

# The *H19* methylation imprint is erased and re-established differentially on the parental alleles during male germ cell development

Tamara L. Davis<sup>+</sup>, Grace J. Yang, John R. McCarrey<sup>1</sup> and Marisa S. Bartolomei<sup>§</sup>

Howard Hughes Medical Institute and Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA and <sup>1</sup>Department of Genetics, Southwest Foundation for Biomedical Research, San Antonio, TX 78228, USA

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**Differences in DNA methylation distinguish the maternal and paternal alleles of many imprinted genes. Allele-specific methylation that is inherited from the gametes and maintained throughout development has been proposed as a candidate imprinting mark. To determine how methylation is established in the germline, we have analyzed the allelic methylation patterns of the maternally expressed, paternally methylated *H19* gene during gametogenesis in the mouse embryo. We show here that both parental alleles are devoid of methylation in male and female mid-gestation embryonic germ cells, suggesting that methylation imprints are erased in the germ cells prior to this time. In addition, we demonstrate that the subsequent hypermethylation of the paternal and maternal alleles in the male germline occurs at different times. Although the paternal allele becomes hypermethylated during fetal stages, methylation of the maternal allele begins during perinatal stages and continues postnatally through the onset of meiosis. The differential acquisition of methylation on the parental *H19* alleles during gametogenesis implies that the two unmethylated alleles can still be distinguished from each other. Thus, in the absence of DNA methylation, other epigenetic mechanism(s) appear to maintain parental identity at the *H19* locus during male germ cell development.**

## INTRODUCTION

Genomic imprinting is a mammalian-specific phenomenon whereby the expression of a subset of genes is dependent on parental origin (1). To achieve parent-of-origin-specific expression, the maternal and paternal alleles must be marked such that the transcriptional machinery can distinguish the two alleles and regulate their expression accordingly. Analysis of imprinted genes in human and mouse has led to the hypothesis that methylation of cytosine residues in CpG dinucleotides comprises at least one part of the imprinting mark. Many

imprinted genes contain regions that are differentially methylated on the maternal and paternal alleles (1). These methylation differences, which can silence or activate the parental alleles, are critical for the monoallelic expression of imprinted genes (2–4).

The appropriate regulation of imprinted genes requires that the parental allele-specific modifications must be reset in each generation to reflect the sex of the parent. Establishment of the imprint must occur in the germline since this is the only time during development that the male and female genomes are in distinct compartments. Consistent with this idea, parent-specific patterns of differential methylation for some imprinted genes are inherited from the gametes, supporting the hypothesis that methylation functions as the imprinting mark (5–8). Furthermore, experiments analyzing global DNA methylation patterns in the germline reveal that methylation levels change dramatically during embryonic germ cell development. Monk *et al.* (9) observed that 12.5 and 14.5 day post coitum (d.p.c.) germ cells have a reduced level of global DNA methylation compared with DNA in somatic cells. Similarly, examination of the methylation status of several non-imprinted genes revealed that they were unmethylated at 12.5 and 13.5 d.p.c. (10). These data support the idea that epigenetic modifications are removed from the DNA in germ cells such that they regain the totipotency necessary for the development of a new organism.

Several models have been proposed to address how allelic marks are reset in the germline. One model suggests that only the parental allele opposite to the sex of the individual needs to be reset since the other parental allele already possesses the appropriate imprint and remains unchanged. A second model postulates that both parental imprints are erased and reset during germ cell development to reflect the sex of the individual. A number of studies provide evidence in support of this second model. Embryonic germ (EG) cell lines derived from 8.5 and 12.5 d.p.c. primordial germ cells (PGCs) display distinctly different methylation patterns for at least one imprinted gene, the *Igf2r* gene. Whereas maternal-specific methylation of the putative imprinting control region of *Igf2r* (region 2) is observed in half of the 8.5 d.p.c. EG cell lines, no methylation is detected in any of the 12.5 d.p.c. EG cell lines, suggesting

<sup>+</sup>Present address: Department of Biology, Bryn Mawr College, Bryn Mawr, PA 19010, USA

<sup>§</sup>To whom correspondence should be addressed. Tel: +1 215 898 9063; Fax: +1 215 573 6434; Email: bartolom@mail.med.upenn.edu

that parent-specific modifications were erased in germ cells between 8.5 and 12.5 d.p.c. (11). In another study, imprinted loci in the somatic nucleus were demethylated when a somatic cell was fused to an EG cell derived from 12.5 d.p.c. PGCs (12). The authors therefore suggest that there is an active demethylase activity in the EG cells that is dominant to the machinery that maintains methylation in the somatic nucleus. Finally, Brandeis *et al.* (5) analyzed the methylation status of a few CpG dinucleotides in differentially methylated regions of the imprinted genes *Igf2*, *Igf2r* and *H19* during germ cell development. They failed to detect methylation at these sites in 13.5 d.p.c. germ cells, indicating that at least some of the parent-specific methylation is absent in germ cells.

To understand how imprinting is established, it is critical to determine when the imprint is set during gametogenesis. We have focussed our analysis on the methylation status of sequence 5' to the mouse *H19* gene, which includes a well-defined 2 kb differentially methylated domain (DMD) that is critical for proper monoallelic expression (13–16). Paternal-specific methylation in this region is inherited from sperm and retained throughout development, indicating that this region may harbor the imprinting mark (7). Previously, we found that the paternal-specific pattern of methylation is set on both the paternal and the maternal *H19* alleles by prophase of meiosis I. We also observed that the acquisition of methylation on the paternal and maternal alleles must be different since 100% of the paternal alleles are fully methylated in spermatogenic stem cells, the primitive type A spermatogonia, whereas the maternal alleles continue to acquire methylation until the onset of meiosis (17). These results raised the possibility that the epigenetic mark on the paternal *H19* allele is not erased in the male germline since methylation differences persist on the parental alleles throughout postnatal spermatogenesis.

