Deletion of the *H19* transcription unit reveals the existence of a putative imprinting control element

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The distal region of mouse chromosome 7 contains a cluster of imprinted genes that includes *H19* and *Igf2* (insulin-like growth factor 2). *H19* is expressed as an untranslated RNA found at high levels in endodermal and mesodermal embryonic tissues. This gene is imprinted and exclusively expressed from the allele of maternal origin. The *Igf2* gene shows a similar pattern of expression but is expressed from the paternal allele. We have generated a targeted deletion of the *H19* transcription unit by insertion of a *neo* replacement cassette. The homozygous mutant animals are viable and fertile and display an overgrowth phenotype of 8% compared with wild-type littermates. This is associated with the disruption of *Igf2* imprinting and the consequent biallelic expression of this gene. A striking feature of the recombinant *H19* allele is the occurrence of a parental imprint set on the *neo* replacement cassette. Therefore imprinting of the *H19* locus is independent of the *H19* gene itself. Taken together with the results of a larger *H19* mutation described previously, this indicates that an imprinting control element is located within the region 10 kb upstream of *H19*.

[Key Words: *H19*; homologous recombination; *Igf2*; genomic imprinting]

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In mammals, normal embryonic development is achieved only in the presence of the maternal and paternal genome (McGrath and Solter 1984; Surani et al. 1984). Extensive research using mice carrying translocations has shown that certain chromosomal regions must carry genes for which the maternal and paternal copy are not functionally identical (Cattanach and Kirk 1985; Beechey and Cattanach 1996). This observation has led to the notion of an imprint set on certain genes during gametogenesis. This results in differential expression of the two parental alleles of these genes, which can occur at certain developmental stages and in certain tissues. Imprinting can therefore be defined by two steps: marking of certain genes at the time the two genomes are separated and recognition of this marking at specific times during embryogenesis. The mechanism by which imprinting is established is as yet unknown, although methylation of CpGs is a possible candidate (Li et al. 1993; Razin and Cedar 1994). Whether this methylation is a cause or only a consequence of imprinting remains unclear (Sasaki et al. 1995).

To date, 17 genes have been shown to be imprinted in mouse and in man (for review, see Barlow 1995; John and Surani 1996). In the distal region of mouse chromosome 7, a cluster of such genes covering ~600 kb has been

described. These genes are *H19*, *Mash2*, and *p57^{Kip2}* (Bartolomei et al. 1991; Guillemot et al. 1995; Hatada and Mukai 1995), which are expressed from the chromosome of maternal origin, and *Igf2* (insulin-like growth factor 2), and *Ins2* (insulin 2), expressed from the chromosome of paternal origin (DeChiara et al. 1991; Giddings et al. 1994; Deltour et al. 1995) (see Fig. 1A). This imprint has also been identified on the human homologs of these genes (Zhang and Tycko 1992; Giannoukakis et al. 1993; Ohlsson et al. 1993; Matsuoka et al. 1996). A remarkable feature of this region is the reciprocal imprint set on the *H19* and *Igf2* genes, one being expressed from the maternal allele and the other from the paternal allele, over a distance of only 80 kb, and their similar pattern of expression in many tissues during embryogenesis (Bartolomei et al. 1991; Zemel et al. 1992).

The *H19* gene is expressed at a very high level in endodermal and mesodermal tissues of the developing mouse embryo (Poirier et al. 1991). After birth, this expression is maintained during several days but then disappears in most tissues with the exception of adult muscle. The 2.5-kb *H19* RNA is spliced and polyadenylated but does not seem to encode a protein (Pachnis et al. 1988; Brannan et al. 1990). Although it has been suggested that *H19* could play a role as a tumor suppressor gene (Hao et al. 1993), no precise function has yet been assigned to this gene, which may play a role through a functional RNA.

