bands, all of which represent *bona fide Ins-2* products, reflect the fact that the products were denatured and separated on a native acrylamide gel. (B) Total RNA was isolated from placentas at E13.5 from crosses of *H19* enhancer heterozygous females to wild-type males. RNase protection was performed with two wild-type (+/+) and four maternal heterozygous (-/+) littermates. *Igf2* RNA served as a control for the amount of RNA (see Fig. 2A).

affected both expressing and nonexpressing tissues similarly.

Discussion

Implications for the enhancer competition model

Mice carrying a deletion in two endoderm-specific enhancers that lie 3' of the *H19* gene exhibit disruptions in the expression of both *H19* and *Igf2*, but on different parental chromosomes, providing compelling evidence

that these enhancers are shared by the genes. Thus, a central prediction of the enhancer competition model to explain the reciprocal imprinting of *Igf2* and *H19* has been met. The model was based on the observation that the two genes were expressed in virtually identical tissues during development, as is evident from the *in situ* hybridization studies in Figure 4, as well as from comparison of previous work by others on the individual genes (Lee et al. 1990; Poirier et al. 1991). The identity of expression of these two genes can now be ascribed to the fact that they utilize the same *cis*-acting regulatory elements, at least in a substantial number of endodermal tissues.

That competition between linked genes for a single enhancer could act as a developmental switch was first demonstrated in the chicken β -globin cluster by Engel and his colleagues (Choi and Engel 1988; Foley and Engel 1992). Between the tandemly arrayed embryonic ϵ - and adult β -globin genes lies a single erythroid-specific enhancer. The preferential expression of the embryonic gene early in development is thought to arise from its inherently stronger promoter. Later in development, with the induction of the adult stage-specific transcription factors, the adult gene becomes the better competitor for the enhancer (Gallarda et al. 1989).

In the case of the competition for enhancers between *H19* and *Igf2*, the difference between the maternal and paternal chromosomes is thought to arise from differential DNA methylation inherited from gametes (Bartolomei et al. 1993; Li et al. 1993; Tremblay et al. 1995). When the *H19* promoter is methylated on the paternal chromosome, the *H19* gene is silenced, thereby permitting *Igf2* expression. The importance of DNA methylation for regulating the imprinting on the paternal chromosome comes from an analysis of mice that lack DNA methyltransferase, the hemimethylase required to maintain DNA methylation (Li et al. 1992, 1993). In mutant embryos, the paternal *H19* gene is reactivated, and the *Igf2* gene is silenced. On the undermethylated maternal chromosome, the *H19* gene competes successfully for the enhancers and thereby excludes expression of *Igf2*. Two possible explanations for the preferential expression of *H19* are that it has the inherently stronger promoter, or it lies closer to the enhancers. We have recently provided compelling data in support of this link between the genes, by establishing that a maternal deletion of the *H19* gene and its 5' flank leads to the activation of the normally silent maternal *Igf2* gene (Leighton et al. 1995).

The enhancer deletion has no effect on the pattern of DNA methylation at the *H19* gene, nor does the deletion affect the paternal-specific methylation in a region upstream of the *Igf2* gene (data not shown). Thus, the enhancers are not required for the establishment or maintenance of the parental-specific methylation at either gene. This result was anticipated from the observation that the somatic DNA methylation patterns of both genes are independent of gene expression (Sasaki et al. 1992; Bartolomei et al. 1993; Ferguson-Smith et al. 1993).

