H19 and *Igf2* monoallelic expression is regulated in two distinct ways by a shared *cis* acting regulatory region upstream of *H19*

Madhulika Srivastava, Sandra Hsieh, Alexander Grinberg, Lisa Williams-Simons, Sing-Ping Huang, and Karl Pfeifer[1]

Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, Bethesda, Maryland 20892 USA

***H19* and *Igf2* are expressed in a monoallelic fashion from the maternal and paternal chromosomes, respectively. A region upstream of *H19* has been shown to regulate such imprinted expression of both genes in *cis*. We have taken advantage of a *loxP*/cre recombinase-based strategy to delete this region in mice in a conditional manner to determine the temporal requirement of the upstream region in initiating and maintaining the imprinted expression of *H19* and *Igf2*. Analysis of allele-specific expression of *H19* and *Igf2* and DNA methylation at the *H19* promoter demonstrates that this region controls the monoallelic expression of the two genes in different ways, suggesting that it harbors two functionally distinct regulatory elements. Continued presence of the region is required to silence maternal *Igf2* in accordance with its proposed role as an insulator. However, it does not have a direct role in keeping the paternal *H19* promoter silenced. Instead, on the paternal chromosome, the upstream element mediates epigenetic modifications of the *H19* promoter region during development, leading to transcriptional silencing of *H19*. Thereafter, its presence is redundant for preventing transcription. Presently, this temporal requirement of the silencing element appears to be a unique *cis* activity in the mammalian system. However, it is likely that other *cis*-acting elements, positive and negative, have the ability to effect stable changes in the chromatin structure and are not constantly required to give signals to the transcriptional machinery.**

[*Key Words*: Genomic imprinting; conditional deletion; DMR; *H19*; *Igf2*]

Received February 24, 2000; revised version accepted March 31, 2000.

Certain loci in the mammalian genome exhibit functional inequivalence of the two alleles. Depending on the parent of origin, some genes are expressed exclusively from the maternal chromosome and others exclusively from the paternal chromosome (Efstratiadis 1994; Bartolomei and Tilghman 1997; Brannan and Bartolomei 1999). To achieve parent-of-origin-specific expression, three aspects are of prime importance. During gametogenesis, a mark must be set to make paternal and maternal alleles distinct from each other at the molecular level. Subsequently, as the embryo develops via cell division and cell differentiation, the distinction must be maintained. Finally, the transcriptional machinery must be able to recognize this distinguishing mark such that it manifests as allele-specific expression. Failure at any of the three steps will lead to loss of parentally imprinted expression.

H19 and *Igf2* are part of a cluster of imprinted genes on mouse chromosome 7 (syntenic to human chromosome 11p15.5). The genes exhibit reciprocity in allele-specific expression. Only the maternal allele of *H19* is expressed (Bartolomei et al. 1991) whereas for *Igf2*, it is the paternal allele that is active (DeChiara et al. 1991). The two genes are almost identical in their spatial and temporal expression patterns. In fact, expression in endodermal tissues has been demonstrated to be dependent on a common set of enhancers located between 7 and 9 kb downstream of the *H19* promoter (Leighton et al. 1995). Also, the imprinting of the two genes is mechanistically linked. Deletion of *H19* and the ~10-kb region upstream of it leads to biallelic expression of *Igf2* (Leighton et al. 1995). Molecular studies implicate sequences upstream of *H19* as important for monoallelic expression of both *H19* and *Igf2*. Maternal chromosome-specific hypersensitivity to nuclease digestion has been demonstrated at two regions that are ~ 2.4 kb and 3.8 kb upstream of the *H19* promoter (Hark and Tilghman 1998; Khosla et al. 1999). Also, this region displays paternal chromosome-specific hypermethylation (Tremblay et al. 1995) that extends

[1]**Corresponding author.**
E-MAIL kpfeifer@helix.nih.gov; FAX (301) 402-0543.

from approximately –4.0 kb to –2.0 kb. The differential methylation patterns are evident at the gamete stage and are retained through development (Tremblay et al. 1997). Therfore, methylation of this region has been suggested to be responsible for controlling the imprinted expression of *H19* and *Igf2*. Strong support in favor of this role also comes from mutant studies in which DNA methyltransferase gene (*dnmt*) has been deleted. In these mutants, *H19* was shown to be expressed in a biallelic manner, whereas *Igf2* expression was completely lost (Li et al. 1993).