To investigate further the difference in the temporal establishment of the imprinting mark on the maternal and paternal *H19* alleles, we have analyzed fetal germ cells. Surprisingly, we found a complete erasure of methylation on both parental *H19* alleles in embryonic germ cells, followed by temporally distinct patterns of methylation acquisition on the maternal and paternal alleles. Our results indicate that the unmethylated maternal and paternal alleles of *H19* are not identical and that parental identity is retained in the absence of methylation.

## RESULTS

### Paternal-specific methylation in the *H19* DMD is erased in embryonic germ cells

Our previous analysis of *H19* methylation patterns during spermatogenesis in postnatal male mice demonstrated that the maternal and paternal alleles are differentially methylated prior to the onset of meiosis (17). To investigate the origin of this differential methylation, we have extended our analysis to earlier stages of gametogenesis. Mouse PGCs are specified between 6.5 and 7.5 d.p.c. in the posterior end of the primitive streak and migrate extensively between 8 and 11 d.p.c., eventually populating the embryonic gonads (18). In female embryos, the germ cells enter meiosis by 13.5 d.p.c. and arrest as diplotene oocytes in meiosis I. In contrast, germ cells in the developing testis enter mitotic arrest between 13.5 and 15.5 d.p.c. The prospermatogonia remain non-dividing until after

birth, when they resume mitosis and differentiate into spermatogenic stem cells (19). In the current study, germ cells were isolated from the embryonic gonads of 13.5, 14.5, 15.5 and 18.5 d.p.c. embryos. All analyses were conducted using F<sub>1</sub> hybrid embryos to enable differentiation of the maternal and paternal *H19* alleles via strain-specific DNA polymorphisms.

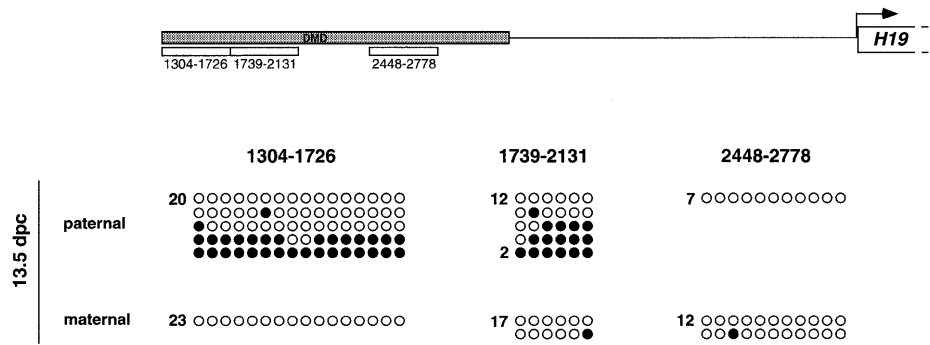
We focussed our analysis on the methylation status of the *H19* DMD. This region is postulated to harbor the imprinting mark since paternal-specific methylation is inherited from sperm and maintained throughout development and targeted deletion of the region results in a loss of imprinted expression and a reduction of methylation across the *H19* locus (13,16). The methylation status of *H19* was first examined in purified populations of 13.5 d.p.c. male embryonic germ cells. At this stage of germ cell development, both bulk DNA and specific genes are significantly undermethylated in embryonic germ cells as compared with somatic tissues (9,10), suggesting that the removal of previous imprinting marks would likely occur by this time.

Bisulfite mutagenesis followed by genomic sequencing was used to assess the methylation status of a significant portion of the DMD (33 CpG dinucleotides spanning 60% of the DMD) (Fig. 1). Based on our previous observation that 100% of the paternal *H19* alleles are hypermethylated throughout spermatogenesis whereas only a subset of the maternal alleles are methylated in spermatogonia with the remainder of the population continuing to acquire methylation through prophase of meiosis I (17), we anticipated that previous parental imprints would not be erased but would be retained in male embryonic germ cells. In contrast to this expectation, 13.5 d.p.c. prospermatogonia clearly demonstrated an absence of methylation on both the maternal and the paternal *H19* alleles (Fig. 1). None of the 54 maternal alleles analyzed had significant methylation in any of the three regions examined within the DMD. Similarly, the majority of paternal alleles were unmethylated across the DMD. Approximately 12% of the paternal alleles displayed significant hypermethylation: these subclones likely derived from somatic cells that contaminated the germ cell preparation, which would contain an unmethylated maternal allele and a methylated paternal allele (see Materials and Methods). The extensive deficiency of methylation in the DMD indicated that methylation is erased from the paternal *H19* allele prior to or during PGC development.