Table 1. Effect of enhancer mutation on growth

Cross	Genotype	Birth		3 weeks	
		weight	EnhΔ/wt	weight	EnhΔ/wt
Maternal inheritance	wt	1.84 ± 0.24 (4)		9.52 ± 0.52 (4)	
	EnhΔ	1.92 ± 0.13 (4)	1.04	9.51 ± 0.73 (6)	1.00
	wt	1.31 ± 0.25 (4)		13.0 ± 0.83 (2)	
	EnhΔ	1.50 ± 0.13 (6)	1.14	14.5 ± 0.98 (2)	1.11
Paternal inheritance	wt	1.64 ± 0.12 (6)		8.62 ± 0.78 (9)	
	EnhΔ	1.20 ± 0.12 (10)	0.73	6.93 ± 0.94 (6)	0.80
	wt	1.48 ± 0.26 (6)		9.22 ± 0.78 (7)	
	EnhΔ	1.04 ± 0.21 (3)	0.70	7.33 ± 1.24 (6)	0.80

The weights of the wild-type (wt) and heterozygous (EnhΔ) progeny derived from two litters each of crosses between female or male heterozygotes to wild-type animals were determined at birth or 3 weeks of age. The average weights are expressed in grams ± s.d. for the number of progeny indicated in parentheses. EnhΔ/wt is the ratio of the average weights of the two progeny classes.

Tissue specificity of the 3' enhancers

The experiments in Figures 2 and 4 provide insight into the role of the 3' enhancers in the overall pattern of expression of the *H19* and *Igf2* genes. The decreases in expression of *H19* and *Igf2* RNAs were most pronounced in liver, in which the primary expressing cell, the hepatocyte, is of endodermal origin. The dramatic and equivalent extent of the decline for both genes implies that the deleted 6.2-kb region is equally necessary for their high-level expression. This conclusion is consistent with experiments conducted with *H19* transgenes containing the 3' enhancers, in which the transgenes were expressed at high levels in liver (Brunkow and Tilghman 1991; Bartolomei et al. 1993). Likewise, the absence of a significant effect in muscle and heart was predicted on the basis of the absence of transgene expression in those tissues.

One inconsistency between the transgene experiments and the enhancer deletion mice was evident in the choroid plexus of the brain. Whereas the enhancer disruption had no effect on expression of either gene in the choroid plexus, transgenes containing the 3' enhancers are faithfully expressed in that tissue (Brunkow and Tilghman 1991; K. Pfeifer and J.R. Saam, unpubl.). This result suggests that additional redundant regulatory ele-

ments for that tissue remain at the locus on the deletion chromosome. The choroid plexus is unusual in another respect in that it exhibits coincident expression of *Igf2* and *H19* on the maternal chromosome (DeChiara et al. 1991; J.R. Saam, unpubl.), in apparent contradiction to the enhancer competition model. One can reconcile the coincident maternal expression of the two genes with the model by proposing the existence of *Igf2*-specific enhancers for which the *H19* gene does not compete.

The lack of a substantial effect of the enhancer deletion on either *H19* or *Igf2* in the extra-embryonic tissues, yolk sac, and placenta, reduced the likelihood that an effect would be observed with either *Ins-2* or *Mash-2*. In fact, *Ins-2* is affected to the same small degree as *H19* and *Igf2* in the yolk sac. That tissue is composed of both endoderm and mesoderm cell types, and it is conceivable that for all three genes the expression in the enhancer deletion is mesoderm specific. On the other hand, it is possible that additional regulatory elements that contribute to yolk sac endoderm expression remain intact on the mutant allele.

Generality of the enhancer competition model

It is increasingly clear that imprinted genes often reside

Table 2. Effect of enhancer mutation on organ size

Cross	Genotype (no. of animals)		Weight					
			heart (%)	lung (%)	liver (%)	kidney (%)	spleen (%)	
Maternal inheritance	wt	(3)	94 ± 14	139 ± 17	376 ± 57	139 ± 6	66 ± 10	
	EnhΔ	(6)	83 ± 10 (88)	130 ± 23 (94)	392 ± 39 (104)	142 ± 17 (102)	69 ± 8 (105)	
	wt	(2)	107	148	492	184	84	
	EnhΔ	(2)	89 (83)	153 (103)	581 (118)	195 (106)	71 (84)	
Paternal inheritance	wt	(9)	80 ± 9	115 ± 13	374 ± 49	88 ± 14	48 ± 13	
	EnhΔ	(6)	62 ± 12 (78)	97 ± 23 (84)	269 ± 57 (72)	48 ± 13 (55)	32 ± 13 (66)	
	wt	(7)	88 ± 25	129 ± 23	385 ± 35	154 ± 16	83 ± 18	
	EnhΔ	(6)	83 ± 25 (94)	94 ± 25 (73)	309 ± 65 (80)	102 ± 21 (66)	48 ± 17 (57)	