Deletion of a part of the differentially methylated region (DMR) encompassing –3.7 kb to –2.1 kb upstream of *H19* resulted in biallelic expression of *H19* when paternally inherited, and biallelic expression of *Igf2* when maternally inherited (Thorvaldsen et al. 1998). This experiment has provided genetic proof of the crucial role played by this region in regulating monoallellic expression of *H19* and *Igf2*. Although these experiments demonstrate the importance of the DMR at the *H19*/*Igf2* locus, they do not determine the exact role of the DMR. The effect of germ-line inheritance of this mutation was examined at the level of transcription and DNA methylation in differentiated tissue. Therefore, loss of monoallelic expression could be interpreted as a failure to set the imprint during gametogenesis, as a failure to maintain the imprint during cell division, or as the loss of a signal important for transcriptional regulation. To address these distinct functions, it is necessary to delete the DMR at different stages of development. A *loxP*/cre recombinase-based strategy (Gu et al. 1993, 1994) was used to delete the DMR in the germ line, in the zygote, and in differentiated tissue to discern its exact role.

Transcriptional regulation at the *H19* locus has been investigated earlier using transgenic mice. These studies demonstrated that sequences downstream of –0.8 kb are sufficient to direct normal *H19* expression (Pfeifer et al. 1996). Sequences between –4 kb and –0.8 kb were necessary to direct monoallelic expression of *H19* transgenes and to induce methylation of the upstream region in a parent-specific manner. However, imprinting of these transgenes was observed only when they were present in multiple copies, suggesting that not all of the genetic information required for the imprinting of the *H19* locus was present on these constructs (Pfeifer et al. 1996; Elson and Bartolomei 1997). More recent studies have shown that BAC transgenes carrying the sequences –7.0 kb to +133 kb are imprinted even when present in single copies (C. Kaffer and M. Srivastava, in prep.). Thus, upstream sequences only up to –7.0 kb were inferred to be necessary and sufficient for imprinting *H19* and plausibly *Igf2*. DMR^{flox} mutants were generated in which *loxP* sites flank the region –7 kb to –0.8 kb upstream of *H19* (Fig. 1), enabling the deletion of this region dependent on expression of cre recombinase. The region includes the entire DMR (–4 to –2 kb), both clusters of hypersensitive sites (–3.8 and –2.4 kb), and all of the sequences sufficient for imprinting single-copy transgenes. On the basis of the analysis of these conditional mutants of mice, we demonstrate that the region

