Deletion of the H19 differentially methylated domain results in loss of imprinted expression of H19 and Igf2

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Differentially methylated sequences associated with imprinted genes are proposed to control genomic imprinting. A 2-kb region located 5' to the imprinted mouse H19 gene is hypermethylated on the inactive paternal allele throughout development. To determine whether this differentially methylated domain (DMD) is required for imprinted expression at the endogenous locus, we have generated mice harboring a 1.6-kb targeted deletion of the DMD and assayed for allelic expression of H19 and the linked, oppositely imprinted Igf2 gene. H19 is activated and Igf2 expression is reduced when the DMD deletion is paternally inherited; conversely, upon maternal transmission of the mutation, H19 expression is reduced and Igf2 is activated. Consistent with the DMD's hypothesized role of setting up the methylation imprint, the mutation also perturbs allele-specific methylation of the remaining H19 sequences. In conclusion, these experiments show that the H19 hypermethylated 5' flanking sequences are required to silence paternally derived H19. Additionally, these experiments demonstrate a novel role for the DMD on the maternal chromosome where it is required for the maximal expression of H19 and the silencing of Igf2. Thus, the H19 differentially methylated sequences are required for both H19 and Igf2 imprinting.

[Key Words: Differentially methylated domain; DNA methylation; H19; Igf2; genomic imprinting]

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We have proposed that this autonomous regulation is governed by paternal-specific DNA methylation present at the endogenous and transgenic loci (Bartolomei et al. 1993). The 7 kb of paternal-specific methylation observed in somatic tissues and sperms includes 4 kb of upstream flanking sequence and the H19 structural gene (Bartolomei et al. 1993; Brandeis et al. 1993; Ferguson-Smith et al. 1993). The importance of methylation for the repression of the paternal allele of H19 is underscored by experiments in which imprinted gene expression was characterized in mice that were deficient for the DNA methyltransferase gene Dnmt1 (Li et al. 1993). When analyzed prior to their death, Dnmt1 null mice expressed both alleles of H19, suggesting that DNA methylation is at least required to maintain H19 imprinting. To demonstrate that DNA methylation could also serve a causative role in the marking of the parental alleles and the setting of the parental imprint, we assayed DNA methylation of H19 during embryogenesis, with an emphasis on preimplantation development as this is the time when the embryo undergoes a period of generalized demethylation (Monk et al. 1987; Sanford et al. 1987). A 2-kb region located from −2 to −4 kb relative to the start of transcription is methylated exclusively on the paternal allele throughout development, suggesting that this region is crucial to determining the imprinted expression of H19 (Tremblay et al. 1995, 1997). When this region was deleted from the original imprinted H19 transgene, the new transgenes were expressed and hypomethylated regardless of parental origin (Elson and Bartolomei 1997).

To determine the role of the 2-kb differentially methylated domain (DMD) at the endogenous H19 locus, we have generated mice lacking the DMD. When the DMD deletion allele is transmitted to the progeny from the father, the normally repressed paternal H19 allele is activated and the expression of the linked paternal Igf2 gene is reduced coordinately. In contrast, transcription of the mutant H19 allele by the mother results in reduced expression of the H19 gene with a concomitant activation of the maternal Igf2 allele, revealing a novel regulatory role for this region. Whereas these experiments prove that the DMD is necessary for silencing the paternal H19 allele, they also show that the DMD is essential on the maternal chromosome for the exclusive expression of H19 and the silencing of Igf2. We conclude that the DMD is required on both parental alleles for the reciprocal imprinting of H19 and Igf2.