The weights of the organs of wild-type (wt) and heterozygous (EnhΔ) progeny derived from two litters each of crosses between female or male heterozygotes to wild-type animals were determined at 3 weeks of age. The average weights are expressed in mg ± s.d. for the number of progeny indicated. The percentage is the EnhΔ average weight/wt average weight × 100.

in proximity to other reciprocally imprinted genes. In humans, the Prader-Willi and Angelman syndromes are two tightly linked genetic disorders associated with deletions of chromosome 15q11-13 (Nicholls 1993). When the deletions are inherited paternally, Prader-Willi syndrome is observed, whereas maternal inheritance leads to the distinctly different symptoms of Angelman syndrome. It is postulated that the region harbors both maternally and paternally expressed genes, although to date only paternally expressed genes have been found in the Prader-Willi region (Ozcelik et al. 1992; Reed and Leff 1994; Sutcliffe et al. 1994; Wevrick et al. 1994). No Angelman candidate gene has been identified. Of interest to this discussion is the number of small deletions that lie in the Prader-Willi region have been identified in Angelman as well as Prader-Willi patients, suggesting that this region is controlling the expression of both the maternally and paternally expressed genes (Buiting et al. 1995).

A third cluster of reciprocally imprinted genes maps to a 300-kb region on the proximal end of chromosome 17 in the mouse and includes the maternally expressed *Igf2r* gene and the paternally expressed *Mas* proto-oncogene (Barlow et al. 1991; Villar and Pedersen 1994). At the moment, nothing is known about their possible connection to one another.

Imprinting and growth control

Several explanations have been proposed for the acquisition of genomic imprinting in eutherian mammals (Barlow 1993; Varmuza 1993; Thomas 1994; Varmuza and Mann 1994). To date, the most compelling model has been provided by Haig and his colleagues, who suggest that imprinting evolved in mammals because of the conflicting interests of maternal and paternal genes within a litter (Moore and Haig 1991). In mammals, which are primarily nonmonogamous, the mother provides significant maternal resources to the offspring. Successful passage of paternal genes into the next generation is best ensured by having the embryos consume maternal resources, even if by so doing the fitness of her future litters is compromised. The mother's interests are best served by distributing her resources among litters.

Haig's model predicts that paternally expressed genes will promote embryonic growth, whereas maternal genes will act to restrain the use of maternal resources. As such, it is in remarkably good agreement with the function of the *Ins-2*, *Igf2*, and *H19* genes. Mouse embryos are exquisitely sensitive to the levels of the paternally expressed *Igf2*: A complete loss-of-function of the growth factor leads to a 40% reduction in birth weight (DeChiara et al. 1991), whereas a partial loss of function in mice that inherit a paternal *H19* enhancer deletion yields animals with a 30% reduction in birth weight (Table 1). By 3 weeks of age, the animals have partially compensated for the reduction of IGFII and are 80% normal sized. We presume that the postnatal increase in relative growth is the result of the fact that the major source of

IGFII production shifts from the fetal liver, an affected tissue, to the skeletal muscle, an unaffected tissue (J.R. Saam, data not shown). Two maternally expressed genes have been shown to reduce the effective IGFII concentration in embryos: *H19* by virtue of its role in the transcriptional silencing of the maternal *Igf2* gene (Leighton et al. 1995), and *Igf2r*, which acts as a sink for the growth factor. Fetuses that lack the receptor are ~30% larger than normal, have elevated circulating levels of IGFII, and die around birth (Lau et al. 1994; Wang et al. 1994). It has been argued that the lethality is the consequence of a gain of function of the effective concentration of circulating IGFII on the basis of the observation that the lethality can be abrogated by paternal inheritance of an *Igf2* null mutation (Filson et al. 1993; Wang et al. 1994).