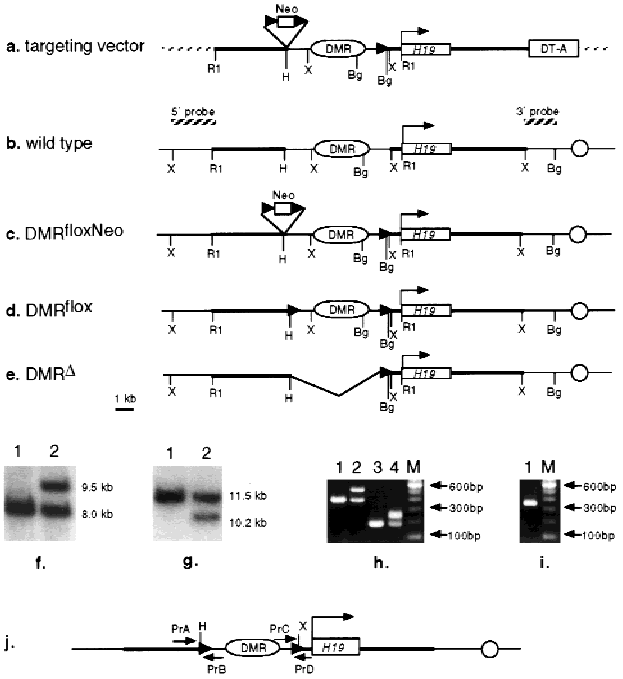

Figure 1. Strategy for conditional deletion of the –7.0-kb to –0.8-kb region upstream of *H19*. (*a*) Targeting vector; (*b*) wild-type chromosome; (*c*) targeted chromosome, $DMR^{floxNeo}$; (*d*) targeted chromosome DMR^{flox} after excision of the Neo^r selection marker; (*e*) the DMR^{Δ} chromosome after cre recombinase-mediated excision at specific stages. (*f*,*g*) Southern hybridization of ES cell DNA to confirm the correctly targeted clones ($DMR^{floxNeo}$). Genomic DNA isolated from the ES cells was digested with *Xba*I (*f*) and hybridized to an *Eco*RI/*Xba*I fragment from the 5′ end of the targeted locus. Clones that have undergone homologous recombination (lane *2*) yield a 9.5-kb band in addition to a 8.0-kb band derived from the endogenous non-targeted allele (lane *1*). Similarly, *Bgl*II digests of the genomic DNA (*g*) hybridized to an *Xba*I/*Bgl*II fragment from the 3′ end of the targeted locus yield 11.5-kb and 10.2-kb bands from the wild-type (lane *1*) and targeted alleles (lane *2*), respectively, due to the presence of the additional *Bgl*II site at the –0.8-kb position. (*h*) DNA amplified from ES cell clones (DMR^{flox}) correctly recombined by cre recombinase in vitro. The DMR^{flox} allele was a result of cre recombinase-mediated excision of the Neo resistance gene. This was identified by amplifying the region around the –7.0-kb *Hin*dIII site (primers PrA and PrB). The endogenous locus (lane *1*) gives a PCR product of 387 bp and the presence of the *loxP* site as a result of the correct excision event increases the size of this fragment to 520 bp. Presence of the other *loxP* site at –0.8-kb *Xba*I was confirmed by amplifying the region around it (Primers PrC and PrD). Wild-type (lane *3*) and *loxP* carrying DNA samples yield PCR products of 177 and 234 bp, respectively. The correct clones carrying the DMR^{flox} allele (lanes *2*, *4*) carry the *loxP* sites at both of these positions. (*i*) DNA amplified from genomic DNA of neonates (DMR^{Δ}), in which DMR has been excised due to in vivo expression of cre recombinase under the control of different promoters (described in text). The excision was verified by PCR amplification (primers PrA and PrD) to give a 340-bp product. The authenticity of all of the PCR products was confirmed by sequencing. (*j*) Positions of the primers used for the PCR- based amplifications shown in *h* and *i*. Enhancers (circles), *loxP* sites (solid triangles), regions used as flanks (thick lines), transcription start site for *H19* (arrow above *H19* gene) and fragments used as probes (hatched lines above *b*) are shown. (DTA) Diptheria toxin A gene; (Bg) *Bgl*II; (H) *Hin*dIII; (R1) *Eco*RI; (X) *Xba*I; (M) DNA size markers.

harbors two regulatory elements that act in distinct ways to regulate monoallelic expression of *Igf2* and *H19* due to parental imprinting.