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Figure 1. Targeted disruption of the *H19* gene. (A) Position of the imprinted genes in the 600-kb cluster on mouse chromosome 7. *Igf2* and *Ins2* are at a distance of 80 and 95 kb, respectively; *Mash2* at 230 kb, and *p57^{KIP2}* at ~600 kb upstream from *H19* (John and Surani 1996). (B) Restriction map of the genomic *H19* region, the targeting vector, and the predicted structure of the disrupted allele. The coding regions are depicted by black boxes. The dashed line represents plasmid sequences. The *EcoRI*–*SalI* genomic fragment deleted in the targeting vector extends from –50 bp to +3 kb relative to the start of transcription. The transcriptional direction of the *H19*, *neo*, and *tk* genes is indicated by arrows. The *neo* and *tk* transcripts are under the control of the pMC1 promoter (Py virus enhancer and *tk* promoter). The 5' and 3' external probes (gray boxes) were used to identify the recombinant alleles following *EcoRI* digestion of genomic DNA. (R) *EcoRI*; (B) *Bam*HI; (Bg) *Bgl*II; (S) *Sal*I; (N) *Not*I. (C) Southern analysis of ES clones. Genomic DNA was digested with *EcoRI* and hybridized with the 5' external probe. The sizes of the endogenous (4-kb) and disrupted (5-kb) allele fragments are shown. The arrow shows ES clone 19, which is heterozygous and was used in blastocyst microinjections. (D) Southern analysis of tail DNA from F₁ animals. Genomic DNA was digested with *EcoRI* and hybridized with a *neo* probe that detects a 5-kb fragment, and with the 3' external probe that detects 11-kb endogenous and 8-kb mutated fragments. (+/–) Heterozygous; (+/+) wild-type animals.

The generation of mice carrying a null mutation of this gene can elucidate its role and possibly give further insight into the mechanism of imprinting. Mice carrying a 13-kb deletion encompassing both the *H19* gene and its upstream sequences have been described previously

(Leighton et al. 1995a). These mice are viable and fertile and show an increase in weight that is linked to biallelic expression of *Igf2*. We have created mice harboring a smaller deletion covering only the transcription unit of the gene. We report here that this 3-kb deletion also affects the growth of the mutant animals, although to a lesser extent, and that in contrast with the previous work, the *neomycin* resistance gene (*neo*) cassette that has been inserted at the *H19* locus is imprinted. This implies that the signals required for the imprint are maintained in these mutant mice and that the *H19* gene itself or its RNA product is not involved in the imprint of this locus. Given the difference between the two deletions, we also propose that a putative imprinting control element is located in the region 10 kb upstream of the *H19* gene.

Results

Targeted disruption of the *H19* gene

The replacement construct that has been used to create a null mutation of the *H19* gene by homologous recombination harbors a 3-kb deletion of the entire transcription unit between the 5' *EcoRI* site and the 3' *SalI* site of the genomic DNA. All five exons of the gene are deleted, as well as an *Sp1* site and the TATA box in the promoter region of *H19*. The vector contains a 2-kb fragment of 5' homology and a 6.5-kb fragment of 3' homology. The expected homologous recombinant allele results in a 3-kb deletion replaced by the 1.1-kb *neo* cassette. Both the *neo* and the herpes simplex virus thymidine kinase (*tk*) cassettes are under the control of the pMC1 promoter, composed of a Polyoma (Py) enhancer (allowing expression in ES cells) and a *tk* promoter; contain a poly(A) addition signal, and are inserted in opposite orientation with respect to the *H19* transcription direction (Fig. 1B).

After electroporation of the CK35 ES cell line, 4 out of 60 clones displayed a correctly targeted allele as determined by Southern analysis (Fig. 1C). The 5' external probe revealed a 5-kb fragment from the mutant allele and a 4-kb fragment from the wild-type allele in *EcoRI*-digested genomic DNA from these clones. The 3' external probe revealed 8-kb and 11-kb *EcoRI* fragments from the mutant and wild-type alleles, respectively (data not shown).

One clone (cl 19) was injected into blastocysts and the resulting male chimeras were mated with MF1 or C57BL/6 females to produce heterozygous F₁ animals. These animals were tested for the presence of the *neo* gene by PCR on rapid-tail DNA preparations (as described in Materials and Methods). *EcoRI*-digested DNA was also analyzed by Southern blot using 5' (data not shown), 3', and *neo* probes (Fig. 1D), showing the expected size fragments. Only one copy of the *neo* gene was detected in these animals, indicating a single insertion site of the targeting construct. These results confirm the presence of a correctly targeted allele, with the entire 3-kb transcription unit of the *H19* gene being replaced by the *neo* cassette.

Paternal and maternal transmission of the mutation

The *H19* gene is imprinted and expressed only from the maternal allele. Therefore heterozygous F_1 offspring will display two phenotypes: (1) Mice in which the null allele is paternally inherited will carry a wild-type *H19* allele on the maternal chromosome that is normally expressed: These animals, called paternal heterozygotes (*pat het*), should display a wild-type phenotype; and (2) maternal heterozygotes (*mat het*), in which the null allele is maternally inherited, carry the wild-type *H19* allele on the paternal chromosome that is not expressed: *mat het* should display the same phenotype as homozygous mutants. This nomenclature was proposed previously for the *Igf2* heterozygous mutants (DeChiara et al. 1990).