**Results**

**Targeted disruption of the H19 upstream differentially methylated domain**

The region from approximately −2 to −4 kb relative to the start of H19 transcription is methylated in sperm, unmethylated in oocytes, and preferentially methylated on the paternal allele throughout development (Tremblay et al. 1995, 1997; O’leik and Walter 1997). To test the role of this DMD at the endogenous locus, we deleted most of the DMD by gene targeting in embryonic stem (ES) cells and generated mice with the deletion. As shown in Figure 1b, a targeting vector was constructed in which 1.6 kb of the DMD was replaced by a neomycin resistance (neo) gene flanked by loxP sites. The deletion removes 48 of the CpG dinucleotides that we have proposed to be essential for conferring imprinted expression (Tremblay et al. 1995, 1997). The remaining DMD sequence includes five differentially methylated CpG dinucleotides located 5′ to the targeted deletion.

Because the function of a putative imprinting regulatory element was being tested, it was important to eliminate any new regulatory elements introduced by the neo gene cassette. Therefore, following the identification of correctly targeted cells lines, two independent clones were chosen for a second electroporation with a vector encoding Cre recombinase to derive clones that deleted the neo gene (Fig. 1c,d). Cells (with and without the neo gene) were injected into C57BL/6j host blastocysts and mice inheriting the targeted allele were selected for subsequent breeding. The mutant mice were maintained by breeding to C57BL/6j mice. For analysis of allelic imprinting patterns, the heterozygous DMD mutant mice were mated with a strain of mice (B6CAST–H19, (Tremblay et al. 1995)) in which the portion of distal chromosome 7 harboring the imprinted genes of interest was derived from Mus musculus castaneous. Heterozygous and homozygous DMD mutant mice were obtained in the predicted Mendelian ratio and were viable and fertile.

**Paternal inheritance of the DMD deletion**

To determine the effect of the DMD deletion on the expression of imprinted genes, mice that inherited the mutant allele (H19\textsuperscript{DMD}) from the father were first tested for H19 expression (Fig. 2a). When the livers from neonatal heterozygous mice were analyzed by RNase protection, the normally silent paternal H19 allele (Fig. 2a, lanes 8–10) was activated to a level of ~60% of that observed for the maternal wild-type allele, whereas H19 expression from the maternal allele was unaffected (Fig. 2a, lanes 11–13). The analysis of other tissues showed that the level of activation of the mutant paternal H19 allele varied according to tissue type, with gut derivatives exhibiting a moderate level of activation, whereas in muscle derivatives activation was nearly equivalent to that for liver (data not shown). These results indicate that deletion of the DMD eliminated sequences that were repressive to H19 gene expression. The deletion did not, however, completely activate H19 expression to levels observed for the wild-type maternal allele.

Because transcription of the Igf2 and H19 genes is linked (Leighton et al. 1995a, b), the effect of the DMD deletion on Igf2 expression was examined. Paternal transmission of the mutant H19\textsuperscript{DMD} allele caused repression of the paternally inherited Igf2 gene (Fig. 2b, cf. lanes 10–12 and lanes 7–9). When the level of paternal allele expression in the liver of neonatal heterozygous mice was compared to that of wild-type littermates, a
ingly, transmission of the mutant allele and expression from the wild-type paternal allele was also tested for the DMD on mouse chromosome 7 are indicated. The gray box corresponds to the 2-kb DMD, which is located -2 to -4 kb relative to the start of H19 transcription. (c) The endodermal enhancers located at -9 and -11 kb. (b) From top to bottom: the linearized targeting vector, the endogenous H19 locus, the targeted H19 locus (H19DMDneo), and the targeted H19 locus after removal of PGK-neo using CRE-IoxP recombination (H19DMD). The linearized vector includes Bluescript II KS (thick line), the diphtheria toxin A gene (open box, DT), PGK-neo (open box, neo) flanked by IoxP sites (black vertical bars), 5’ H19 sequence (thin black line) and H19 gene sequence extending into the third exon (solid boxes). Arrows indicate direction of transcriptional orientation of PGK-neo and H19. The positions of the restriction sites and the external probes (EcoRV, EcoRI and BamHI/Stu) used to analyze the targeted clones are indicated. (c,d) ES cell clones were screened by Southern blot analysis for the targeting event. Genomic DNA was digested with EcoRV and hybridized to the 5’ probe EcoRV/EcoRI (c) or with Stu and hybridized to the 3’ probe BamHI/Stu (d). The DNA samples shown include the parental ES cell DNA (+/+), targeted clones with the neo’ gene (neoR+), and a targeted clone in which the neo’ gene was excised (neoR-). Molecular sizes (in kb) are indicated to the right. Of the 85 G418-resistant clones analyzed, four were correctly targeted to the H19 locus.