Results

Generation of conditional mutants

To generate a mutation upstream of the *H19* gene, a targeting vector (Fig. 1) was constructed that carried a *loxP* site at the –0.8-kb *Xba*I site and a neomycin-resistance gene flanked by *loxP* sites at the –7.0-kb *Hin*dIII site. Distances given are relative to the transcriptional start of *H19*. Correctly targeted clones of embryonic stem cells were identified by Southern hybridization. Cell lines heterozygous for this mutation, $DMR^{floxNeo}$, were subsequently electroporated with a supercoiled plasmid that directs the expression of cre recombinase. Resultant clones were analyzed using a PCR-based strategy to identify the recombination event that resulted in the excision of the neomycin-resistance gene without deleting the sequences between the –7.0-kb *Hin*dIII and –0.8-kb *Xba*I sites. Consequently, the region between the *Hin*dIII and *Xba*I sites was flanked by *loxP* sites (Fig. 1), generating the DMR^{flox} allele. Thus, mice generated from such cell lines carried an allele of chromosome 7 in which the –7.0- to –0.8-kb region upstream of *H19* could be deleted dependent on the in vivo expression of cre recombinase to generate DMR^{Δ}. Successful deletion was monitored by a PCR assay (see Materials and Methods) and quantified, as needed, by Southern analysis.

Strategy for the assay of allele-specific expression

For all experiments, matings were set up such that two types of progeny were obtained. Experimental progeny carried a mutant allele of the DMR on a *domesticus* chromosome and a wild-type allele on a *castaneus* chromosome. Control littermates also carried a *domesticus* chromosome and a *castaneus* chromosome, but not the mutation under investigation.

To analyze the relative expression from the two chromosomes, we designed a single nucleotide primer extension (SNuPE) assay taking advantage of polymorphisms between *domesticus* and *castaneus* mice at the *H19* locus. RNA from the tissue of interest was reverse transcribed and amplified for *H19*. A primer immediately upstream of the polymorphic base (+2395 of *H19* relative to the transcription start) was extended using radioactively labeled dATP or dGTP in the absence of any other nucleotides. When the *H19* primer is extended, relative dATP and dGTP incorporations give an estimate of cDNA derived from *domesticus* and *castaneus* alleles, respectively, and hence reflect the relative expression of *H19* from the two parental alleles.

To authenticate the ability of the SNuPE assay to estimate the relative contribution of the two alleles, we assayed DNA samples from pure *domesticus* and pure *castaneus* mice and also their F_1 progeny. As expected, pure *domesticus* DNA incorporated dATP and pure *castaneus* DNA incorporated dGTP, whereas F_1 DNA incorporated both of these nucleotides (Fig. 2a). Theoretically, incorporation of dGTP and dATP in F_1 DNA should be equivalent. Instead, we noticed a bias in favor of dATP. This bias could be attributed to a higher affinity of *Taq* polymerase for dATP compared with dGTP and/or to a difference in the specific activity of the two nucleotides. Considering this bias, we noted the requirement to include multiple F_1 controls with each assay that we performed. On the basis of the relative incorporation of the two nucleotides by the F_1 DNA, a correction factor was deduced specifically for each experiment (see Materials and Methods).

Next, DNA amplified from pure *domesticus* and pure *castaneus* animals was mixed in known proportions (*castaneus:domesticus* ratio varying from 8:1 to 1:8) and subjected to SNuPE (Fig. 2b). We observed a linear increase in the dATP incorporation and a linear decrease in the dGTP incorporation as the relative proportion of *domesticus* DNA increased. Relative incorporation of the two nucleotides was calculated as A/(A+G) taking the F_1 correction into account and compared with the theoretically expected A/(A+G) values. The observed values were found to be comparable with the expected values, indicating that the SNuPE assay does reflect the relative concentration of DNA from the two alleles. Thus, we conclude that the SNuPE assay is capable of quantifying the relative abundance of RNA from *domesticus* and *castaneus* alleles in a given RNA sample and, therefore, enables an estimate of the paternal and maternal contributions to total *H19* RNA.