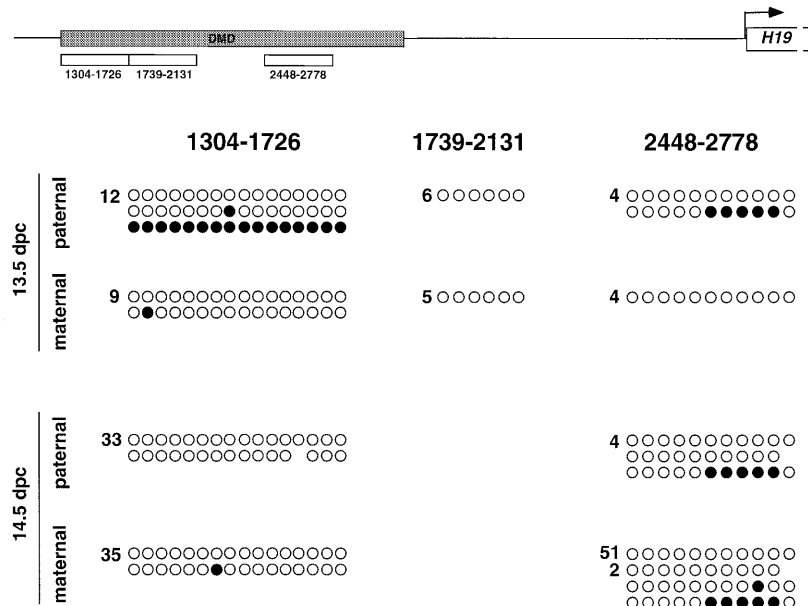
Since germ cell development is identical in males and females until the sexual differentiation of the gonads at 12.5 d.p.c. we predicted that methylation would also be erased in the female germline. The methylation status of *H19* was therefore analyzed in female germ cells at 13.5 and 14.5 d.p.c. Consistent with our prediction, no methylation was found within the DMD on either the maternal or the paternal *H19* alleles (Fig. 2). Combined, these data indicated that parent-specific methylation was erased early during germ cell development.

### The *H19* DMD acquires methylation on the paternal allele before the maternal allele in male germ cells

To determine when methylation was first acquired at the *H19* locus during development of the male germline, the methylation status of 27 CpGs within the *H19* DMD in mitotically arrested prospermatogonia isolated at 14.5, 15.5 and 18.5 d.p.c. was examined using bisulfite mutagenesis followed by sequence



**Figure 1.** Methylation status of the *H19* differentially methylated domain in 13.5 d.p.c. male embryonic germ cells. At the top is a schematic representing sequences upstream of the *H19* promoter (arrow). The filled box on the line represents the 2 kb differentially methylated domain (DMD). The open boxes below the line indicate the regions in which methylation was analyzed. Thirty-three CpGs were analyzed by bisulfite mutagenesis and sequencing, spanning 60% of the DMD (13,45). Cytosines are located at the following positions: 1330, 1360, 1362, 1372, 1374, 1391, 1397, 1538, 1546, 1568, 1617, 1621, 1624, 1638, 1645, 1668, 1756, 1809, 1967, 1993, 1999, 2036, 2468, 2474, 2490, 2496, 2513, 2642, 2651, 2653, 2655, 2679, 2710; numbering is in accordance with GenBank accession no. U19619. Each line corresponds to a single strand of DNA and each circle represents a CpG dinucleotide on that strand. A filled circle designates a methylated cytosine and an open circle corresponds to an unmethylated cytosine. The number of strands observed with a given methylation profile (if greater than one) is indicated to the left of each line. Paternal and maternal alleles were distinguished during sequence analysis by DNA polymorphisms. No non-CpG methylation was detected in the region analyzed.

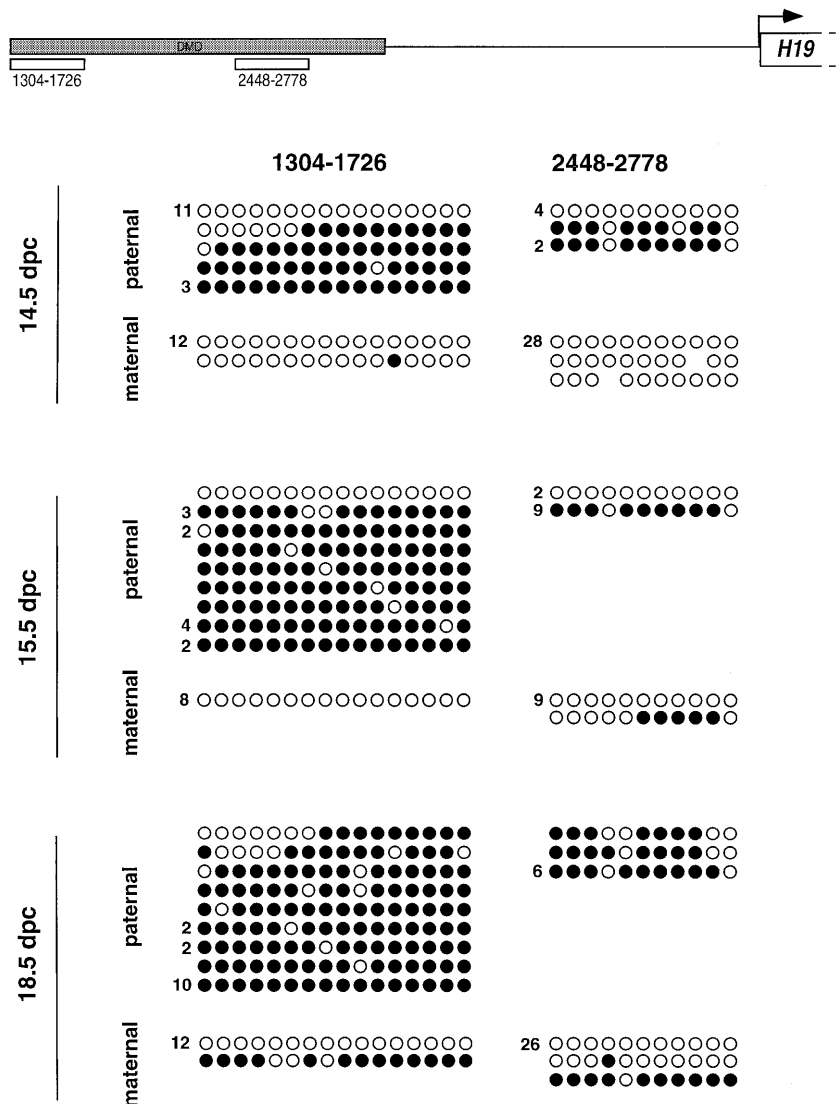


**Figure 2.** Methylation status of the *H19* DMD in 13.5 and 14.5 d.p.c. female embryonic germ cells. Circles represent individual CpG dinucleotides, as described in the legend to Figure 1. The absence of a circle indicates incomplete sequence on that strand. No non-CpG methylation was detected.