66% reduction in Igf2 RNA was observed (Fig. 2d). As noted for H19, the reduction in Igf2 expression varied according to tissue (data not shown). Consistent with the decrease in Igf2 expression, the weights of the heterozygous littermates were on average 93% that of their wild-type littermates. Whereas reduction of Igf2 expression in liver is concordant with experiments proving that activation of Igf2 and H19 liver is concordant with experiments proving that activation of Igf2 and H19 was also observed upon paternal transmission of the mutation, these results indicate that deletion of the DMD resulted in a true competition for the endodermal enhancers. These results additionally demonstrate that the DMD has a previously unsuspected positive regulatory function for the exclusive expression of the maternal H19 allele.

Maternal inheritance of the DMD deletion
The effect of maternal transmission of the DMD mutation was also tested for H19 and Igf2 expression. Surprisingly, transmission of the mutant H19DMD allele through the maternal germ line resulted in the reduced expression of the H19 gene (Fig. 2a, cf. lanes 4–7 with lanes 1–3). When quantified by RNase protection, the expression of H19 in neonatal livers was approximately half that observed in wild-type littermates (Fig. 2c). To determine if Igf2 was affected by the maternally derived DMD mutation, allelic Igf2 expression levels were assayed. The normally silent maternal Igf2 allele was activated to about one-third the level of the wild-type paternal allele and expression from the wild-type paternal allele was unaffected (Fig. 2b, lanes 2–6). Additionally, when compared to their wild-type littersmates, the mice that inherited the mutant allele maternally were on average 17% larger, which is consistent with activation of maternal Igf2. Thus, a reduction in the level of H19 expression on the mutant maternal allele was accompanied by an activation of the maternally derived Igf2 gene and a slight increase in weight. Because the coordinated expression of H19 and Igf2 was also observed upon paternal transmission of the mutation, these results indicate that deletion of the DMD resulted in a true competition for the endodermal enhancers. These results additionally demonstrate that the DMD has a previously unsuspected positive regulatory function for the exclusive expression of the maternal H19 allele.

Methylation analysis of the targeted H19 alleles
We have proposed that the DMD harbors an imprinted mark in the form of paternal-specific methylation (Tremblay et al. 1995, 1997). Because not all of the differentially methylated sequences at the H19 locus were removed by the DMD deletion, it was of interest to determine the effect of the deletion on the methylation of the remaining CpG dinucleotides. The CpG dinucleotides located in the promoter-proximal region and the 5’ portion of the H19 structural gene are preferentially
methylated on the paternal allele late in gestation [see Fig. 3d, sites −500 and +501 bp (Bartolomei et al. 1993; Brandeis et al. 1993; Ferguson-Smith et al. 1993; Tremblay et al. 1997)]. In contrast, the 3' portion of the H19 structural gene is equally methylated on both alleles [see Fig. 3d, sites downstream of +501 bp (Ferguson-Smith et al. 1993)], as are sites 5' of the 2-kb DMD [Fig. 3d, upstream of −4000 bp (Tremblay et al. 1997)]. To determine whether the methylation of the H19 promoter and structural gene was affected by the mutation, neonatal liver DNA from reciprocal heterozygotes was digested with PvuII and StuI and the methylation-sensitive restriction enzyme HpaII and subjected to Southern analysis (Southern 1975). The wild-type M. castaneus allele lacks a PvuII site, which enabled the distinction between the mutant C57BL/6 H19 allele (3.2 kb) and the wild-type M. castaneus H19 allele (3.4 kb) (Bartolomei et al. 1993). When the mutation was transmitted to the progeny by the mother, the methylation of the H19 promoter and structural gene appeared unaffected (Fig. 3b, cf. lanes 4 and 6). In contrast, the deletion of the DMD on the paternally inherited allele resulted in the hypomethylation of the HpaII sites in the promoter region (Fig. 3b, PvuII/StuI fragment in lanes 8 and 10). Similarly, a HhaI site in the promoter was not methylated on the mutant paternal allele (data not shown). These results were consistent with methylation analysis in homozygous mutant animals in which the promoter was hypomethylated on both alleles (Fig. 3b, lane 13). Thus, deletion of the DMD from the paternal allele was accompanied by a loss of methylation in the sequences surrounding the promoter, causing the mutant paternal allele to resemble the wild-type maternal allele (Fig. 3d).