A similar assay was used for estimation of the relative

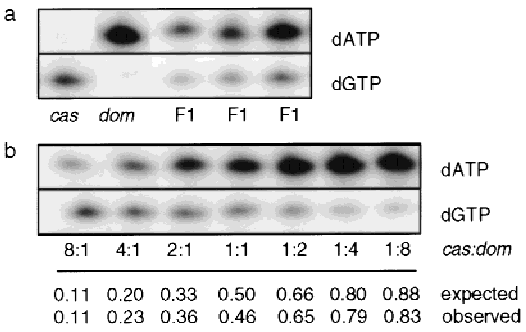

Figure 2. SNuPE assays for *H19* with control DNA templates. (*a*) DNA from *castaneus* (*cas*), *domesticus* (*dom*) and from *castaneus* × *domesticus* F_1 (F1) mice. *Castaneous* DNA incorporates dGTP and *domesticus* DNA incorporates dATP. F_1 DNA, representing a 1:1 mix of the *castaneus* and *domesticus* templates, incorporates both of the nucleotides. dATP incorporation in F_1 is higher than dGTP incorporation, although, theoretically, it should be equivalent (see text). The experimentally observed relative incorporation of the two nucleotides by F_1 DNA was used to derive a correction factor (see Materials and Methods) for dGTP incorporation. (*b*) *Castaneus* and *domesticus* DNA mixed in known proportions as indicated, amplified, and assayed by SNuPE. The expected values of A/(A+G) on the basis of relative concentrations of *domesticus* and *castaneus* DNA used in the mix are compared with those observed.

expression of *Igf2* from the maternal and paternal alleles. In this case, a primer immediately upstream of the polymorphic base at position +440 of exon 6 was extended. This primer incorporates dGTP from the *domesticus* allele and dATP from the *castaneus* allele.

Effect of loxP *sites insertion on expression of* H19 *and* Igf2

Because the insertion of *loxP* sites may, even without deletion of the intervening sequences, interfere with allele-specific expression of *H19* and *Igf2*, we investigated *H19* and *Igf2* expression in DMR^{flox} mutants. *Domesticus* DMR^{flox} males were mated to wild-type females carrying a *castaneus* allele at the *H19/Igf2* locus. In $+/DMR^{flox}$ neonates, expression of *H19* continues to be solely maternal (Fig. 3b; Table 1). $DMR^{flox}/+$ progeny from a reverse cross, with maternal inheritance of the DMR^{flox}, exhibited normal imprinting of *Igf2* (Fig. 3g; Table 1), that is, solely paternal expression was observed. Thus, insertion of *loxP* sites did not lead to activation of either paternal *H19* or maternal *Igf2*. In addition, the overall levels of expression of *H19* and *Igf2* in $+/DMR^{flox}$ and $DMR^{flox}/+$ neonates were unaltered in liver, heart, and muscle as determined by Northern hybridization (data not shown).

Analysis of allele-specific expression of H19

Deletion of the DMR in the entire mouse, including the germ-line tissues, was afforded by mating DMR^{flox} males with females expressing cre recombinase during early embryogenesis under the control of the *EIIa* promoter (Lakso et al. 1996). Mutants heterozygous for the deleted allele $DMR^{\Delta G}$ (for germ-line deletion) were then mated to wild-type mice homozygous for the *castaneus* allele of *H19*. When the deletion was inherited paternally, activation of paternal *H19* was observed (Fig. 3c; Table 1). As suggested by the biallelic expression, an increase in the total *H19* RNA was also observed by Northern analysis (data not shown). These results confirm the role of the DMR in imprinted expression of *H19* as reported earlier (Thorvaldsen et al. 1998).

To address the role of the DMR after gametogenesis, we examined *H19* expression in mice in which the DMR was intact in germ cells but was deleted after fertilization. Zygotes were obtained by mating males carrying the DMR^{flox} allele with females homozygous for the wild-type *castaneus* allele. These zygotes were injected with plasmid pCAGGS-cre to induce transient expression of cre recombinase from the β-actin promoter (Araki et al. 1995). Neonates in which the DMR was successfully deleted were identified. $DMR^{\Delta Z}$ mutants (for zygotic deletion), like $DMR^{\Delta G}$ mutants, showed an activation of the paternal *H19* (Fig. 3d; Table 1). As described earlier, setting up of the imprint during gametogenesis is normal in DMR^{flox} males. Hence, these results demonstrate that the DMR plays a crucial role in maintaining the imprint of the locus or in transcriptional silencing.