analysis. A significant number of hypermethylated strands were detected in 14.5 d.p.c. prospermatogonia. At this stage, 38% (9 of 24) of paternal *H19* strands were methylated in the DMD (Fig. 3). By 15.5 d.p.c., 89% of the paternal alleles were hypermethylated within the DMD and hypermethylation of the paternal allele was retained in the male germline at 18.5 d.p.c. and postnatally (Fig. 3) (17). These data indicated that the paternal *H19* allele acquired a paternal-specific pattern of hypermethylation in the DMD over a 2 day period, spanning 14.5 and 15.5 d.p.c.

In contrast, the maternal *H19* allele remained unmethylated in the DMD throughout embryonic/fetal germ cell development (Fig. 3). Unlike the paternal allele, no methylation was

observed on the maternal allele at 14.5 or 15.5 d.p.c. At 18.5 d.p.c., 2 of the 31 maternal strands examined were hypermethylated, suggesting that the maternal alleles were beginning to acquire methylation in male germ cells just prior to birth. Although the fraction of methylated maternal alleles was small, it was significant since maternal *H19* alleles are hypomethylated in somatic cells (13,14). Our previous data demonstrated that the maternal allele does not become fully hypermethylated until prophase of meiosis I (17), indicating that the acquisition of methylation on the maternal allele spans an extended period during male germ cell development, initiating in mitotically arrested prospermatogonia at 18.5 d.p.c., but is not complete until after the onset of meiosis.



**Figure 3.** Methylation status of the *H19* DMD in 14.5, 15.5 and 18.5 d.p.c. prospermatogonia. At these three stages, 27 CpGs were analyzed. Circles represent individual CpG dinucleotides, as described in the legend to Figure 1. For each stage analyzed, data obtained from paternal alleles are shown above data obtained from maternal alleles. The absence of a circle indicates incomplete sequence on that strand. No non-CpG methylation was detected.

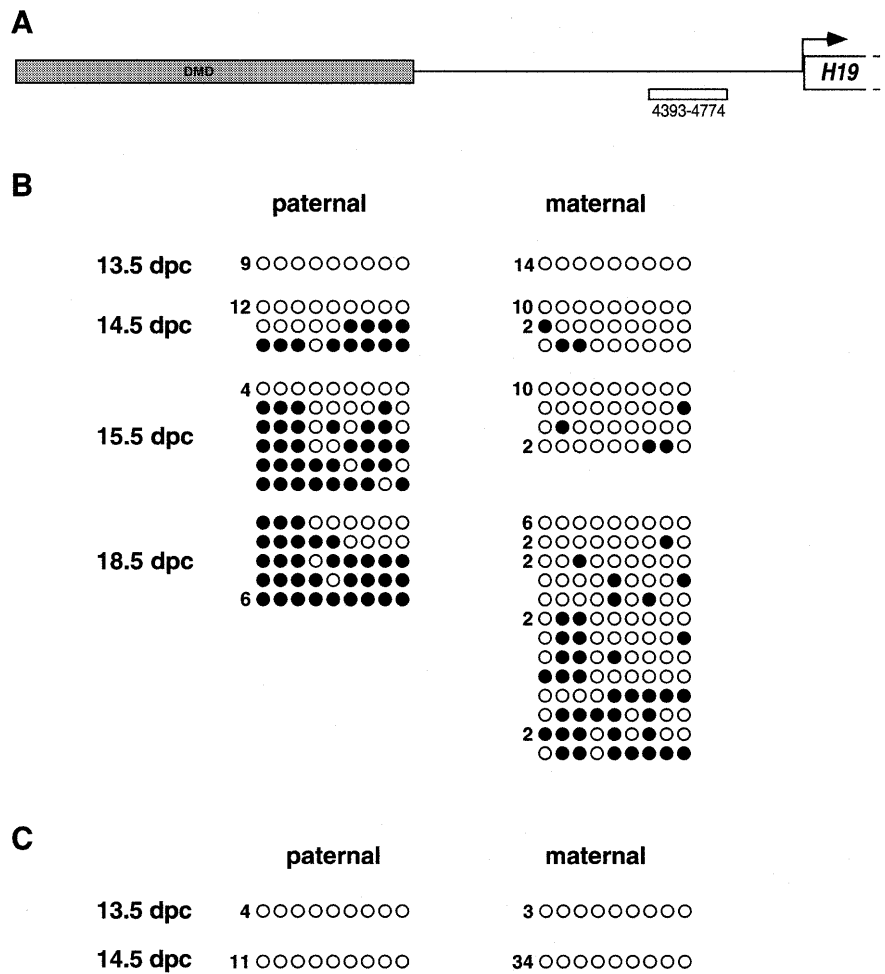
### Sequence outside the DMD acquires methylation differentially on the parental *H19* alleles in male germ cells

Differential methylation at the *H19* locus extends to sequence beyond the DMD (20,21). However, the methylation within these regions, which includes sequence between the DMD and the *H19* promoter as well as the *H19* transcription unit, is more labile: the paternal-specific methylation that is inherited from sperm is erased at the blastocyst stage and is re-acquired by mid-gestation (13,14). Since methylation in these regions varies more with fluctuations in global DNA methylation, we determined when the sperm-specific pattern of methylation is established on the parental alleles.