Next, we examined the methylation status of upstream CpG dinucleotides that remained on the mutant allele (Fig. 3a, sites between −4500 and −500 bp). The HhaI site located immediately 5' to the deletion and the two HhaI sites located between the promoter and the
DMD were analyzed using SacI polymorphic fragments (Fig. 3a). In wild-type animals, the sites were hypermethylated on the paternal allele but hypomethylated on the maternal allele (Fig. 3c, lanes 5, 6, 9, 10). However, the mutant H19DMD allele was hypermethylated whether it was maternally or paternally transmitted (Fig. 3c, lanes 7 and 8 and 11 and 12, respectively). This hypermethylation of the mutant allele was also evident in homozygous mutant animals (Fig. 3c, lanes 15 and 16). The remaining HpaII sites in this upstream region were also hypermethylated on both mutant alleles (data not shown). Thus, whereas the DMD deletion was associated with the hypomethylation of cytosine residues surrounding the promoter, CpG dinucleotides located upstream from the promoter were partially methylated on the mutant alleles (Fig. 3c and data not shown). Taken together, these results indicate that the methylation status of the mutant alleles reflects neither the maternal nor paternal wild-type pattern (Fig. 3d). Rather, the mutant allelic methylation pattern was intermediate between the two wild-type parental alleles and no longer parental-specific.

We also compared the methylation status of sperm DNA isolated from wild-type and homozygous mutant adult males generated from F1 heterozygous intercrosses. The methylation state of the H19 promoter, structural gene, and remaining 5' sequence was unchanged with the removal of the DMD sequence. Specifically, the wild-type and ΔDMD sperm DNA were similarly unmethylated in the H19 promoter region (Fig. 3b, lanes 14 and 15) and were similarly methylated at HhaI sites immediately 5' and 3' of the deleted sequence (data not shown). These data indicate that removal of DMD sequence did not perturb the acquisition of the sperm-specific methylation pattern in the remaining H19 sequence.

