Epigenetic mechanisms underlying the imprinting of the mouse *H19* gene

Marisa S. Bartolomei,¹ Andrea L. Webber, Mary E. Brunkow,² and Shirley M. Tilghman

Howard Hughes Medical Institute and Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544 USA

The expression of the *H19* gene is governed by parental imprinting in mammals. *H19*, an unusual gene encoding an RNA with no known function, is exclusively expressed from the maternal chromosome. In mouse, it lies 90 kb downstream from the *Igf2* gene, which encodes a fetal-specific growth factor, insulin-like growth factor II, and is expressed primarily from the paternally inherited chromosome. In this report we have utilized interspecific hybrid mice to identify male-specific DNA methylation of a 7- to 9-kb domain surrounding the *H19* gene and its promoter. This allele-specific methylation could function as a mark to suppress transcription of the *H19* paternal allele. Consistent with this proposal, the *H19* promoter displayed an open chromatin conformation only on the relatively unmethylated active maternal allele. In contrast, a cell type-specific enhancer that lies outside the methylation domain is hypersensitive to restriction enzyme digestion in nuclei on both maternal and paternal chromosomes. That the allele-specific methylation domain, coupled to the two *H19* enhancers, contains all the information necessary for its imprinting was tested by examining two transgenic lines containing an internally deleted *H19* transgene. Both displayed paternal-specific methylation of the transgene and maternal-specific expression. Although neither line has been tested in an inbred genetic background, and therefore the action of complex modifiers cannot be formally excluded, the result suggests that the sequences necessary for the imprinting of *H19* have been identified.

[Key Words: Imprinting; *H19*; insulin-like growth factor II; DNA methylation; chromatin; transgenic mice]

Received April 27, 1993; revised version accepted July 14, 1993.

The male and female genomes are functionally non-equivalent in mammals, due in part to the presence of imprinted genes (McGrath and Solter 1983, 1984; Surani et al. 1984, 1986). These genes display unequal levels of expression from the two alleles, depending on the parent from which the allele is inherited. To date, four imprinted genes have been identified in the mouse. The insulin-like growth factor type-2 receptor (*Igf2r*) maps to the *T-associated maternal effect* (*Tme*) locus on mouse chromosome 17 and is maternally expressed (Barlow et al. 1991). Its ligand, the gene encoding insulin-like growth factor II (*Igf2* or *IGFII*) and the *H19* gene, which lie 90 kb apart on the distal end of chromosome 7 (Zemel et al. 1992), are both imprinted, but in opposite directions. *H19* encodes a maternally expressed RNA of unknown function (Bartolomei et al. 1991), whereas the fetal-specific growth factor *IGFII* is paternally expressed (DeChiara et al. 1991). The imprinted gene identified most recently, the small nuclear ribonucleoprotein polypeptide N gene, is also found on chromosome 7 but at least 40 cM proximal to the other two genes. Its expression is exclusively paternal (Leff et al. 1992). Thus far,

two of the four genes, *H19* (Rachmilewitz et al. 1992; Zhang and Tycko 1992) and *Igf2* (Rainier et al. 1993), have been shown to be imprinted in humans as well.

A central problem in imprinting is how the transcriptional machinery of the cell discriminates between the maternal and paternal alleles. It has been assumed that the alleles must be marked differently, presumably during gametogenesis when the maternal and paternal genomes are in separate compartments. One promising candidate for such a mark is the methylation of CpG dinucleotides in DNA. This covalent modification of DNA has been implicated in the perpetuation of the imprinting state in X chromosome inactivation, on the basis of the observation that genes on the inactive X chromosome are more highly methylated than their counterparts on the active X (for review, see Riggs and Pfeifer 1992). In addition a set of transgenes in mice exhibit parent-of-origin differences in DNA methylation, and in at least one instance, the methylation acts to silence the expression of the transgene (Hadchouel et al. 1987; Reik et al. 1987; Sapienza et al. 1987; Swain et al. 1987; Chaillet et al. 1991; Sasaki et al. 1991; Ueda et al. 1992). In all but one of these transgenic mouse lines, the transgene becomes methylated after passage through the female germline.

The possible role of differential DNA methylation in the imprinting of endogenous genes has been assessed for

Present addresses: ¹Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104; ²Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario M5G 1X5 Canada.

two of the endogenous imprinted genes. Stoger et al. (1993) localized a region in an intron of the *Igf2r* gene that is methylated on the expressed maternal allele. This region was also methylated in oocyte DNA but not in sperm DNA, a requirement for a candidate for the imprinting signal. Similarly, Sasaki et al. (1992) located a region ~3 kb upstream of the first exon in the *Igf2* gene that is methylated on the expressed paternal allele. Although these results are counter to the expectation that the silent allele would be methylated, it is possible that the methylated region could be preventing the binding of a repressor or facilitating the binding of an activator.