The heterozygous offspring from the male chimeras inherit the mutated allele paternally, and these *pat het* mice were identical to wild-type mice. In the F_2 offspring from these mice, we found that *pat het*, *mat het*, and homozygous animals were all viable and fertile. The only observed phenotype was an increase in weight of the *mat het* and homozygous animals compared with their wild-type littermates. The increase ranged between 4% and 12% with a mean of 8%, estimated on 100 animals (5-day neonates) from 12 litters. Mice from different backgrounds were analyzed and all displayed the same significant increase, as estimated by Student's *t*-test ($P < 0.01$). This weight difference was also maintained in adult animals (Table 1; Fig. 2).

Northern analysis on muscle and liver RNA from 5-day neonates showed expression of *H19* in *pat het* mice. No *H19* transcript was detected in *mat het* (Fig. 3A,B) and in homozygous mutants (data not shown). This result confirms that the *H19* targeted allele is correctly mutated and that the imprint is maintained on the normal allele because the paternal *H19* allele is not expressed in *mat het* animals.

Expression of the neo gene

The same Northern blots were hybridized with a *neo* probe: *neo* transcripts were detected in *mat het* but not in *pat het* animals, which carry the *neo* gene on the paternally transmitted allele (Fig. 3C,D). These results show that the *neo* cassette that has been inserted at the *H19* locus is subject to imprinting. The mutation that was introduced into these animals has therefore left intact the signals necessary for this imprint.

RNAs from adult tissues of *mat het* animals (kidney, liver, muscle, testis, and thymus) were hybridized with a *neo* probe (Fig. 4); expression of *neo* was detected only in muscle, therefore suggesting that the *neo* gene is under the same spatio-temporal regulation as the *H19* gene it has replaced.

DNA methylation analysis of the 5' upstream region

It has been shown previously that the 5' upstream region of the *H19* gene is differentially methylated on the maternal and paternal allele (Bartolomei et al. 1993; Tremblay et al. 1995). To analyze the methylation profile of this region in the *H19 mat het* and *pat het* mice, genomic DNA from neonatal liver was digested with *EcoRI* or with *EcoRI* and *HpaII* and hybridized to the 5' external *BglIII-BamHI* probe (Fig. 5A,B). Double digestion of wild-type DNA shows a fully methylated 4-kb fragment (paternal allele) and several *HpaII* fragments (maternal allele). In *mat het* and *pat het* DNA samples, 4-kb (wild-type allele) and 5-kb (targeted allele) *EcoRI* fragments are detected. In *mat het* DNA, the 4-kb paternal fragment was resistant to *HpaII* digestion, indicating that the *HpaII* sites were methylated on the paternal allele, whereas the 5-kb maternal fragment was sensitive to *HpaII*. Similarly, in *pat het* DNA samples, the 5-kb paternal fragment was resistant to *HpaII* digestion (the ob-

Table 1. Weight of mutant animals

Background	5 day neonates (litter no.)	Genotypes		Percent
		<i>mat het</i>	wild type	
MF1/129 × C57	1	2.16 ± 0.03 (n = 3)	1.98 ± 0.04 (n = 7)	8
	2	3.92 ± 0.07 (n = 4)	3.63 ± 0.07 (n = 6)	7.4
	3	4.61 ± 0.07 (n = 6)	4.42 ± 0.09 (n = 4)	4
	4 and 5	3.97 ± 0.10 (n = 7)	3.48 ± 0.12 (n = 9)	12
	6	3.74 ± 0.12 (n = 5)	3.46 ± 0.04 (n = 5)	7.4
	7	3.57 ± 0.11 (n = 4)	3.15 ± 0.05 (n = 2)	11.8
C57/129 × C57	8	3.73 ± 0.08 (n = 3)	3.46 ± 0.08 (n = 3)	7
C57/129 × 129	9	3.65 ± 0.05 (n = 4)	3.36 ± 0.08 (n = 3)	7.7
		homozygotes	wild type	
C57/129	10	4.86 ± 0.06 (n = 3)	4.28 ± 0.15 (n = 5)	12
		<i>pat het</i>	wild type	
129 × C57/129	11 + 12	2.90 ± 0.16 (n = 5)	3.0 ± 0.11 (n = 11)	-3
	adults	<i>mat het</i>	wild type	
C57/129 × 129	females	28.2 ± 1.3 (n = 8)	25.1 ± 1.2 (n = 6)	11
	males	24.2 ± 2.0 (n = 2)	22.2 ± 0.9 (n = 6)	8