To address this question, the methylation status of nine CpGs located from -480 to -860 bp relative to the *H19* promoter was analyzed at each of the stages described above using the bisulfite mutagenesis and sequencing assay (Fig. 4A). Similar to what was observed within the DMD, the promoter proximal

region was completely unmethylated on both the paternal and the maternal *H19* alleles in 13.5 d.p.c. male germ cells (Fig. 4B). In addition, this region lacked methylation in 13.5 and 14.5 d.p.c. female germ cells (Fig. 4C). These results confirmed our conclusions from the analysis of the DMD: parent-specific methylation patterns are erased across the *H19* locus in embryonic germ cells.

The differential acquisition of methylation on the paternal and maternal *H19* alleles that we observed within the DMD in male germ cells was also detected in the promoter proximal region. The paternal allele acquired hypermethylation in the male germline during fetal germ cell development. It remained unmethylated at 14.5 d.p.c. By 15.5 d.p.c., half of the paternal alleles were hypermethylated and, by 18.5 d.p.c., all of the alleles were methylated (Fig. 4B; Table 1). Therefore, the acquisition of methylation in the promoter proximal region of paternal *H19* alleles followed a similar pattern to that observed in the DMD. It is interesting to note that the promoter proximal

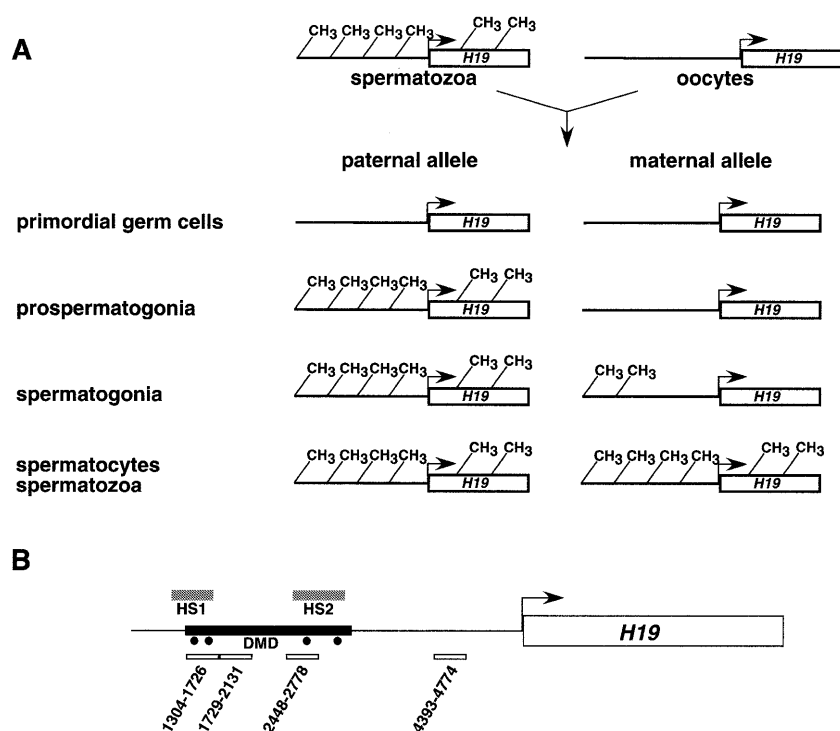


**Figure 4.** Methylation status of individual DNA strands in a region outside the DMD, proximal to the *H19* promoter. (A) Schematic representing sequences upstream of the *H19* promoter. The shaded box represents the DMD. The open box indicates the region analyzed. Nine CpGs are contained in the region extending from 4393 to 4774 (4446, 4556, 4560, 4564, 4654, 4706, 4726, 4731, 4751; GenBank accession no. U19619), which is located at -480 to -860 bp relative to the start of transcription. (B) Data are from 13.5 d.p.c. male embryonic germ cells and 14.5, 15.5 and 18.5 d.p.c. prospermatogonia. Circles represent individual CpG dinucleotides, as described in the legend to Figure 1. Paternal alleles are shown on the left, maternal alleles on the right. A numerical representation of these data is found in Table 1. (C) Data are from 13.5 and 14.5 d.p.c. female embryonic germ cells. No non-CpG methylation was detected in this region.

**Table 1.** The percentage methylation of promoter proximal CpGs in male embryonic germ cells

Embryonic stage (d.p.c.)	Allele	Nucleotide position of cytosine <sup>a</sup>								
		4446	4556	4560	4564	4654	4706	4726	4731	4751
13.5	Paternal	0	0	0	0	0	0	0	0	0
	Maternal	0	0	0	0	0	0	0	0	0
14.5	Paternal	7	7	7	0	7	14	14	14	14
	Maternal	15	8	8	0	0	0	0	0	0
15.5	Paternal	56	56	56	22	33	22	44	44	22
	Maternal	0	7	0	0	0	0	14	14	7
18.5	Paternal	100	100	100	80	80	80	80	80	80
	Maternal	14	41	50	5	36	9	27	18	18

<sup>a</sup>Numbering is in accordance with GenBank accession no. U19619.