This report describes allele-specific epigenetic modifications of the mouse *H19* gene, using F₁ hybrids between two inbred strains of mice to distinguish the parental origin of the genes. A paternal-specific domain of DNA methylation was identified over a 7- to 9-kb region that extends from ~5 kb upstream of the start of transcription to the polyadenylation signal. Within this domain there was a striking difference in the chromatin structure of the promoter of the maternal and paternal alleles, with the transcribed maternal allele displaying a more open conformation. In contrast, the enhancer region, located ~5 kb downstream of the polyadenylation signal displays no allele specific methylation. Similar results have been obtained recently by Ferguson-Smith et al. (1993), examining mice with maternal disomies of the *Igf2-H19* locus. Unlike the promoter, the *H19* enhancers are equally accessible to restriction enzyme digestion on both alleles, suggesting that both maternal and paternal enhancers are engaged in transcription. That the DNA containing the allele-specific DNA methylation is suffi-

cient to invoke the appropriate parental imprint was demonstrated by analyzing two transgenic mouse lines carrying the domain. Both lines displayed paternal-specific DNA methylation of the transgene as well as maternal-specific RNA expression, mimicking the behavior of the endogenous *H19* gene.

Results

Strategy for analyzing allele-specific methylation

To determine whether there is a difference in the methylation patterns of the parental alleles of the *H19* gene, an assay was developed that allowed them to be distinguished. Restriction fragment length polymorphisms (RFLPs) between two different subspecies of mice, the *Mus musculus domesticus* strain C57BL/6J and *Mus musculus castaneus* (*M. castaneus*), were identified throughout the *H19* gene. Hybrid F₁ mice were then generated and their DNA was analyzed using the polymorphic restriction enzyme and a second enzyme, *HpaII*, which will not cut DNA at its recognition sequence if the internal CpG is methylated. Because each allele produced a characteristic restriction fragment, differences in their methylation status were easily detected. Figure 1 shows the RFLPs and probes used to analyze the methylation of a 29-kb region containing the *H19* gene and its flanking sequences.

Methylation analysis of the *H19* structural gene

The methylation status of a region extending from 900 bp upstream of the *H19* gene to 700 bp 3' of the gene was

Figure 1. RFLPs at the *H19* locus. A 29-kb region surrounding the *H19* transcription unit (darkly shaded box), drawn in a 5' → 3' direction, is shown in the center. The promoter is denoted by the position of an *EcoRI* site. The two enhancers are designated by shaded ovals. The *SfiI* site (Sf) used in the chromatin study of the enhancers is designated above the second enhancer. A DNase I hypersensitive site is indicated by a large arrow. Hybridization probes that were used for genomic DNA analysis are depicted above the restriction map by shaded rectangles. Beneath the restriction map are shown the restriction site polymorphisms used to distinguish the C57BL/6J and *M. castaneus* alleles. Each polymorphic region is represented by two lines. The top line corresponds to the C57BL/6J-specific fragment (or fragments) while the bottom line corresponds to an *M. castaneus*-specific fragment (or fragments). The approximate site of the polymorphism is designated by a vertical line and asterisk on the allele that contains the extra site. (Bottom) A summary of the region that displays paternal-specific methylation.

investigated using a *PvuII* polymorphism detected with the RS probe (Fig. 1). As shown in Figure 2A (lanes 1,2), *PvuII* digestion generated a 4.4-kb fragment in C57BL/6J derived genomic DNA and a 4.6-kb fragment in *M. castaneus* DNA. When neonatal liver DNA from the progeny of the cross of a C57BL/6J female and a *M. castaneus* male was digested with *PvuII* and the methylation-sensitive enzyme *HpaII* (Fig. 2A, lane 7), ~60% of the paternally derived *M. castaneus PvuII* fragment was resistant to digestion, indicating that it was methylated at all 11 *HpaII* sites found within the 4.6-kb fragment (Fig. 2C). The two most intense bands located immediately below the full-length *PvuII* band (Fig. 2A, lanes 5–8; Fig. 2B, lanes 2–9) correspond to partially methylated fragments from the inactive allele that result from the incomplete methylation of three *HpaII* sites located around the cap site (data not shown). These account for the majority of the other 40% of the paternally inherited allele.

In contrast, the maternally derived C57BL/6J fragment was digested with *HpaII* and was therefore unmethylated at one or more of the *HpaII* sites. The hypermethylation of the *M. castaneus* allele was dependent on its paternal inheritance, as it was digested by *HpaII* when it was inherited maternally (Fig. 2A, lane 8), whereas the paternally derived C57BL/6J allele was now resistant to *HpaII* cleavage.

As a further control, *MspI* has the same recognition sequence as *HpaII* but digests DNA regardless of the methylation status of the internal CpG. The identical *MspI*–*PvuII* digestion patterns in all DNAs in Figure 2A, lanes 9–12, demonstrate that the allele-specific differences observed with *HpaII* cannot be attributed to differences in the distribution of the CCGG recognition sequences in the two mouse strains.

Paternal-specific methylation of *H19* is found in all somatic cells and embryonic stem cells

The experiments in Figure 2A demonstrate that the inactive paternal allele of the *H19* gene is hypermethylated relative to the active maternal allele. The experiments utilized DNA from neonatal liver, where the maternal allele is actively transcribed. Thus, it is possible that the methylation differences observed are the consequence of the differences in transcription on the two chromosomes, rather than the cause of the difference. In an attempt to address this issue, the pattern of DNA methylation around the *H19* gene was examined in brain, where the expression of *H19* is restricted to the choroid plexus and leptomeninges during prenatal development (J.R. Saam and S.M. Tilghman, unpubl.). As these two tissues constitute a small percentage of cell mass of the brain, they do not contribute significantly to the pattern of methylation seen in total genomic DNA. The pattern observed in either neonatal or adult brain was essentially identical to that of neonatal liver (Fig. 2B, lanes 3,5,7,8), suggesting that the selective methylation of the paternal allele does not require maternal gene expression.