Figure 2. Growth of heterozygous and homozygous progeny carrying the *H19* null mutation. Histograms represent the weight of heterozygous or homozygous vs. wild-type 5-day neonates and adults in individual litters from different backgrounds (see Table 1).

served 4.2-kb fragment results from the presence of a partially methylated *HpaII* site in the *neo* gene) and the 4-kb maternal fragment was sensitive to *HpaII*. Similar results were obtained with muscle DNA (data not shown). In conclusion, the paternal allele is methylated in both *mat het* and *pat het* animals, suggesting that the observed imprint of the paternal *neo* gene is correlated with allele-specific methylation of the 5' upstream region.

Expression of the *Igf2* gene

The *H19* gene is located at a distance of 80-kb from the *Igf2* gene. To analyze a possible interaction between these two genes, we performed a cross between *H19* heterozygous

females and *Igf2* heterozygous males (kindly provided by A. Efstratiadis, Columbia University, New York, NY). These *Igf2* heterozygous mice result from a targeted disruption of the *Igf2* gene; it was shown that *pat het* animals, which do not express any *Igf2*, because *Igf2* is expressed only from the paternal allele, show a 30% decrease in weight (DeChiara et al. 1990).

This cross gives rise to four genotypes corresponding to different epigenotypes as indicated in Figure 6A. The [*H19*⁺/*Igf2*⁻] animals, having received a normal *H19* allele from their mother and the *Igf2* null mutation from their father, should display an *Igf2* *pat het* phenotype. The [*H19*⁻/*Igf2*⁺] animals, having received the *H19* null mutation from their mother and a normal *Igf2* allele from their father, should display an *H19* *mat het* phenotype. The [*H19*⁻/*Igf2*⁻] double heterozygous animals should allow the analysis of *Igf2* expression in the absence of *H19*.

The progeny obtained were genotyped by digestion of tail DNA with *EcoRI* and Southern analysis using a *neo* probe. The *H19* targeted allele was revealed by a 5-kb diagnostic fragment and the *Igf2* targeted allele by an 11-kb diagnostic fragment (data not shown).

Analysis of the weight of 5 day neonates in each group showed the following results (Fig. 6B): [*H19*⁺/*Igf2*⁻] animals were 30% lighter than wild-type [*H19*⁺/*Igf2*⁺] ones, as expected because these animals are *Igf2* *pat het*. The [*H19*⁻/*Igf2*⁻] animals were 7% lighter than their wild-type littermates ($P < 0.1$). The [*H19*⁻/*Igf2*⁺] animals are not shown because of a low sample number in this particular cross; however, in other crosses, as shown in Table 1, *H19* *mat het* animals were on average 8% heavier than wild-type animals.

Figure 3. Expression of *H19* and *neo* genes in neonatal heterozygous mice. (A,B) Northern analysis of 5-day neonate muscle (A) and liver (B) RNA with the *H19* probe. Genotypes of the mice are indicated at top; sizes of the transcripts are indicated in middle. (C,D) Northern analysis of 5-day neonate muscle (C) and liver (D) RNA. The same A and B filters were hybridized with the *neo* probe.

Figure 4. Northern analysis of adult tissues (6 months old) from a *mat het* animal. The *neo* probe was hybridized to RNA from the indicated tissues. A *GAPDH* probe was used as control. Sizes of the transcripts are indicated at right.

Figure 5. Analysis of DNA methylation of the 5' upstream region of the *H19* gene. (A) Liver genomic DNAs from wild-type, *mat het*, and *pat het* 5-day neonates were digested with *EcoRI* (E) or with *EcoRI* and *HpaII* (EH) and separated on 1% agarose gels. The Southern blots were hybridized with the *BglII*-*BamHI* 200-bp probe shown in B. Molecular sizes are indicated on left. (B) Restriction map of the 5' region of *H19*. (Top) The wild-type 4-kb *EcoRI* fragment upstream from the *H19* gene. (Bottom) The 5-kb *EcoRI* fragment resulting from the replacement of *H19* by the *neo* cassette. The *BglII*-*BamHI* 200-bp probe is indicated below. (R) *EcoRI*; (H) *HpaII*; (Bg) *BglIII*; (H) *HpaII*.