**Figure 5.** (A) Summary of methylation changes at the *H19* locus during male germ cell development. Methylated cytosines are represented by CH<sub>3</sub>. The paternal-specific methylation that is inherited from the sperm is erased in primordial germ cells and is re-acquired at different times on the paternal and maternal *H19* alleles. (B) Schematic depicting the relationship between the *H19* DMD, the nuclease-hypersensitive sites and the CTCF binding sites. The black box on the line represents the DMD. The open boxes beneath the line represent the regions in which methylation was analyzed. The grey boxes above the line represent the two nuclease-hypersensitive sites (28) and the filled circles beneath the line indicate the location of the CTCF-binding sites (30,31,35). Cytosines 1360, 1362, 1372, 1374, 1391, 1397, 1617, 1621, 1624, 1638, 1645, 2642, 2651, 2653, 2655 and 2679 are within CTCF-binding sites (numbering is in accordance with GenBank accession no. U19619).

region acquired methylation subsequent to methylation within the DMD, supporting the theory that the DMD harbors the imprinting mark and that methylation of the promoter proximal region is a secondary event.

In contrast, whereas the maternal *H19* allele was not fully methylated during embryonic germ cell development, it acquired a basal level of methylation (Fig. 4B; Table 1). No methylation was detected on the maternal *H19* alleles at 14.5 or 15.5 d.p.c. By 18.5 d.p.c., the maternal allele acquired some methylation in the promoter proximal region. This low level of methylation was reminiscent of the hypomethylation observed on the maternal allele in this region in somatic tissue from mid-gestation embryos (13). Therefore, the maternal allele acquired a maternal-like pattern of hypomethylation in the promoter proximal region by 18.5 d.p.c., but did not achieve the paternal pattern of hypermethylation until the germ cells entered pachytene in meiosis I (17).

## DISCUSSION

Our previous analysis indicated that differential methylation of the maternal and paternal *H19* alleles may persist during early male germ cell development: the maternal allele does not become fully methylated until the spermatocytes enter meiosis (17). In this study, however, we have found that both alleles are unmethylated in embryonic germ cells at 13.5 d.p.c. Despite the lack of methylation on both alleles in germ cells from mid-

gestation embryos, we propose that the alleles remain non-equivalent since they subsequently acquire methylation at distinct times in the male germline (Fig. 5A). Our data provide direct evidence that the parental alleles of *H19* retain their identity in the absence of methylation differences.

### Absence of methylation at the *H19* locus in embryonic germ cells

Global DNA methylation levels vary dramatically during mouse development. Methylation levels are significantly reduced during two developmental stages, pre-implantation and germ cell development (9,22–25). In this first case, gamete-specific methylation patterns are largely erased in the blastocyst. It is critical for imprinted genes to maintain their parental identity during this time. Therefore, methylation must be maintained during pre-implantation development if it is the imprinting mark that designates the parental alleles. It has been demonstrated that a number of imprinted genes including *H19*, *Igf2r* and *Snrpn* retain their allele-specific methylation in pre-implantation embryos (5–8).

PGCs exhibit limited, if any, methylation. This lack of methylation is hypothesized to be part of the process that restores germ cells to a totipotent state (9). In contrast to pre-implantation embryos where parental identity must be conserved when DNA methylation is minimal, parental identity must be erased and re-established for at least one parental allele in germ cells. In support of this proposal,

experiments with 12.5 d.p.c. EG cells indicate that imprinted genes are hypomethylated in PGCs (11,12). Additionally, analysis of a few CpGs in various imprinted genes reveals that they are unmethylated in 13.5 d.p.c. embryonic germ cells (5).

We have found that the *H19* locus lacks methylation in 13.5 d.p.c. PGCs. Our analysis included 33 CpGs within the DMD, a region that is required for imprinted expression (16). Importantly, it has been demonstrated that cytosines within the DMD remain methylated on the paternal allele during the genome-wide demethylation that occurs in the blastocyst (7,14,15), emphasizing that this region can escape demethylation that other loci are subject to and supporting the idea that the differential methylation ensures the maintenance of the imprint. We also examined the methylation of nine CpGs proximal to the *H19* promoter, a region not postulated to harbor parental-specific imprints, and determined that these CpGs are also devoid of methylation in 13.5 d.p.c. PGCs. Although these experiments do not address whether methylation is erased in earlier populations of PGCs or whether germ cells ever exhibit any methylation prior to 13.5 d.p.c., they do demonstrate that methylation is lacking at a time when parental alleles are likely being reset to reflect the sex of the individual.

#### The maternal and paternal *H19* alleles are non-equivalent in the absence of methylation

The lack of methylation on both alleles at the *H19* locus in 13.5 d.p.c. male germ cells indicates that the parental alleles are equivalent at this time. However, our results suggest that parental identity is retained in the absence of methylation since the paternal *H19* allele acquires methylation before the maternal allele. By 15.5 d.p.c., all DNA corresponding to the paternal *H19* allele is completely methylated, whereas the maternal allele remains unmethylated. Methylation is first detected on a subset of maternal alleles a few days later, a process that continues until the onset of meiosis when both *H19* alleles exhibit the pattern of methylation characteristic of mature male gametes.