As a further test of this premise, the methylation pattern of the *H19* gene in embryonic stem cells was examined. These cells derive from the inner cell mass of the mouse embryo and do not express the *H19* gene (Poirier et al. 1991). Although the parental origin of the DNA bands could not be determined because the cell line used was derived from a homozygous 129/J mouse, once again the characteristic pattern of protected and digested bands was observed (data not shown). Finally, the pattern in adult liver DNA, at a time when the maternal allele has

Figure 2. Analysis of DNA methylation of the *H19* structural gene. (A) Genomic DNA was digested either with *PvuII* (lanes 1–4), *PvuII* and *HpaII* (lanes 5–8), or *PvuII* and *MspI* (lanes 9–12) as indicated, size fractionated on a 1.2% agarose gel, and hybridized to the RS probe as described in Materials and methods. The DNAs were isolated from C57BL/6J (B), *M. castaneus* (C), the progeny of C57BL/6J females and *M. castaneus* males (BXC), and the progeny of *M. castaneus* females and C57BL/6J males (CXB). Molecular mass standards in kb are shown at left. (B) The same as A, except that DNAs were isolated from sperm, neonatal liver (NLI), neonatal brain (NBr), adult liver (ALi), and adult brain (ABr) and digested with *PvuII* and *HpaII*. (C) Positions of the *HpaII* (H) sites that were assayed using the *PvuII* (P) polymorphism. The middle *PvuII* site is not present in *M. castaneus* DNA. The transcription start site is indicated with an arrow. Other restriction sites shown in the diagram are *EcoRI* (R) and *SaII* (S).

been repressed ~600-fold, remains unchanged from that in neonatal liver (Fig. 2B, lanes 6,9).

Methylation analysis of the region 5' to the *H19* gene

To ask whether the differential methylation of the maternal and paternal alleles extended to regions beyond the promoter and transcribed portion of the gene, 10 kb of DNA 5' to the promoter was analyzed. A polymorphic 3.7-kb *SacI* fragment in C57BL/6J DNA which lies immediately 5' to the 4.6-kb *PvuII* fragment (Fig. 1 and Fig. 3B), was resistant to *HpaII* digestion when it was paternally inherited but was digested to completion when the parent of origin was the mother (Fig. 3A, lanes 7,8). In contrast to the gene body pattern in which the paternal allele exhibits partial methylation at a few of the *HpaII* sites, the 3.7-kb region appears to be 100% methylated on the paternal allele.

The analysis was extended farther 5' by taking advantage of an *AccI* polymorphism at -6.0 kb that yielded a 4.0-kb fragment in C57BL/6J DNA and a 2.6-kb fragment in *M. castaneus* DNA. This region is partially methylated in neonatal liver (Fig. 3C, lanes 1-11); however, there was no significant difference in the methylation status of the maternal and paternal alleles (cf. lanes 7 and 8). The conclusion from the methylation analysis of the *SacI* and

AccI fragments is that one border of differential methylation lies between 4 and 6 kb upstream of the start of transcription.

Methylation analysis of the region 3' of the *H19* gene

The region immediately 3' of the polyadenylation signal was then analyzed for allele-specific differences in DNA methylation. This region contains two enhancers that are important for the expression of the *H19* gene in endodermal derivatives (Yoo-Warren et al. 1988), as well as a strong DNase I hypersensitive site detected in neonatal liver nuclei ~2 kb 3' of the enhancers (Brunkow and Tilghman 1991). The methylation status of this region was determined using three polymorphic fragments, *SphI*, *NsiI*, and *PvuII* (Fig. 1). In all three cases, both the active, maternal allele and the silent, paternal allele of the *H19* gene were partially methylated in an identical manner (Fig. 4), indicating that there is no differential methylation of the two alleles in this region. It therefore appears that the 3' border of the differential methylation is within 700 bp of the polyadenylation signal of the *H19* gene.

Methylation analysis of sperm DNA

For the paternal-specific methylation domain to serve as

Figure 3. Analysis of DNA methylation of the region 5' to the *H19* gene promoter. Neonatal liver DNAs from C57BL/6J (B), *M. castaneus* (C), C57BL/6J × *M. castaneus* F₁ hybrids (BXC), and *M. castaneus* × C57BL/6J F₁ hybrids (CXB) were digested with the enzyme producing the RFLP alone (lanes 1-4) or with that enzyme plus *HpaII* (lanes 5-8) or *MspI* (lanes 9-12 in A and lanes 9-11 in C). The samples were size fractionated on a 1% agarose gel and treated as described in Materials and methods. To the left of each autoradiograph are shown the molecular size standards. (A) The region immediately 5' to the promoter was assayed using a *SacI* polymorphism and the 4.0 R1 probe. The 3.7-kb C57BL/6J fragment and the 2.4-kb *M. castaneus* fragment are indicated at left. Lane 13 contains sperm DNA digested with *SacI* and *HpaII*. (B) (H) The *HpaII* sites located in the 5' region; (R) *EcoRI* sites. Transcription of *H19* begins at the arrow indicated. The extra *SacI* (S) site that is present in *M. castaneus* DNA is designated with an asterisk (*). (C) The region 5' to the 4-kb *EcoRI* fragment was assayed using an *AccI* polymorphism and the 4.8 R1 probe. Lanes 12 and 13 contain sperm DNA digested with *AccI* (lane 12) and *AccI* plus *HpaII* (lane 13).