To correlate these observations with the level of *Igf2* transcription, Northern analysis was performed on muscle RNA from 5-day neonates (Fig. 6C). [*H19*⁺/*Igf2*⁻] animals (*Igf2 pat het*) showed expression of *H19* and no expression of *Igf2* mRNA, as reported before (DeChiara et al. 1990). Unexpectedly, [*H19*⁻/*Igf2*⁻] animals, which carry only one *Igf2* allele of maternal origin, expressed *Igf2* transcripts. However, the level of *Igf2* was only 25% compared with 100% in wild-type animals (Fig. 6D). [*H19*⁻/*Igf2*⁺] animals (*H19 mat het*) displayed higher amounts of *Igf2* transcripts (125%) than wild-type animals and no *H19* transcript.

The maternal *Igf2* allele, which is normally silent, is therefore expressed in the absence of the maternal *H19* gene in *cis*. However, the maternal *Igf2* level of expression is lower than the wild-type paternal *Igf2* level. This correlates with the partial rescue of the *Igf2* phenotype, as indicated by the 7% weight reduction of these animals compared with wild-type animals.

Discussion

The chromosomal regions subject to imprinting appear to contain clusters of genes displaying parental-specific differential expression. This has been shown not only for

the distal region of mouse chromosome 7, containing the *H19-Igf2* locus, but also in a more central region of this chromosome containing the *Snrpn* gene, homologous to the Prader-Willi-Angelman syndrome locus on human chromosome 15 (Nicholls 1994). X chromosome inactivation is yet another example of long-range control, because the paternal X chromosome is preferentially inactivated in extraembryonic tissues and the *Xist* gene is

Figure 6. Analysis of the progeny from a cross between *H19*^{-/+} females and *Igf2*^{+/-} males. (A) Schematic representation of expected progeny. Genotypes of the progeny are represented in the rectangles and abbreviated epigenotypes are indicated below. The *Igf2* null allele was generated by replacing exon1 by the *neo* cassette, whereas the entire *H19* gene is deleted in our animals. (M) Maternal allele; (P) paternal allele. (B) Weight analysis of the different epigenotypes. Histogram representing the weight of 5-day neonates from each epigenotype (n, number of animals). Percentage of decrease in weight compared with wild-type animals [*H19*⁺/*Igf2*⁺] (100%) is shown at the top of each bar. (C) Northern analysis of 5-day neonate muscle RNA from the four epigenotypes. The size of the *H19* transcript and of the two main *Igf2* transcripts detected in muscle RNA is shown on the right. An 18S oligonucleotide probe was used to estimate the levels of RNA in each sample. (D) The corresponding histogram, obtained from the Northern analysis of several animals, represents the percentage of *Igf2* transcripts for each epigenotype compared with wild-type level (100%). (n) Number of RNA samples analyzed.

imprinted (Kay et al. 1993, 1994). The imprinting mechanism must result from a complex gene regulation over large distances, involving chromatin structure, *trans*-acting factors, and *cis*-acting sequences. The targeted disruption of the *H19* gene has enabled the study of some aspects of the regulatory mechanism involved in imprinting.

We have generated null mutant animals in which only the 3-kb transcription unit of the *H19* gene has been deleted. We will refer to this mutation as the *H19*^{Δ3} allele. Our results have shown that this loss of function mutation does not affect embryogenesis of the mouse: Although *H19* is first detected in the trophectoderm cells of the blastocyst and is then expressed at high levels in endodermal and mesodermal tissues of the mid-gestation embryo, it does not appear to play a fundamental role in development. The null mutant mice are viable and fertile, and after one year of breeding, no other obvious phenotype has been detected apart from a weight increase of the maternal heterozygous and homozygous animals compared with wild-type littermates. This gene might be subject to redundancy and other genes might be accomplishing the same function. There is, however, no indication of a multigene family related to the *H19* gene. It is also possible that the sheltered breeding conditions in which the mice are maintained do not allow the detection of a more severe phenotype in these mutant animals. Alternatively, it could be that this gene must be repressed in certain tissues to allow full development of the embryo. To address this question requires experiments involving ectopic expression of *H19* in nonexpressing tissues of the embryo.