**Figure 3.** Methylation analysis of H19 in heterozygous and homozygous DMD mutant mice. (a) The location of the HpaII (H) and HhaI (Hh) sites with respect to the deleted DMD sequence. The 5' H19 DMD sequence, deleted between the KpnI (K) and HindIII (Hd) sites, and H19 are represented by boxed regions. (P) PvuII; (R) EcoRI; (Stu) Stul; (S) SacI. (*) The polymorphic PvuII site that is found in C57BL/6J and H19DMD; (**) The polymorphic M. castaneus SacI site. The RSt probe used in b and the ScK probe used in c are indicated beneath the line. The position of the sites, relative to the start of transcription, are indicated (in bp) above the gene line. (b) The methylation status of HpaII sites in the H19 PvuII/Stul fragment is assessed with the RSt probe. DNA from C57BL/6J (B) mice, B6CAST–H19 (C) mice, progeny of a H19DMD heterozygous female mated to a B6CAST–H19 male (lanes 3–6), progeny from a B6CAST–H19 female mated to a H19DMD heterozygous male (lanes 7–10) and progeny of a H19DMD heterozygous female mated to a H19DMD heterozygous male (lanes 11–16) were analyzed. DNA was digested with PvuII and Stul and, in lanes indicated, HpaII (H) or MspI (M). The genotypes of the assayed DNA from the specific mating are indicated above the lanes. DNA from neonatal livers (lanes 1–13, 16) and adult sperm (lanes 14, 15) were assayed. The M. castaneus–specific PvuII/Stul fragment (C, 3.4 kb) and the C57BL/6 and H19DMD–specific PvuII/Stul fragment (B, ΔDMD, 3.2 kb) are noted at left, with size markers in kb shown at right. (c) DNA from mice described in b was analyzed for HhaI methylation in the 5' H19 SacI fragment using the ScK probe. DNA was digested with SacI and, in lanes indicated, HhaI (Hh). The genotypes of the DNA samples are indicated above the lanes. The C57BL/6J (3.8 kb), B6CAST–H19 (1.5 kb) and the H19DMD (2.2 kb) specific SacI fragments are indicated at left. (d) Summary of the parental-specific methylation status of HpaII and HhaI sites on wild-type (top line) vs. H19DMD (middle line) alleles. The taller and shorter lollipops represent the HhaI and the HpaII sites, respectively. (Solid circles) Fully methylated sites; (open circles) unmethylated sites; (striped circles) sites that are methylated on a subset of the alleles, as determined from previous studies (bisulfite sequence and Southern analyses) and data presented herewith; (shaded circles) sites that are partially methylated. The paternal-specific methylation status is presented above each allele; the maternal-specific methylation status is presented below each allele.
Analysis of H19<sup>DM</sup>neo alleles

The experiments described above used mice in which the neo<sup>+</sup> gene was excised. To determine if the perturbation of imprinted gene expression was caused solely by the absence of the DMD or if the spacing change imposed by the deletion was responsible for altered gene-expression patterns, mice in which the neo<sup>+</sup> gene remained at the H19 locus were examined. Because the size of the neo<sup>+</sup> gene was similar to that of the deleted DMD fragment, inclusion of the neo<sup>+</sup> gene preserved spacing of the H19 upstream elements. As observed for the H19<sup>DM</sup>neo alleles, both the H19 and Igf2 genes were expressed on the maternal and paternal H19<sup>DM</sup>neo alleles in neonatal liver (Fig. 4). The neo<sup>+</sup> gene did not affect H19 expression levels on either parental allele (Fig. 4a, data not shown), supporting the proposed role of the DMD as both a positive and negative regulator of H19 gene expression. However, the presence of the neo<sup>+</sup> gene caused a less dramatic increase in the expression of Igf2 on the mutant maternal allele (Fig. 4b, lanes 2,3). That is, in the presence of the neo<sup>+</sup> gene, Igf2 was activated to ~6% that of the wild-type paternal allele, whereas in the absence of the neo<sup>+</sup> gene, Igf2 expression on the mutant maternal allele increased to an average of one-third the level of the wild-type paternal allele (Fig. 4b, lanes 1–3 and 4–6, respectively). These results suggest that the neo<sup>+</sup> gene interfered with the activation of Igf2 on the mutant maternal allele and are consistent with studies of regulatory elements at other loci demonstrating that the neo<sup>+</sup> gene regulatory elements affected gene expression (Fiering et al. 1995).

The imprinting of the neo<sup>+</sup> gene was also assessed in RNA isolated from livers of heterozygous and homozygous H19<sup>DM</sup>neo neonatal mice. Northern blot analysis showed that the neo<sup>+</sup> expression levels were similar in the maternal and paternal H19<sup>DM</sup>neo heterozygous mutants and twofold higher in the homozygous mutants (data not shown), demonstrating neo<sup>+</sup> expression was not imprinted. Thus, in contrast to previous experiments in which the neo<sup>+</sup> gene was used to replace the H19 transcription unit and was imprinted (Ripoche et al. 1997), the sequences remaining at the H19<sup>DM</sup> locus were not sufficient to imprint the neo<sup>+</sup> gene.