Figure 4. Analysis of DNA methylation in the region 3' to the *H19* gene. A 15-kb region 3' to the structural gene was analyzed using a set of three RFLPs (*SphI*, *NsiI*, and *PvuII*; see Fig. 1). The sources of the genomic DNA samples are indicated at the top with the enzymes used in the digestions. The abbreviations are as described in the legend to Fig. 2. (A) Hybridization of the 1.5-kb *SphI* fragment to genomic DNA; (B) hybridization of the *NsiI* probe to genomic DNA. Lanes 11–13 contain sperm DNA digested with *NsiI* (lane 11), *NsiI* and *HpaII* (lane 12), and *NsiI* and *MspI* (lane 13). (C) Hybridization using the 7.0 R1 probe.

the mark that silences the paternal *H19* allele, it should be present in sperm DNA. In Figure 2B, lane 1, the extent of DNA methylation of the structural gene in sperm DNA is illustrated. A single band is detected with *PvuII*–*HpaII* digestion, which upon further restriction mapping was identified as a 3.4-kb fragment beginning at an unmethylated *HpaII* site ~200 bp 3' of the cap site of the *H19* gene and proceeding to the *PvuII* site 3' of the gene. The unmethylated *HpaII* site corresponds to one of the closely linked partially methylated sites detected in ~40% of the paternal DNA of neonatal and adult tissues (Fig. 2A, lanes 5–8; Fig. 2B, lanes 2–9). The other partially methylated site in somatic DNA, which is actually two very closely spaced *HpaII* sites at the cap site of the gene, is also unmethylated in sperm DNA (data not shown). Further 5' to the gene, the C57BL/6J-specific 3.7-kb *SacI* fragment that is fully methylated in paternally derived neonatal DNA is also fully methylated in sperm DNA (Fig. 3A, lane 13). Thus, the region of allele-specific methylation of the *H19* gene is heavily methylated in sperm DNA, consistent with its acting as a stably inherited mark.

The methylated domain in sperm DNA extends beyond that identified on the paternal allele in somatic cells. Sperm DNA is also heavily methylated farther 5', as well as 3', of the *H19* gene (Fig. 3C, lanes 12,13; Fig. 4B, lanes 11–13).

Analysis of the chromatin structure of the *H19* gene promoter

If the 7- to 9-kb domain, over which DNA methylation is maintained in an allele-specific manner, is acting as the epigenetic mark to suppress transcription of the *H19* gene on the paternal chromosome, then one would ex-

pect that the two alleles within this domain might assume different chromatin configurations. To test this idea, the chromatin structure of the promoter region of the two alleles was investigated. Rather than using the sequence-nonspecific enzyme DNase I to probe the relative accessibility of regions of DNA in chromatin, *EcoRI*, which cleaves between two Sp1 consensus sequences at –50 bp in the *H19* promoter (Pachnis et al. 1988), was employed (Liau et al. 1986). Nuclei from C57BL/6J mice, *M. castaneus* mice, and F₁ hybrids were treated with *EcoRI* for various lengths of time, and following purification, the DNA was digested with *PvuII* and hybridized to a 692-bp probe (PR in Fig. 1) to reveal the same RFLP used in the methylation analysis of the promoter. The *EcoRI*-resistant C57BL/6J and *M. castaneus* alleles, indicative of a closed configuration, yielded products of 2.5- and 2.7-kb, respectively, (B and C, respectively, in Fig. 5). However, if the chromatin is in an open conformation and *EcoRI* has access to the DNA, smaller fragments are generated (HS B and HS C in Fig. 5A, lanes 25 and 26). As shown in the first two panels of Figure 5A, digestion of nuclei isolated from homozygous animals yielded the expected hypersensitive products within 5–10 min of digestion. When nuclei from F₁ hybrid animals were treated with *EcoRI*, the only *EcoRI* cleavage product detected was derived from the maternal allele. This was true whether the mothers were C57BL/6J (Fig. 5A, BXC panel) or *M. castaneus* (Fig. 5A, CXB panel). Therefore, only the maternally derived active allele is in an open chromatin configuration at the promoter.

The degree of hypersensitivity of the two promoters was also examined in brain, which does not express the *H19* gene. Figure 5B shows the results obtained from brain nuclei of progeny of C57BL/6J females and *M. castaneus* males. Surprisingly, the brain displayed a pattern

Figure 5. Hypersensitivity of the *H19* promoter in chromatin. (A) Liver nuclei from mice 7–15 days old were subjected to increasing periods of *Eco*RI digestion followed by DNA purification and complete digestion with *Pvu*II, as well as *Hind*III, to decrease the sizes of the products and increase their resolution on agarose gels. The DNA was then subjected to gel electrophoresis, transfer to filters, and hybridization to a 692-bp *Pvu*II–*Eco*RI fragment (PR probe; see Fig. 1). Liver nuclei were obtained from C57BL/6J [B (lanes 1–6)]; *M. castaneus* [C (lanes 7–12)]; C57BL/6J females $\times$ *M. castaneus* male F₁ mice [BXC (lanes 13–18)] and *M. castaneus* female $\times$ C57BL/6J male F₁ mice [CXB (lanes 19–24)]. Lanes 1, 7, 13, and 19 represent control reactions with no *Eco*RI added; lanes 25 and 26 show the predicted hypersensitive products specific to each strain. The positions of the full-length, resistant products (2.5 kb for C57BL/6J; 2.7 kb for *M. castaneus*) and the hypersensitive products [692 bp for C57BL/6J (HS B); 882 bp for *M. castaneus* (HS C)] are indicated by arrows. Arrows to the left refer to lanes 1–6; arrows to the right refer to lanes 7–26. (B) Brain nuclei from C57BL/6J female $\times$ *M. castaneus* male F₁ mice were treated as in A.

similar to that observed for liver, in that the maternal allele was selectively hypersensitive.