Several studies have revealed a modification of the imprint in embryonal human tumors related to the Beckwith-Wiedemann syndrome such as Wilms' tumors and rhabdomyosarcomas (Ohlsson et al. 1993; Moulton et al. 1994; Steenman et al. 1994; Reik et al. 1995; Taniguchi et al. 1995). In these tumors, expression of *H19* is no longer detected and the adjacent *Igf2* gene is frequently expressed biallelically. These observations could be related to the tumor suppressor role proposed for *H19* (Hao et al. 1993). It is noteworthy that mutant animals lacking the *H19* gene have not shown any incidence of tumors, although they are now over a year of age. The animal model that we have generated suggests that absence of *H19* expression is not sufficient to induce tumors in mice.

H19 expression is strongly down-regulated in adult mice and is found only in muscle tissues. Expression analysis of the *neo* cassette that has replaced the *H19* transcription unit has shown that the only adult tissue of mutant mice in which the *neo* transcripts are detected is muscle. Regulatory elements involved in the correct spatio-temporal expression of the *H19* locus have thus been maintained in these mutant animals. Interestingly, this suggests that these regulatory elements can override the tissue-specificity of the *Py* enhancer, because one of the target organs for polyoma virus is the kidney (Dawe et al. 1987; Rochford et al. 1990).

One striking observation is the imprint of the *neo* cas-

sette in heterozygous animals. The *neo* gene is expressed only when inherited from the maternal allele in neonatal muscle and liver, and no expression is detected in *pat het* animals, which carry the *neo* cassette on the paternal allele. Signals responsible for inducing a closed chromatin configuration on the paternal allele must have been maintained in the *H19*^{Δ3} mutation, such that the *neo* cassette that has replaced the *H19* transcription unit is imprinted. A similar regulation was described previously for an X-linked *neo* transgene that was subject to correct imprinting and X inactivation (Dandolo et al. 1993) and for the *neo* cassette introduced in the *Igf2* mutation (DeChiara et al. 1991). Recent transgene experiments have suggested that the structural *H19* gene or the *H19* RNA itself may be involved in its own imprint (Pfeifer et al. 1996; Elson and Bartolomei 1997). Our present study, however, clearly shows that in the context of the chromatin structure of chromosome 7, neither the *H19* DNA sequence nor transcription of the *H19* gene itself is required for establishment or maintenance of imprinting at this locus.

In addition, our results are in contrast with the targeted disruption of *H19* described previously (Leighton et al. 1995a), in which biallelic expression of *neo* was observed. In these animals, a 13-kb deletion encompassing not only *H19* but also 10-kb of the 5' upstream region of the transcription unit had been generated. We propose to refer to this latter mutation as the *H19*^{Δ13} allele. Taken together, these results show that the 10-kb sequence located 5' to the *H19* gene is required for the imprint at the *H19* locus (Fig. 7). This suggests that the *H19* 5' upstream region contains an imprinting control element. This 5' region has been shown to display allele-specific methylation correlated with differential expression of *H19* and to include several repeats of a (G)GGGG-TATA consensus sequence (Bartolomei et al. 1993; Ferguson-Smith et al. 1993; Tremblay et al. 1995). Analysis of DNA methylation of this region in the *H19*^{Δ3} mutants showed that only the paternal allele is methylated. Allele-specific methylation has thus been maintained in the mutant animals.

Among known imprinted genes, three have been identified that encode a possible functional RNA: *H19*, *IPW* (Wevrick et al. 1994), and *Xist*. It has been shown that the *Xist* gene, exclusively expressed from the inactive X chromosome, is involved in the inactivation of adjacent genes (Clemson et al. 1996; Lee et al. 1996; Penny et al. 1996; Herzing et al. 1997; Lee and Jaenisch 1997). One could suggest a possible similarity in the role of these genes, being involved in the down-regulation of expression of chromosomal domains, and acting through a *cis* effect of their RNA: *Xist* on genes of the inactive X chromosome and *H19* on the *Igf2-Ins2* region. The *H19* RNA itself may thus regulate the silencing of the *Igf2* gene by a *cis* effect. A possible example of this effect is the presence of biallelic *Igf2* expression in the absence of *H19* expression in certain human tumors (as mentioned above).