The implication of these observations is that evolution is acting to finely regulate the expression of the *Igf2* gene. To date, the longest surviving 9-month-old loss-of-function *H19* mice show no phenotype other than somatic overgrowth, which can be attributed to overexpression of *Igf2* (Leighton et al. 1995). That is, the only function that can be ascribed to *H19* is the regulation of *Igf2* imprinting. Whether the *H19* gene performs this function by acting solely as a transcriptional foil for *Igf2*, or whether the RNA product itself plays a role, remains to be seen.

Materials and methods

Preparation of the enhancer targeting vector

Genomic clones containing the *H19* 3' flanking sequences were isolated from a 129Sv/J-derived bacteriophage genomic library obtained from Stratagene. A targeting vector was constructed that contained a 4.3-kb *SalI*-*XbaI* *H19* genomic DNA fragment corresponding to +3 to +7.3 kb relative to the start of transcription, a 1.6-kb PGKneoBpA (neo) cassette (Soriano et al. 1991), a 6.2-kb *Bam*HI-*Bgl*III *H19* genomic DNA fragment from +13.5 to +19.7 kb, and the herpes simplex virus thymidine kinase gene (tk). The vector was linearized at a unique *SalI* site before electroporation into ES cells.

Derivation of mice carrying the enhancer deletion

W9.5 ES cells (Lau et al. 1994) were grown on neo^r primary embryonic fibroblasts as described (Abbondanzo et al. 1993). Cells (3×10⁷) were collected in 0.8 ml of phosphate-buffered saline (PBS) and electroporated with a single pulse of 250 μF and 320 V with 25 μg of linearized targeting vector. Drug selection in 350 μg/ml of G418 and 0.2 μM FIAU was initiated 24 hr later. Eight days after electroporation, individual colonies were isolated and grown up, and genomic DNA was prepared. The DNAs were digested with *Eco*RI, pooled in groups of 3, and separated by electrophoresis through a 1% agarose gel. The DNA was transferred to a nitrocellulose filter (Southern 1975) and hybridized to a 3-kb *Eco*RI-*SalI* fragment containing the *H19* structural gene, labeled by random hexamer priming (Feinberg and Vogelstein 1983). Of 120 clones screened, 4 displayed the 9.4-kb *Eco*RI fragment corresponding to the mutation. Upon further analysis, only one clone (E2.2) maintained a 1:1 ratio of band intensities of wild-type to mutant at the *H19* locus. These cells were injected into C57Bl/6J blastocysts (Stewart 1993),

and the resulting male chimeras were bred to C57Bl/6J females to obtain germ-line transmission of the mutation.

DNA was extracted from tail biopsies of the progeny of the chimeric mice and digested with *EcoRI* or *XmnI*. The DNAs were separated on a 0.8% agarose gel, transferred to nitrocellulose, and hybridized to the 3-kb *EcoRI*–*SalI* fragment (Probe A; *EcoRI* digests) or a 200-bp PCR product amplified from a region 3' of the DNA included in the targeting vector (probe B, Fig. 1; *XmnI* digests). The forward primer was 5'-GATCTCATCCGG-GAAAGAGC-3', and the reverse primer was 5'-GTTATCT-CAANACCCAAAGGG-3'.