This result raised the possibility that the selective *Eco*RI cleavage observed in nuclei was not a function of differences in the chromatin structure but, rather, the ability of *Eco*RI to digest methylated versus unmethylated DNA. To rule this out, naked DNA from an F₁ hybrid mouse was digested for short periods with *Eco*RI. No difference was observed in the rates of cleavage of the two alleles, indicating that they are equally digested in the absence of chromatin protein [data not shown]. Thus, in both expressing and nonexpressing tissues, the chromatin structure at the *H19* promoter is showing allele-specific differences in accessibility.

Analysis of the chromatin conformation of the *H19* gene enhancers

To ascertain whether the *H19* enhancers also exhibit differential sensitivity to nucleases, the relative cleavage rate of an *Sfi*I site within the downstream enhancer was tested in neonatal liver nuclei of F₁ hybrid mice. DNA was then isolated and digested with *Nsi*I which detects an RFLP between the two parental strains (Fig. 1). As indicated in Figure 6, *Sfi*I cleavage of the C57BL/6J chromosome yielded a 4.1-kb fragment, whereas a 2.7-kb fragment was released on the *M. castaneus* allele within 5 min of digestion. In an F₁ hybrid between a C57BL/6J female and a *M. castaneus* male, both alleles showed

Figure 6. Hypersensitivity of *H19* enhancer II in chromatin. (A) Liver nuclei from mice 7–15 days old were subjected to increasing periods of *Sfi*I digestion followed by DNA purification and complete digestion with *Nsi*I. The DNA was treated as described in Materials and methods and hybridized to a 1.6-kb *Sfi*I–*Eco*RI fragment (*Sfi*-R probe; see Fig. 1). Liver nuclei were obtained from C57BL/6J, [B (lanes 1–6)]; *M. castaneus*, [C (lanes 7–12)]; and C57BL/6J female $\times$ *M. castaneus* male F₁ hybrid mice [BXC (lanes 13–18)]. Lanes 1, 7, and 13 represent control reactions with no *Sfi*I added; lanes 19 and 20 show the predicted hypersensitive products specific to each strain. The positions of the full-length, resistant products (5.7 kb for C57BL/6J; 4.3 kb for *M. castaneus*) and the hypersensitive products [4.1 kb for C57BL/6J (HS B); 2.7 kb for *M. castaneus* (HS C)], are indicated by arrows.

equivalent hypersensitivity at the *Sfi*I cleavage site. The equivalent hypersensitivities of the maternal and paternal *H19* gene enhancers imply that they are not directly mediating the allele-specific expression of *H19*.

To prove that the *Sfi*I digestion reflected hypersensitivity rather than general cleavage or loss of nuclear integrity, the rate of cleavage of an *Sfi*I site approximately midway between the two genes was examined in the same DNA series. This intergenic region has not been implicated in the regulated expression of either *Igf2* or *H19* and is therefore an appropriate control for general cleavage. At that site, equivalent cleavage to that observed at the enhancer was detected after only 30 min of digestion (data not shown).

The hypersensitivity of the maternal promoter of the *H19* gene in brain nuclei was unexpected, given its silent state in that tissue. To ask whether the enhancers were also hypersensitive, brain nuclei were isolated and cleaved with *Sfi*I for varying lengths of time. The rate of cleavage of the *Sfi*I site within the distal enhancer in brain nuclei was equivalent to that observed for the unrelated site between the genes (data not shown). Thus, in a tissue where the *H19* gene is not expressed, the enhancers do not exhibit hypersensitivity.

Demonstration of the imprinting of two transgenic mouse lines

The combined results from the DNA methylation and hypersensitivity studies pointed to a 7- to 9-kb domain, including the *H19* gene and its promoter region, as important for controlling its imprinting. Previously, we had generated several lines of transgenic mice containing most of this domain, including 4 kb of 5'-flanking DNA, an internally deleted *H19* structural gene, and 8 kb of 3'-flanking DNA (Brunkow and Tilghman 1991). Although the expression of the transgene had been examined in these mice, the studies were not conducted in a way that would have revealed parental-specific effects.

Closer inspection of two of these transgenic lines revealed that in both cases the transgene was imprinted in a very similar manner to the endogenous gene. The results from one of these lines (699) are presented in Figure 7. Male and female mice that were homozygous for the transgene were bred to DBA/J mice, and livers from the progeny were analyzed for both DNA methylation and RNA expression of the transgene. As shown in Figure 7A, the paternally derived transgenic DNA (lanes 8–13) was generally more highly methylated than the maternally derived DNA (lanes 1–7). In addition, the amount of RNA that was expressed from each animal correlated closely with its methylation status, in that those animals with highly methylated DNA expressed little or no transgenic RNA, whereas the progeny with relatively unmethylated DNA were high expressers of the *H19* transgene (Fig. 7B). In both lines, anomalous animals were observed. For example, the animals represented in lanes 6 and 7 were derived from transgenic mothers, yet their methylation pattern and expression level was more characteristic of the animals deriving their transgene