The results obtained from the cross between *H19*^{Δ3} and *Igf2* heterozygous mutant animals have shown that

Figure 7. Schematic representation of the *H19-Igf2* locus in *H19^{wt}*, *H19^{Δ3}*, and *H19^{Δ13}* animals. Only maternal alleles of each animal are shown in the diagram. The arrows show the direction of transcription. The *EcoRI* fragment (–10 kb to –50 bp) containing the putative imprinting control element is shown below by a bold line. The endoderm enhancers (E) at +9 and +11 kb are shown by circles. (R) *EcoRI*; (S) *SalI*. *H19^{Δ3}* animals display imprinted expression of the *neo* gene, whereas *H19^{Δ13}* animals display biallelic expression of this gene.

a regulatory link can be established between these two genes. Absence of a maternally expressed *H19* gene is associated with expression of the normally silent adjacent *Igf2* gene on the same chromosome. This loss of imprint of *Igf2* resulting in its biallelic expression is correlated with the observed weight increase of the *H19* mutant animals. Disruption of *Igf2* imprint has now been observed in two cases (Fig. 7): (1) In these *H19^{Δ3}* mutants, the level of maternal *Igf2* expression corresponds to 25% of the paternal level and is correlated with an 8% weight increase and only partial rescue of the *Igf2* weight phenotype in *H19^{Δ3}-Igf2* double heterozygous mutant animals; (2) in the *H19^{Δ13}* mutant animals described by Leighton et al. (1995a), the level of expression of maternal *Igf2* is similar to paternal *Igf2* in muscle and is correlated with a 27% weight increase and with total rescue of the *Igf2* mutant phenotype in double *H19^{Δ13}-Igf2* heterozygous mutant animals. Because the *H19^{Δ3}* deletion of the transcription unit leads to only limited loss of imprint, it appears that *Igf2* transcription is not controlled exclusively by *cis* expression of the *H19* gene. Taken together with the results described for the *H19^{Δ13}* mutation, this suggests that the region 10 kb upstream of *H19* itself is also involved in controlling *Igf2* expression and that the *H19* region is a complex structure that contains at least two elements involved in the control of adjacent genes: One is the *H19* gene itself and the other is the 5' upstream region of the gene.

It must be taken into account, however, that the difference between the phenotypic consequences of these two mutations could also reflect differences in the constructs used for targeting. First, the two deletions could result in disruption of the chromatin structure of the region, thus leading to different levels of maternal *Igf2* transcription. Second, it has been suggested that *H19* and *Igf2* compete for a common enhancer (Leighton et al. 1995b). Because the *H19* upstream sequences have been deleted in both *H19^{Δ3}* and *H19^{Δ13}* mutations, the enhancer may now interact with the *Igf2* promoter on the maternal allele. However, it may also interact with the replacement genes, and the relative strength of the *Py-tk* (in the *H19^{Δ3}* mutation) and *Pgk* (in the *H19^{Δ13}* mutation) promoters of the *neo* cassettes could account for the observed different levels of maternal *Igf2* expression (Fig. 7).

In conclusion, the *H19^{Δ3}* null mutation of the *H19* gene has shown that this gene is dispensable during embryonic development and leads to a phenotype of larger animals as a result of biallelic expression of the adjacent *Igf2* gene. Absence of the *H19* transcription unit modifies the imprint of the *Igf2* gene. The replacement *neo* cassette shows the same pattern of parental imprinting and tissue-specific expression as *H19*. Taken together with the *H19^{Δ13}* mutants reported previously, our results suggest that the region 10 kb upstream of *H19* carries a putative imprinting control element. Further experiments using subtle mutations in this upstream region or in the RNA sequence itself of the *H19* gene should allow a more precise definition of the regulatory events involved in the control of this imprinted region.

Materials and methods

Disruption of the *H19* gene.

An *H19* genomic clone was isolated from a 129 λFixII mouse genomic library (Stratagene) and the phage insert was subcloned into pBluescript KSII. The targeting vector carries a deletion of the *EcoRI-SalI* fragment encompassing the *H19* transcription unit and 50 bp of the promoter (Fig. 1B). It is composed from left to right of pBluescript, a 2-kb *BamHI-EcoRI* fragment from the 5' end of the gene (–2 to –50 bp), the pMC1-*neo* cassette (Stratagene) inserted in opposite orientation, a 6.5-kb *SalI-BglII* fragment from the 3' end of the gene (from +3 to +9.5 kb) and the pMC1-*tk* cassette. The vector was linearized at a unique *NotI* site and electroporated into embryonic stem (CK35 ES) cells.