To investigate the role of the DMR in transcription per se, we examined *H19* expression in mice in which the region was deleted from the paternal chromosome late in development, at the final steps of differentiation. Such mice were obtained by mating males carrying the DMR^{flox} allele with females expressing cre recombinase under the control of the muscle creatin kinase (MCK) promoter (Bruning et al. 1998) and carrying *castaneus* alleles of *H19*. The MCK promoter is expressed in terminally differentiated skeletal and cardiac muscle cells (Lyons et al. 1991; Sternberg et al. 1988) and has been demonstrated to cause cre recombinase-mediated excision at a high efficiency in these tissues (Bruning et al. 1998). Southern analysis demonstrated that excision of

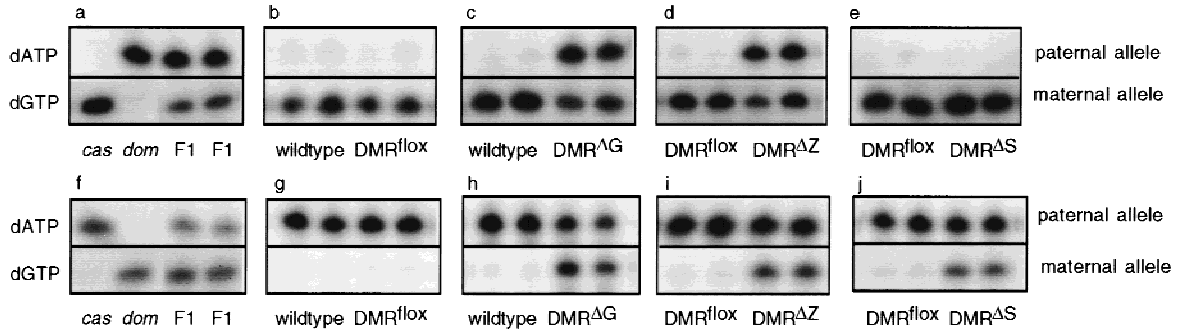

Figure 3. Effect of the DMR deletion mutations on *H19* and *Igf2* allele-specific expression. Skeletal muscle RNA samples were DNase treated, amplified for *H19* and *Igf2* by RT–PCR, and analyzed by SNuPE assays. (*a*,*f*) Demonstration of the efficiency of the assay for *H19* and *Igf2* respectively, by testing pure DNA templates, *castaneus* (*cas*), *domesticus* (*dom*), and DNA from *castaneus* × *domesticus* F_1 mouse (F1), which represents a 1:1 mix of the templates. On the basis of a polymorphism between *castaneus* and *domesticus* alleles, the relative incorporation of the radiolabeled nucleotides dATP and dGTP during primer extension gives an estimate of relative abundance of the DNA from the two parental alleles. *H19* expression was analyzed in neonates carrying a mutated paternal allele (*b–e*). *Igf2* expression was analyzed in neonates carrying mutated maternal allele (*g–j*). Control littermates are either wild-type or DMR^{flox} as indicated. The experimental chromosome (paternal for *H19* and maternal for *Igf2*) is always *domesticus*, whereas the other chromosome is wild-type *castaneus*. For each RNA sample analyzed, RT-minus controls were used. These exhibited no amplification products, demonstrating the absence of any DNA contamination in the DNase treated RNA samples.

Table 1. *Allele specific expression of* H19 *and* Igf2 *in DMR mutants*

	% contribution of paternal allele total *H19* mRNA	% contribution of maternal allele to total *Igf2* mRNA
Wild type	2.5 ± 0.2	4.0 ± 0.9
DMR^{flox}	2.9 ± 0.3	4.7 ± 1.3
$DMR^{\Delta G}$	42.9 ± 6.2	34.7 ± 5.0
$DMR^{\Delta Z}$	29.8 ± 2.5	40.5 ± 1.3
$DMR^{\Delta S}$	1.7 ± 0.4	20.0 ± 0.2