Figure 7. Imprinting of transgenic mouse line 699. Mice homozygous for the *H19* transgene were mated to DBA/J mice, and the livers from 2-day-old progeny were assayed for DNA methylation and RNA expression of the transgene. DNA and RNA in lanes 1–7 are from the progeny of a transgenic (TG) female mated to a DBA/J male; DNA and RNA from the progeny of a TG male are shown in lanes 8–13. (A) Ten micrograms of genomic DNA was digested with *Pvu*II (C) or *Pvu*II and *Hpa*II (lanes 1–13), size fractionated on a 1% agarose gel, and hybridized after transfer to nitrocellulose to the RS probe (see Fig. 1). Arrows at left show the location of the transgene that is not digested with *Hpa*II (undigested TG) and the location of the primary product obtained with complete *Msp*I digestion (*Msp*I). (B) RNase protection assay using 3 μ g of total RNA. The location of the transgene-specific RNA product is indicated at left by TG, and the location of the endogenous *H19* RNA is designated endog. In lanes 1–5 the expression of the transgenic RNA is so high that the endogenous *H19* RNA is obscured.

from fathers. These exceptions may indicate that the entire region required to establish the endogenous *H19* gene imprinting pattern is not present. In that case, the inclusion of additional 5'-flanking DNA and/or the restoration of the 1 kb deleted from the structural gene should eliminate the variability in the patterns with female inheritance. Alternatively, multiple transgene copies may partially override the imprinting machinery, as both lines contain >30 copies of the transgene.

Discussion

Parental imprinting of a gene requires a mechanism whereby the transcriptional machinery can distinguish the maternal and paternal alleles of the gene. Differential

DNA methylation was a logical choice, primarily because this modification could fulfill all the requirements for a mark. That is, it could be assembled in different patterns in the male and female germ lines by virtue of testis- or ovary-specific DNA methylases. The parental-specific methylation must be maintained through the period after fertilization when genome-wide demethylation of nonimprinted genes has been demonstrated (Monk et al. 1987; Chaillet et al. 1991; Kafri et al. 1992). The difference could be maintained through many somatic cell divisions through the action of DNA methyltransferase, whose substrate is hemimethylated DNA (Gruenbaum et al. 1982; Bestor and Ingram 1983). It could be erased in the next generation with the action of a DNA demethylase. Finally, the presence of methyl groups on the binding sites for regulatory proteins had been shown to affect transcription (Busslinger et al. 1983).

The first evidence that allele-specific gene expression was accompanied by differential methylation of the target gene came from studies of mice carrying transgenes. Swain et al. (1987) showed that a transgene containing both prokaryotic and eukaryotic DNA segments, none of which were derived from an authentically imprinted gene, was expressed exclusively from the paternal chromosome. The silent maternally inherited copies were highly methylated. Since then, additional examples have been reported, all but one of which is maternally methylated (Hadchouel et al. 1987; Reik et al. 1987; Sapienza et al. 1987; Sasaki et al. 1991; Ueda et al. 1992).

We examined ~29 kb of DNA surrounding the *H19* gene and identified a 7- to 9-kb domain encompassing ~4- to 6-kb of the 5' region of the gene, along with the structural gene itself, which displayed paternal-specific DNA methylation (see Fig. 1). If this domain is the mark, then it acts negatively to suppress *H19* transcription on the paternally inherited chromosome. Sasaki et al. (1992) have conducted a similar survey of the *Igf2* gene and found no comparable domain on the maternal chromosome at that locus. Rather, they found paternal-specific methylation at one site ~3 kb upstream from the most proximal promoter of the *Igf2* gene (see Fig. 8). If that is also a mark, then it must act positively to facilitate transcription of *Igf2* on the paternal chromosome.

Thus, as indicated in Figure 8, all of the allele-specific methylation of DNA identified to date at the *H19/Igf2* loci is paternally derived. This is not generalizable to all endogenous imprinted genes, however. The gene encoding the binding protein for IGFI, *Igf2r*, which is expressed exclusively from the maternal chromosome (Barlow et al. 1991) like *H19*, has a maternal-specific island of DNA methylation within the gene itself that is maintained during the genome-wide demethylation after fertilization (Stoger et al. 1993). Thus imprinting may accommodate both male- and female-specific DNA methylation.

The paternal-specific methylation of DNA surrounding the *H19* gene in all somatic cells examined was not observed for all *HpaII* sites within the domain. At least two sites at the *H19* promoter displayed heterogeneity

Figure 8. The enhancer competition model to explain the opposite imprinting of *H19* and *Igf2*. The *H19* and *Igf2* genes are indicated by the boxes, with the horizontal arrows indicating the transcribed alleles. The two *H19* enhancers are designated by solid circles. The positions of allele-specific methylation of the paternal chromosome are indicated by the CH₃ symbol; the hypersensitive regions detected in chromatin are indicated by the double-lined vertical arrows. The single-lined arrows leading from the enhancers indicate the engagement of the enhancers, with the *H19* gene on the maternal allele and the *Igf2* gene on the paternal allele. The data for the modifications at *Igf2* are taken from Sasaki et al. (1992); the data for *H19* are taken from this paper.

with respect to methylation, in both expressing and non-expressing cells. When the pattern of methylation of this region was examined in sperm DNA, it was found that at least one of those sites was completely unmethylated. Thus, the heterogeneity at these clustered sites does not reflect a diminution in paternally inherited methylation but, rather, variation in methylation that occurs after fertilization.