The time at which methylation is first detected at the *H19* locus in male germ cells coincides with that observed at other loci. Kafri *et al.* (10) demonstrated that a series of non-imprinted genes were unmethylated in 13.5 d.p.c. male germ cells but acquired methylation at varying times between 15.5 and 18.5 d.p.c. Similarly, it was also shown that an imprinted transgene was unmethylated in 13.5 d.p.c. male germ cells but established a distinctive methylation pattern by 17.5 d.p.c. in prospermatogonia (26). Indeed, there appears to be a global increase in methylation levels in germ cells in the late fetus. Using an antibody to 5-methylcytosine, Coffigny *et al.* (25) determined that euchromatin transitions from a demethylated to a strongly methylated state between 16 and 17 d.p.c. Finally, intracisternal A particle endogenous retroviruses lack methylation in 13.5 d.p.c. germ cells but are hypermethylated in prospermatogonia (27). Taken together these results suggest that at least one DNA methyltransferase is induced to methylate sequences in the male germline during the period between 14.5 and 18.5 d.p.c.

Although the paternal *H19* allele is methylated at approximately the same time as many other DNA sequences, the maternal allele remains resistant to methylation for several additional days. It therefore appears that the paternal allele is

initially in a more receptive state for DNA methyltransferase activity than is the maternal allele which must apparently undergo some type of change before it is capable of being methylated. Although it is conceivable that all paternally derived DNA is more receptive to DNA methylation, we favor the idea that imprinted genes uniquely display this property. One possibility for how the alleles acquire methylation differentially is that the initial chromatin structure of the maternal *H19* allele differs from that of the paternal allele such that it does not allow access of DNA methyltransferase to its CpG dinucleotide substrates. In support of this proposal, recent studies have highlighted differences in chromatin structure between the maternal and paternal *H19* alleles in somatic cells. Specifically, the maternal *H19* allele harbors two nuclease-hypersensitive regions within the DMD, designated as HS1 and HS2 (28,29) (Fig. 5B). The individual hypersensitive regions (as well as the entire DMD) have now been shown to act as boundary elements that are hypothesized to mediate the allele-specific expression of the linked and oppositely imprinted *H19* and *Igf2* genes (30–32). According to this boundary model, putative boundary proteins bind to the hypersensitive sites and isolate the *H19* transcription unit and enhancers. This permits the exclusive expression of the *H19* gene on the maternal chromosome. On the paternal chromosome, the *H19* gene and DMD are hypermethylated preventing boundary formation and thereby allowing *Igf2* exclusive access to the shared enhancers (33). Recently, the zinc finger DNA-binding protein CTCF, a protein previously demonstrated to bind the chicken  $\beta$ -globin insulator (34), has been shown to bind to sequence defined by the hypersensitive sites within the DMD (30,31,35). Specifically, the CTCF-binding sites correspond to short repeats in the mouse DMD that are conserved in rat and human *H19* 5' sequences (30,31,36–38). Because CTCF will not bind *in vitro* if the binding sites are methylated (30,31), it is possible that this protein maintains maternal allelic identity during male germ cell development. In this report, 16 of the 33 cytosines analyzed reside in three of the four CTCF-binding sites and all of these cytosines exhibit the parental allele-specific differences in methylation acquisition described above.

We propose the following model to explain differential acquisition of methylation in male germ cells. The maternal allele of *H19* is epigenetically marked by CTCF or other proteins in the female germline. This maternal allelic imprint is maintained in the early embryo and in embryonic germ cells. Although DNA methyltransferase is able to methylate the paternal allele of *H19* as well as other genomic DNA in prospermatogonia, proteins that bind to the boundary element initially prevent access of the DNA methyltransferase to the maternal *H19* allele. Later in gestation, maternal allelic identity is lost, possibly by a reduction in affinity or degradation of the proteins that bind to the maternal allele, thereby allowing access of DNA methyltransferase. Thus, both parental *H19* alleles are fully methylated by the time germ cells enter meiosis I in spermatocytes and the paternal-specific imprint is set. Interestingly, it is at this same time during spermatogenesis that a shift from asynchronous to synchronous replication of *H19* alleles is observed (39). Further experiments examining chromatin structure and CTCF in germ cells will be required to test this model.

## MATERIALS AND METHODS

### Mice

C57BL/6J and *Mus musculus castaneus* mice were purchased from the Jackson Laboratory (Bar Harbor, ME). To facilitate the isolation of F<sub>1</sub> hybrid mice for the bisulfite analysis of embryonic germ cells, a strain of mice that served as the source of the *M.m.castaneus* allele [B6(CAST-H19)] was used (7). Natural matings between C57BL/6J females and B6(CAST-H19) males were used to produce embryos for the isolation of PGCs and prospermatogonia.

### Isolation of germ cells from 13.5 and 14.5 d.p.c. embryos

Purified populations of male and female germ cells were obtained from 13.5 and 14.5 d.p.c. embryonic gonads by immunoaffinity purification (40). Embryos at each stage were sexed by gonadal morphology: the developing testicular cords are easily recognized in male embryos by 13.5 d.p.c. (41). The embryonic gonad was separated from the mesonephros, digested with 1 ml of trypsin-EDTA [0.25% trypsin, 1 mM EDTA, 1× phosphate-buffered saline (PBS)] for 5 min at 37°C, washed with PBS-DNase [5 mM EDTA, 0.5% bovine serum albumin (BSA), 20 µg/ml DNase, 1× PBS], manually triturated to a single cell suspension in 180 µl and shaken for 10 min at room temperature. Twenty microliters of TG-1 antibody (42) was added to the single cell suspension and the solution was incubated for 30 min at 4°C with occasional mixing. The cells were washed twice with 150 µl of PBS-DNase, resuspended in 180 µl of PBS-DNase and incubated with 20 µl of rat anti-mouse IgM MiniMACS beads (Miltenyi Biotec, Auburn, CA) for 30 min at 4°C with occasional mixing. The cells were then passed over an MS(+) separation column (Miltenyi Biotec). The column was washed three times with 500 µl of PBS-DNase and once with 500 µl of 0.5% BSA in PBS. The cells were eluted in 1.5 ml of 0.5% BSA in PBS and collected by centrifugation for 2 min at 2500 r.p.m. An aliquot was removed for the assessment of germ cell purity prior to freezing the cells in liquid nitrogen in preparation for the isolation of genomic DNA.