Discussion

We proposed previously that paternal-specific DNA methylation in the region located from ~2 to ~4 kb relative to the start of H19 transcription is involved in the imprinting of the mouse H19 gene (Tremblay et al. 1997). Absence of this region in a normally imprinted mouse transgene results in paternal-independent expression and hypomethylation of the derivative transgene (Elson and Bartolomei 1997), indicating that the DMD is crucial for suppression of a paternally transmitted transgene. This element also has unique properties in Drosophila where it acts as a silencer, despite the absence of DNA methylation in the Drosophila genome (Lyko et al. 1997).

To test the role of the DMD at the endogenous H19 gene locus, we have deleted most of this region using gene-targeting technology. Paternal transmission of the mutant allele resulted in activation of H19 expression, a concomitant reduction in Igf2 expression, and a reduction in methylation of the remaining CpG dinucleotides at the H19 locus (Fig. 5). These results are consistent with the transgenic experiments and support the hypothesis that the DMD represses transcription of the paternally derived H19 allele. The negative regulatory role of the paternally derived DMD is likely caused by its hypermethylation that could either act directly by preventing the binding of factors that establish a transcriptionally competent state or could act indirectly through the methyl-CpG-binding protein MeCP2, or other unknown proteins with analogous activities, which subsequently recruits histone deacetylases and represses transcription (Jones et al. 1998b; Nan et al. 1998).

A new finding of this study was the observed decrease in H19 expression together with the concomitant activation of Igf2 upon maternal transmission of the mutant allele, suggesting that the DMD also influences transcription of H19 on the maternal allele positively (Fig. 5). Indeed, chromatin studies of the H19 locus have shown that the DMD is hypersensitive to nucleases exclusively on the maternal chromosome, supporting the concept of a novel maternally derived positive role for this element (Hark and Tilghman 1998). A number of mechanisms could lead to this positive regulatory function. First, the unmethylated DMD might bind transcription factors

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**Figure 4.** Expression of H19 and Igf2 in H19<sup>DM</sup> and H19<sup>DM</sup>neo mice. The genotypes are noted above the lanes and the parental identity of protected fragments are indicated to the right. Neonatal liver RNA (3 µg) is analyzed using allele-specific RNase protection assays. (a) H19 expression in paternal heterozygotes. The RNA isolated from a wild-type and a mutant littermate, generated from mating a B6(CAST–H19) female with a heterozygous H19<sup>DM</sup>neo male, is assayed in lanes 1 and 2, respectively. The RNA isolated from the progeny of a mating between a B6(CAST–H19) female and a heterozygous H19<sup>DM</sup>neo male is assayed in lanes 3–5. (b) Igf2 expression in maternal heterozygotes. RNA from wild-type and mutant neo littermates (lanes 1 and 2 and 3, respectively) and wild-type and mutant neo littermates (lanes 4 and 5 and 6, respectively) produced from the reciprocal mating performed in a is assayed.
that promote the expression of H19. In the case of the mutant maternal allele, the decreased expression of H19 may be caused by the loss of positive transcriptional elements rendering H19 less effective in utilizing the endoderm-specific enhancers. These enhancers are then free to drive the expression of Igf2. Thus, although the DMD is not required for transgenic H19 expression (Pfeifer et al. 1996; Elson and Bartolomei 1997), possibly because of the absence of the competing Igf2 gene, it appears to be essential for the optimal and exclusive expression of H19 at the endogenous locus. Second, the maternal DMD may form a unique chromatin configuration that promotes transcriptional activity. Third, the DMD could act as a selector that directs transcription either toward H19 or Igf2. Fourth, the DMD may function to repress Igf2 transcription on the maternal allele, thereby indirectly affecting H19 expression. Thus, in the absence of the DMD on the maternal allele, Igf2 expression is derepressed, which reduces access of the enhancers to H19. Finally, Tilghman and colleagues have suggested that the DMD acts as a domain boundary or a chromatin insulator which isolates the H19 promoter and endodermal enhancers and blocks the Igf2 gene from accessing these enhancers on the maternal chromosome (Webber et al. 1998). Originally identified in Drosophila, boundary elements insulate a gene and its regulatory elements from position effect variegation and can block gene expression when placed between a gene and its enhancers (Kellum and Schiedl 1991, 1992). The proposal that the DMD functions as a domain boundary in mouse is supported by experiments in which Igf2 is preferentially expressed on maternally derived chromosomes in which the H19 endodermal enhancers were removed from their normal location and placed between the H19 and Igf2 genes and upstream of the DMD (Webber et al. 1998). Although these latter experiments could be explained by distance effects or the placement of the enhancers in a new chromatin environment, taken together, the gene-targeting experiments would argue in favor of the DMD acting as a domain boundary. Formal proof of this model will require the demonstration that the DMD insulates gene expression at a heterologous locus.