Quantitation of RNA

Total cellular RNA was isolated from the organs of neonatal and 3-week-old progeny by LiCl–urea extraction (Auffray and Rougeon 1980). Allele nonspecific RNase protection probes for *H19* (Brunkow and Tilghman 1991) and *Igf2* (DeChiara et al. 1991) were synthesized in vitro in the presence of [³²P]CTP, purified by gel electrophoresis, and hybridized together with RNA at 50°C overnight. The samples were digested with 50 µg/ml of RNase A and 2 µg/ml of RNase T1 for 1 hr at 30°C, and the products were separated on a 7.5% acrylamide, 7 M urea gel and visualized by autoradiography. The *H19* and *Igf2* allele-specific probes were generated as described (Bartolomei et al. 1991; Leighton et al. 1995), except the *H19* template was linearized with *SmaI* instead of *XbaI* to generate a smaller probe.

The allele-specific *Ins-2* PCR-based assay (Giddings et al. 1994) was executed as described (Leighton et al. 1995) except the number of cycles in the PCR was varied from 25 to 35 to ensure that the signal was in the linear range of amplification.

A 450-bp *HindIII* fragment from the 5'-untranslated region of the *Mash-2* gene (Guillemot et al. 1994) was subcloned into Bluescript KSII. The plasmid was linearized with *AccI* and transcribed in the presence of [³²P]CTP with T7 RNA polymerase. Gel-purified probe (1 × 10⁵ cpm) was mixed with E13.5 placenta RNA, incubated at 55°C overnight, digested with 40 µg/ml of RNase A, and 2 µg/ml of RNase T1 at 23°C for 1 hr and the products were analyzed as above.

In situ hybridization

Embryos were isolated at E13.5 and fixed overnight in 4% paraformaldehyde in PBS. Embryos were put in 12%, 16%, and 18% sucrose solutions for 2 hr each. The embryos were then frozen in OCT media (Tissue Tek) and stored at –80°C. Ten-micrometer sections were cut on a cryostat and stored at –20°C.

The *H19* probe was generated from a *BamHI*–*SmaI* fragment from the first exon cloned into pBluescript II KS (Stratagene). This plasmid was linearized with *XhoI* and transcribed in the presence of [³⁵S]CTP and T7 polymerase (Promega). The *Igf2* vector contained a 400-bp *XbaI*–*BamHI* fragment from the 3'-untranslated region of the gene, cloned into pBluescript II KS. For the antisense strand, T3 polymerase (Promega) was used on the vector linearized with *NotI*.

The in situ hybridization protocol was based on the whole mount protocol described by Conlon and Rossant (1992). Sections were fixed in 4% paraformaldehyde in PBS for 20 min and then washed in PBS twice for 5 min each. The slides were immersed for 10 min in 0.1 M triethanolamine (pH 8) to which 0.5 ml of acetic anhydride was added. The sections were then washed sequentially in 100 mM Tris-HCl (pH 8), 100 mM glycine, PBS; and 0.15 M NaCl for 5 min each. Slides were dehydrated through a 30%, 50%, 70%, 85%, 95%, and 100% ethanol series for 5 min each and then air-dried. The hybridization so-

lution was 50% formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 8), 5 mM EDTA, 10% dextran sulfate, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.5 mg/ml of yeast tRNA, and 10 mM dithiothreitol. To 100 µl of hybridization solution, 1 × 10⁶ cpm of probe was added and applied to each slide. The slides were covered with parafilm and incubated at 65°C overnight. Slides were washed for 5 min in 50% formamide, 0.3 M NaCl, and 0.03 M sodium citrate. They were washed twice in this same solution at 65°C for 30 min and then in RNase buffer [0.5 M NaCl, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA] for 10 min at 25°C and then three times for 10 min at 37°C. Slides were treated in RNase buffer with 25 µg/ml of RNase A and 100 U/ml of RNase T1 for 1 hr at 37°C. After RNase treatment the slides were washed at 65°C for 1 hr in 50% formamide, 0.3 M NaCl, and 0.03 M sodium citrate followed by 0.3 M NaCl, 0.03 M sodium citrate and 0.015 M NaCl, 0.0015 M sodium citrate for 10 min each at 25°C. Slides were air-dried and then dipped in autoradiography emulsion. *H19* slides were left in the dark for 24 hr, whereas *Igf2* slides were left for 72 hr before development.

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