Although the presence of parental-specific domains of DNA methylation within these imprinted genes is suggestive of a mark, they do not prove that methylation per se, or these methyl groups in particular, function in that capacity. Li et al. (1992) have recently generated a mouse containing a targeted mutation in the DNA methyltransferase gene. The substrate for this enzyme is hemimethylated DNA (Gruenbaum et al. 1982; Bestor and Ingram 1983). Thus, it has been proposed to function as the maintenance methylase required to maintain domains of DNA methylation in dividing cells. The phenotype of homozygous progeny is embryonic lethality, between 7.5 and 10 days of gestation. Transcription of *H19* and *Igf2* is activated in the extraembryonic endoderm by day 4.5 and in the embryo proper by day 7.5 (Lee et al. 1990; Poirier et al. 1991). Thus, the test of whether methylation affects the allele-specific expression of these genes will come from an examination of these methyltransferase-deficient embryos.

The allele-specific methylation of the *H19* promoter coincides with a marked difference in the accessibility of a restriction enzyme to the DNA on the two chromosomes. The likeliest explanation for this difference is that the methyl groups on the paternal chromosome block the binding of transcription factors and thereby leave the promoter in a closed conformation. The very different chromatin conformations of the two *H19* promoters provides strong support for this assumption.

The differential chromatin conformations of the two *H19* promoters is in marked contrast to the equivalent hypersensitivity detected at the distal enhancer. The two known *H19* enhancers lie outside the domain of allele-specific DNA methylation. Their equal accessibility to *Sfi*I digestion suggests that the regulation of the imprinted state is being achieved through mechanisms that act at the promoter of the gene and not through the enhancers.

The equal accessibility of the maternal and paternal enhancer to *Sfi*I digestion also raises the question of the function of the apparently active enhancer on the silent paternal chromosome. We have suggested that the enhancer is directing transcription of *Igf2* on the paternal chromosome (Fig. 8) (Bartolomei and Tilghman 1992; Zemel et al. 1992). On the basis of the very similar patterns of expression of these genes during embryogenesis and fetal development (Lee et al. 1990; Poirier et al. 1991), we proposed that the opposite imprinting of these two tightly linked genes is achieved by their competing for a set of shared regulatory elements. Given the results in Figures 2 and 3, we would suggest that on the paternal chromosome the inhibition of *H19* transcription by the allele-specific DNA methylation domain allows the enhancers 3' of the *H19* gene to engage in *Igf2* transcription. On the apparently unmarked maternal chromosome, either the relative strength of the accessible *H19* promoter and/or its proximity to the enhancers biases the competition in *H19*'s direction.

Several additional observations are consistent with this model. First, in contrast to our results with *H19*, the promoter regions of the *Igf2* alleles do not display differential sensitivities to digestion by DNase I, as assessed by comparing nuclei from normal animals and those carrying a maternal duplication of the distal end of chromosome 7 (Sasaki et al. 1992). This result is not entirely unexpected, given the low levels of *Igf2* RNA that can be detected in fetuses with maternal disomies (Sasaki et al. 1992). That is, the imprinting of the maternal *Igf2* allele is leaky, relative to *H19*, where no paternal transcripts have ever been observed. The absence of signs of stringent transcriptional regulation at *Igf2* is consistent with all imprinting at this locus being mediated by the DNA methylation at the *H19* gene. That is, both promoters of *Igf2* are capable of assuming active conformations. Whether they transcribe at a high rate simply depends on their ability to engage the *H19* enhancers. The only tissues in which the imprint of *Igf2* has been lost, the choroid plexus and the leptomeninges, are the only tissues identified to date in which *H19* is down-regulated relative to *Igf2* (J.R. Saam and S.M. Tilghman, unpubl.). According to the model, in the absence of *H19* transcription on the maternal chromosome, both alleles of *Igf2* are transcribed by default. Finally, no regulatory elements have been identified in the 15-kb region between the 5' end of the *Igf2* gene and the 3' end of the upstream *insulin-2* gene, based on the lack of appropriate expression of a transgene in which this region has been fused to a *lacZ* reporter gene (A. Efstratiadis, pers. comm.).

The enhancer competition model, as outlined in Fig-

ure 8, predicts that the *H19* gene, together with the allele-specific DNA methylation domain and the 3' enhancers, should be sufficient to impart imprinting on a transgene. This prediction was verified in two independent lines of transgenic mice that carried a significant amount of the domain. Unlike the majority of fortuitously imprinted transgenes that are maternally methylated, the *H19* transgene displayed paternal DNA methylation and maternal expression. This imprinting was independent of the genetic background in which it was measured, as the imprinting was observed in both progeny outcrossed to CD1 mice, as well as mice that had been inbred for three generations into DBA/2J (data not shown). Thus, the imprinting cannot be explained by the presence of a single dominant genetic modifier, as has been observed for other transgenes (Hadchouel et al. 1987; Sapienza et al. 1989; Allen et al. 1990; Engler et al. 1991), although the presence of complex genetic modifications cannot be ruled out until the transgenes are on completely inbred backgrounds. The appropriate imprinting of the *H19* transgene confirms that the elements required for its allele-specific expression are localized to a small region surrounding the structural gene. These results should greatly facilitate the identification of the specific sequences responsible for imprinting.

Materials and methods

Mice

C57BL/6J, DBA/J, and *M. musculus castaneus* mice were purchased from The Jackson Laboratory.