The experiments described in this report support the original model proposing that the reciprocal imprinting of the H19 and Igf2 genes is mediated by a competition for the shared set of endodermal enhancers (Fig. 5 [Bartolomei and Tilghman 1992]). Mice harboring the DMD deletion express H19 and Igf2 from the mutant chromosome, with enhanced expression of one gene accompanied by a coordinate decrease in the expression of the other gene. The canonical example for this type of regulation is the promoter competition in the chicken β-globin gene complex, where the switch from the embryonic α-globin to the adult β-globin is achieved through a competition for the β-globin enhancer (Choi and Engel 1988; Foley and Engel 1992). Similar to the compensatory expression changes observed in our mutant mice, mutations that attenuated adult β-globin expression were accompanied by an increase in the expression of α-globin (Foley and Engel 1992). Recent experiments by Jones and colleagues indicate that the competition by H19 and Igf2 promoters for the endodermal enhancers may not be mediated strictly by the promoters and DMD alone (Jones et al. 1998a). In experiments in which the H19 transcriptional unit was replaced with the luciferase gene, luciferase was expressed at variable levels on the paternal allele, whereas the expression and imprinting of Igf2 was maintained at wild-type levels. One interpretation of these experiments is that the RNA-coding portion of the H19 gene is also required for linked competition of these genes.

Our study does not address the mechanism by which H19 and Igf2 share enhancer elements at the cellular level. For example, in the paternal mutant heterozygote, H19 and Igf2 may be expressed simultaneously from the mutant allele. Alternatively, each cell makes a choice: some cells may exclusively express Igf2 from the mutant paternal allele and other cells may exclusively express H19. It is also possible that each cell expresses both
genes from the mutant paternal allele but only one gene is expressed at a given time. The latter is analogous to the flip-flop model of gene regulation that has been proposed to explain how distal control elements allow the simultaneous expression of γ- and β-globin in early development (Wijgerde et al. 1995). Future studies of our mutants at the cellular level will discriminate between these possibilities.

Finally, we have determined that deletion of the DMD on one chromosome does not affect the expression or methylation of the genes on the wild-type chromosome. Unlike experiments showing that Igf2 transgenes can transactivate the endogenous Igf2 gene and lead to Beckwith-Wiedemann-related symptoms (Sun et al. 1997), the activation of either H19 or Igf2 on the mutant chromosome does not affect the expression of their counterparts on the wild-type chromosome. Furthermore, after three generations of breeding the mutant mice, the only observed phenotypes in animals with alterations in H19 and Igf2 expression are subtle size effects that are consistent with the changes in Igf2 expression. No phenotypic consequences reminiscent of Beckwith-Wiedemann have been noted. Additionally, homozygous mutant animals have no apparent phenotype, possibly because the total expression of H19 and Igf2 in the neonatal livers of homozygous animals is similar to that of wild-type animals (data not shown).

In conclusion, we have demonstrated that the DMD has multiple roles in regulating the imprinting of the H19 gene. Our hypothesis that the differential methylation serves as the allelic mark is strengthened by the observation that deletion of the DMD on both chromosomes renders them indistinguishable by the criteria employed in these studies. As expected, the DMD mediates a repressive effect on the transcription of the paternally derived chromosome, presumably through its hypermethylation. Additionally, we have shown that the DMD permits the exclusive expression of H19 on the maternal chromosome, possibly through the binding of positive regulatory factors, a unique chromatin configuration, inhibition of Igf2, or through the assembly of a domain boundary which prevents access of Igf2 to the enhancers. Future studies will elucidate the factors responsible for the multiple roles of this complex element.