Germ cell purity was determined by staining an aliquot of cells for alkaline phosphatase activity. Germ cells were allowed to settle on poly-L-lysine coated slides in a humidified chamber for 30 min at room temperature. The cells were then washed twice with 1× PBS, fixed in 4% paraformaldehyde (in PBS) for 20 min at room temperature, washed twice with distilled H<sub>2</sub>O, washed once with 25 mM Tris-malate pH 9.0 and incubated in alkaline phosphatase staining solution for 30–45 min (25 mM Tris-malate pH 9.0, 8 mM MgCl<sub>2</sub>, 1 mg/ml Fast Red TR salt, 0.4 mg/ml naphthyl phosphate). Germ cell identity was confirmed by the appearance of red cells in the eluate and purity of each population was determined by counting a sample of at least 100 cells from each sample purified; for most stages, more than one independent germ cell purification was performed. Purity of germ cells was determined to be 93 and 89% for 13.5 d.p.c. male germ cells; 96% for 13.5 d.p.c. female germ cells; 89 and 77% for 14.5 d.p.c. male prospermatogonia; 93 and 97% for 14.5 d.p.c. female oocytes.

### Isolation of 15.5 and 18.5 d.p.c. prospermatogonia

Germ cells were isolated from gonads of fetuses at 15.5 and 18.5 d.p.c. using a mini Sta Put method essentially as described (10,43). Briefly, uteri were removed from euthanized timed-pregnant females, and gonads were dissected from fetuses and pooled according to sex as determined by gonadal morphology. Gonadal cells were dissociated by incubation at room temperature in 0.05% trypsin plus 0.53 mM EDTA in Hank's buffered saline solution without Ca<sup>2+</sup> or Mg<sup>2+</sup> (Gibco BRL, Gaithersburg, MD) with pipetting until no cell clumps were visible. The cells were then resuspended in 2 ml of 0.5% BSA in EKRB buffer (44), loaded onto a 50 ml 2–4% BSA gradient in a mini Sta Put chamber and allowed to settle under unit gravity at 4°C for 2 h. The gradient was then fractionated and fractions containing germ cells were identified on the basis of morphology under phase optics and pooled. Purities of each cell population were >85%. After washing, cell pellets were snap frozen in liquid nitrogen in preparation for isolation of genomic DNA.

### DNA purification and bisulfite analysis

DNA was isolated and subjected to bisulfite modification, PCR amplification, subcloning and sequencing as previously described (17), with the following modifications. DNA isolated from a single 13.5 or 14.5 d.p.c. germ cell purification was used in each bisulfite mutagenesis reaction. The mutagenized DNA was resuspended in 20 µl of TE and 1 µl was used for PCR amplification. All mutagenized DNA were subjected to multiple independent PCR amplifications, to ensure recovery of different strands of DNA. In addition, for some of the developmental stages assayed (13.5 and 14.5 d.p.c.), two independently isolated cell samples were analyzed separately to confirm the observed results. The following primer pairs were used for nested amplification of the mutagenized DNA. For 1304–1726, BMsp2t1/BHha1t3 followed by BMsp2t2/BHha1t4; for 1739–2131, BHha2t1/BMsp3t2 followed by BHha2t2/BMsp3t; for 2448–2748, BHha5t2/BHha5t3 followed by BHha5t/BHha5t3; and for 4393–4774, BMsp6t/BMsp7t followed by BMsp6t2/BMsp7t. Primer sequences follow with the nucleotide position of the first base indicated in parentheses (GenBank accession no. U19619):

BMsp2t1, 5'-GAGTATTTAGGAGGTATAAGAATT-3' (1278);  
 BMsp2t2, 5'-GTAAGGAGATTATGTTTATTTTGG-3' (1304);  
 BHha1t4, 5'-CCTCATTAAATCCATAACTAT-3' (1726);  
 BHha1t3, 5'-ATCAAAAACATAACATAAACCCCT-3' (1751);  
 BHha2t1, 5'-ATAGTTATGGGTTTTATGAGG-3' (1706);  
 BHha2t2, 5'-AGGGGTTTATGTTAGTTTTTGATAA-3' (1739);  
 BMsp3t, 5'-ACACCCAAAACCTAATATAAAATTC-3' (2131);  
 BMsp3t2, 5'-CCTCTCAATTAATTTAACT-3' (2153);  
 BHha5t2, 5'-TTGTGAGTGGAAAGATTAAATTTGTTGG-3' (2355);  
 BHha5t, 5'-TAGAGATAGTTAAAGTTAAGGTTTGTATG-3' (2415);  
 BHha5t3, 5'-ATACACACATCTTACCACCCCTATAAATCCC-3' (2748);  
 BMsp6t, 5'-GGTTGAGGATTTGTTAAGGTGTTATTG-3' (4343);  
 BMsp6t2, 5'-GAGTGGTTATGATTGGTTAGTTTTTGAG-3' (4396);  
 BMsp7t, 5'-TAATAACTAATTTAAACACTCCTCACC-3' (4777).

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## NOTE ADDED IN PROOF

The methylation status of *H19* in male germ cells has been independently reported by Sasaki and colleagues: Ueda *et al.* (2000) *Genes Cells*, **5**, 649–659.

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