The CK35 ES cell line was established according to procedures described previously using blastocysts from superovulated 129 female mice (Robertson 1987). These cells are maintained on *neo^r* mouse embryo fibroblasts in the presence of leukemia inhibitory factor (LIF) (10^3 U/ml). They were tested for efficiency of chimerism and germ-line transmission after microinjection into C57BL/6 blastocysts prior to transfection procedures.

Embryonic stem (ES) cells (2×10^7) in 0.5 ml were electroporated with 15 μg of DNA using a Bio-Rad Gene Pulser apparatus at 220 V and 960 μF in PBS or medium. G418 selection (200 μg/ml effective concentration) (GIBCO) was applied 48 hr later and gancyclovir (2×10^{-6} M) (Syntex) was added on day 4 or 5 after electroporation. This double selection provided a 5-fold enrichment in positive clones compared with G418 selection alone. These clones were picked after 8–10 days and grown in 96-well plates. They were then divided into two 24-well plates,

one being frozen directly in serum with 10% DMSO when confluent and the other being kept for DNA extraction.

Genomic DNA from individual clones was prepared, digested with *EcoRI*, separated on 1% agarose gels, blotted to Hybond N⁺ (Amersham) filters, and hybridized to ³²P-labeled random primed probes (Sambrook et al. 1989). Hybridization was performed in Church buffer at 65°C overnight (Church and Gilbert 1984). Blots were washed in 0.4× SSC, 0.5% SDS for 30 min at 65°C.

The 5' external probe is a *BglII*–*BamHI* 200-bp fragment at –2 kb and the 3' external probe is a *BglII*–*EcoRI* 1-kb fragment from +9.5 to +10.5 kb (Fig. 1B). The *neo* probe is a 1.1-kb *XhoI*–*BamHI* fragment from the pMC1-*neo* plasmid.

Blastocyst injections and mating of chimeric mice

One of the correctly targeted clones (cl 19) was injected into MF1 or C57BL/6 blastocysts. Germ-line transmission was very low with MF1-derived chimeric males (0.5%–3%) and much higher with C57BL/6-derived chimeras (25%–100%). Heterozygous and homozygous animals were obtained from these different crosses.

Rapid testing for heterozygotes

Heterozygous animals were analyzed by a rapid PCR procedure. Tail samples were digested in 200 µl of lysis buffer (50 mM KCl, 10 mM Tris at pH 8.3, 2.5 mM MgCl₂, 4.5% NP40, 4.5% Tween 20, 1 mg/ml of proteinase K). A 35-cycle PCR reaction (94°C for 1 min, 55°C for 30 sec, 72°C for 30 sec) using *neo*-specific oligonucleotides (Dandolo et al. 1993) was directly performed on 2.5 µl of DNA after a preliminary incubation at 95°C for 10 min before adding *Taq* polymerase (Hanley and Merlie 1991).

Northern blot analysis

RNA was extracted from 5-day neonatal or adult tissues using RNAXEL (Eurobio). For Northern analysis, 20 µg of total RNA was denatured at 68°C for 10 min in 50% formamide, 2.2 M formaldehyde, 5 mM sodium acetate, 1 mM EDTA, 20 mM MOPS at pH 7.0 and separated in a 1% agarose, 2.2 M formaldehyde gel in MOPS buffer (Sambrook et al. 1989). The RNAs were transferred to Hybond N⁺ filters in 10× SSC. Hybridizations were performed in Church buffer and filters were washed in 0.4× SSC, 0.5% SDS at 60°C (cDNA probes) or 42°C (oligonucleotide probe). Ethidium bromide staining, 18S, or *Gapdh* probes were used as loading controls. Quantification of Northern was performed using Image master (Pharmacia).

Igf2 transcripts were detected with a rat *Igf2* cDNA probe (Soares et al. 1985) and *H19* and *Gapdh* transcripts were detected with mouse (Fort et al. 1985) and rat (Poirier et al. 1991) cDNA probes, respectively. The 18S probe was an oligonucleotide probe: 5'-AGACGACCGCTCGGGGTCGCG-3'.

DNA methylation analysis

Liver genomic DNAs from heterozygous (*mat het* and *pat het*) and wild-type 5-day neonate animals were digested with *EcoRI* or with *EcoRI* and *HpaII*. DNA samples (20 µg) were separated on 1% agarose gels, transferred to Hybond N⁺, and hybridized to the *BglII*–*BamHI* 200-bp probe as described above.

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Deletion of the H19 transcription unit reveals the existence of a putative imprinting control element.

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