Targeted disruption of the DMD region in ES cells

The vector was linearized at a unique NotI site prior to electroporation into ES cells. E14.1 ES cells (Kuhn et al. 1993) were grown on neomycin-resistant mouse embryonic fibroblasts. ES cells (1.5 × 10^6/ml) were collected in 0.8 ml of phosphate-buffered saline and electroporated with a pulse of 250 V/500 μF (Gene Pulser, Bio-Rad) with 25 μg of linearized targeting vector. Following a 24-hr recovery, the medium was adjusted to 200 μg/ml G418. After 8–10 days growth, G418-resistant colonies were isolated and expanded, and DNA was prepared. The DNA was digested with EcoRV (5′-end confirmation) or Stul (3′-end confirmation) and size fractionated on 0.75% and 1.0% agarose gels, respectively. DNA was transferred to nitrocellulose (Southern 1975) and hybridized to nick-translated external probes (Rigby et al. 1977). The EcoRV/EcoRI probe was used for 5′-end confirmation and the Bambh/Stul probe was used for 3′-end confirmation (Fig. 1b).

The neo cassette was removed by transiently transfecting two independent DMDneo ES cell lines with 25 μg of a plasmid encoding the Cre recombinase (Sauer and Henderson 1990). Correctly excised clones were verified by digestion with Stul or EcoRV, as described above, and by a 350-bp PCR product that was amplified using primers that flank the DMD mutation. The forward primer was 5′-ATCCAGGAGGCATCCGAATT-3′ and the reverse primer was 5′-GTTGCAACAAATGCCTGATCT-3′.

Cells from targeted ES cell clones (with and without the neo gene) were injected into C57BL/6j blastocysts, and the blastocysts were transferred to pseudopregnant female mice. To determine if germ-line transmission of the mutant allele had occurred, chimeras were bred with C57BL/6j mice, and DNA was isolated from tail biopsies of progeny. The Southern blot and PCR analyses described above were used to genotype the mice. To analyze allele expression and methylation patterns, the heterozygous mutant mice were bred to the B6C3F1 strain of mice (Tremblay et al. 1995). These mice have M. castaneus H19 and Igf2 alleles on a C57BL/6 background. For F1 hybrid mice, the maternal parent is designated first.
For quantitation, RNase protection gels were exposed to storage phosphor screens that were scanned on a Storm 840 PhosphorImager (Molecular Dynamics). Band intensities were calculated using ImageQuant Version 1.0 (Molecular Dynamics). After pseudocolor enhancement of the image, bands of interest were traced using the freehand drawing tool. The median pixel values of individual segment boundaries were used as the background values. In all cases, RNase protection assays of the deletion allele produce one protected fragment, whereas assays of the wild-type M. castaneus allele produce two protected fragments. In samples in which biallelic expression was observed, the value of the mutant protected fragment was quantified relative to the two fragments corresponding to the wild-type B6(CAST–M. castaneus) allele. The levels of H19 RNA in ΔDM D maternal heterozygotes and Igf2 RNA in ΔDM D paternal heterozygotes were quantified relative to wild-type levels using rpL32 as an internal control.

DNA isolation and methylation analysis

DNA was isolated from tissues and sperm as described previously (Bartolomei et al. 1993). Genomic DNA (10 µg) was digested with PvuII and Stul in combination with HpaII orMspI to analyze the methylation of the H19 structural gene or with SacI and HhaI to analyze the methylation of upstream sequences. The probes used for the respective analyses were the 2.5-kb EcoRI–Stul (RSI) fragment and the 0.9-kb SacI–KpnI (SCK) 5’ fragment (Fig. 3a).

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