Isolation of genomic DNA

Brain and liver samples were incubated overnight at 55°C in 500 µl of digestion buffer [50 mM Tris-HCl (pH 8.0), 100 mM EDTA, 0.5% SDS, 0.5 mg/ml of proteinase K (Beckman)]. The mixture was then extracted two times with phenol/chloroform (1:1), and NaCl was added to a final concentration of 0.5 M. Following precipitation with 2.0 volumes ethanol and a wash in 70% ethanol, the DNA was resuspended in 100 µl of TE buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA].

Sperm were taken from the cauda epididymis and vas deferens, and genomic DNA was isolated as described previously (Ariel et al. 1991) except that the sample was incubated overnight at 50°C in the presence of 0.5 mg/ml of proteinase K.

Genomic DNA analysis

Ten micrograms of genomic DNA was digested with the appropriate restriction enzyme (or enzymes) and separated by electrophoresis on agarose gels. The DNA was then transferred to nitrocellulose (Millipore) (Southern 1975), and the filters were prehybridized and hybridized to radiolabeled probes prepared by nick translation (Rigby et al. 1977). The filters were washed as described previously (Wahl et al. 1979) and exposed to Kodak XAR-5 X-ray film with intensifying screens at -70°C. For quantification, the blots were exposed overnight to storage phosphor screens and analyzed on a Molecular Dynamics PhosphorImager.

Isolation of nuclei

Neonatal livers from approximately seven mice (7–15 days old) were homogenized in 20 ml of nuclear buffer [0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 0.1 mM EGTA, 15 mM Tris-HCl (pH 7.5), 0.5 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] (Wu 1989). The homogenate was filtered through a gauze pad, divided into two portions, and centrifuged at 7000 rpm in a Sorvall SA-600 rotor for 4 min. The supernatant was removed, and each pellet was resuspended in 10 ml of nuclear buffer. The homogenate was filtered again through a gauze pad and divided into two portions. Each portion was layered over a 25-ml cushion of nuclear buffer/1.7 M sucrose and centrifuged at 11,000 rpm in a Sorvall HB-4 rotor for 20 min. Pelleted nuclei were resuspended in 0.9 ml of digestion buffer [0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 3 mM MgCl₂, 0.05 mM CaCl₂, 0.5 mM DTT, 10 mM Tris-HCl (pH 7.5)] (Liau et al. 1986).

Restriction enzyme digestion of nuclei

The resuspended nuclei were divided into nine 200- μ l reactions. For *Eco*RI digestion, 15 μ l (20 μ l for brain nuclei) of *Eco*RI (20 U/ μ l) was added to eight of the nine reactions to yield a final concentration of 1.5 U/ μ l (2.0 U/ μ l for brain nuclei). Digestion was carried out at 37°C for varying times and was then terminated with the addition of an equal volume of quench buffer (final concentration 12.5 mM EDTA, 0.5% SDS, 25 μ g/ml of proteinase K) (Liau et al. 1986). A control reaction with no *Eco*RI was kept on ice for 60 min, at which time quench buffer was added.

For *Sfi*I digestion, 20 μ l of *Sfi*I (10 U/ μ l) was added to eight of the nine reactions to yield a final concentration of 1.0 U/ μ l. Digestion was carried out as described for *Eco*RI, except that the initial *Sfi*I digestion was done at 50°C. For both sets of reactions, proteinase K treatment was continued for 3 hr or overnight at 37°C. DNA was purified by extraction with phenol/chloroform (1 : 1) and precipitated with 2.0 volumes of ethanol. DNA pellets were washed with 70% ethanol, dried, and resuspended in 100 μ l of TE buffer.

Ten micrograms of DNA was digested to completion with a restriction enzyme (or enzymes) to reveal a RFLP. The DNA was then subjected to Southern blot analysis as described above.

Analysis of transgenic mice

Transgenic mouse line 699 (Brunkow and Tilghman 1991) had been maintained as heterozygotes for several generations by breeding to CD1 mice, and then heterozygotes were intercrossed to generate a line homozygous for the transgene. Males and females were then crossed to DBA/2J, and progeny were sacrificed 2 days after birth, at which time the *H19* gene is highly expressed. Part of the liver was taken for the preparation of genomic DNA, and the rest was used to prepare RNA by the lithium chloride method (Auffray and Rougeon 1980). *H19* RNA was analyzed by an RNase protection assay as described previously (Brunkow and Tilghman 1991). The founder of transgenic line 382 was crossed for two generations to C57BL/6, and subsequently crossed for three generations to DBA/2J. Progeny were tested after one and three backcrosses, and in both cases, imprinting was observed. Imprinting was also observed when the founder of line 382 was crossed twice to CD1 mice.

Acknowledgments

We are grateful to David Tobin and Bob Ingram, who assisted in the identification of RFLPs, and the members of the laboratory

for helpful discussions during the course of this work. We are also indebted to Dr. Judy Cebra-Thomas for assistance in preparing sperm DNA. This work was supported by a grant from the National Cancer Institute (CA44976). S.M.T. is an investigator of the Howard Hughes Medical Institute, and M.S.B. was supported by a postdoctoral fellowship from the U.S. Public Health Service.

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Epigenetic mechanisms underlying the imprinting of the mouse H19 gene.

M S Bartolomei, A L Webber, M E Brunkow, et al.

Genes Dev. 1993, **7**:

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