Samples were analyzed by SNuPE assays. The values are from representative experiments and are an average derived from at least three skeletal muscle RNA samples. Similar results were noted from heart RNA samples in all cases. Pure *castaneous* DNA gives a background of 2 ± 0.5% for *H19* and 5 ± 2% for *Igf2.* The values have not been corrected for this background but are corrected for differences in incorporation of dATP and dGTP by F1 DNA (see Materials and Methods).

the DMR occurred in at least 50% of the cells in the muscle preparations and in a greater proportion of cells in hearts from neonates (data not shown). The partial excision observed was expected because the dissected muscle or heart tissue also contains non-muscle cells coming with connective tissue and blood vessels that should not express MCK*cre*. Strikingly, despite the DMR excision, expression of *H19* in these $DMR^{\Delta S}$ (for somatic deletion) mutants remained completely monoallelic in muscle (Fig. 3e; Table 1) and heart (data not shown). We conclude that the DMR sequences and the imprint they carry are not required to actually prevent transcription of the paternal *H19* allele. Rather, it appears that the DMR in its imprinted state must have directed changes during development, possibly in the *H19* promoter region, that led to silencing of the paternal *H19*.

Analysis of DNA methylation around H19 *promoter*

At the gamete stage, paternal methylation of the *H19* locus is restricted to the DMR. During early embryogenesis, however, the methylated region on the paternal chromosome spreads to encompass the *H19* promoter and structural gene (Bartolomei et al. 1993; Brandeis et al. 1993; Ferguson-Smith et al. 1993; Tremblay et al. 1995, 1997). Considering the strong correlation between the silencing of *H19* and methylation of its promoter region, we investigated the requirement of the DMR for this promoter methylation using restriction enzymes sensitive to methylation (Fig. 4). Genomic DNA derived from $DMR^{\Delta G}$ mutants inheriting the mutation either paternally or maternally was digested with *Bam*HI and *Bgl*II and probed with an *Xba*I/*Bam*HI probe as shown in Figure 4a. The $DMR^{\Delta G}$ allele yields a band of 1.5 kb as opposed to 2.5 kb from the wild-type DNA. When this digest was additionally digested with *Hpa*II or *Hha*I, the wild-type paternal chromosome showed partial resistance to these methylation-sensitive enzymes, whereas the wild-type maternal chromosome was completely digested. In contrast, the $DMR^{\Delta G}$ allele on the paternal chromosome was digested completely and was indistinguishable from a maternal chromosome in this respect. $DMR^{\Delta Z}$ mutants gave identical results (data not shown). The methylation status of $DMR^{\Delta S}$ was investigated by digesting the genomic DNA with *Bam*HI and *Hin*dIII using the *Xba*I/*Bam*HI fragment as a probe. In this case wild-type, undeleted DMR^{flox} and deleted $DMR^{\Delta S}$ alleles give 2.5-, 2.6-, and 1.6-kb bands, respectively. Additional digestion with methylation-sensitive enzymes *Hpa*II and *Hha*I was performed. The paternal $DMR^{\Delta S}$ allele, like the wild-type paternal allele, was partially resistant to *Hpa*II and *Hha*I and thus distinct from the maternal $DMR^{\Delta S}$, indicating that the promoter stays methylated despite the absence of the DMR when deletion is effected in differentiated cells.

The analysis of DNA methylation on the basis of digestion with methylation-sensitive enzymes and Southern hybridization gives an estimate of the population-averaged status of methylation at some specific enzyme sites. Bisulphite-based cytosine methylation analysis was performed to derive information about additional CpG residues in the *H19* promoter region and to determine methylation patterns of individual chromosomes. Genomic DNA from the skeletal muscle of wild-type and mutant neonates was digested with *Bam*HI, treated with bisulphite, amplified, cloned, and sequenced. Bisulphite treatment modifies all unmethylated cytosines to thymidines (Frommer et al. 1992). Methylated cytosines escape this conversion and hence can be recognized directly. Most of the 19 CpG residues between –170 and +167 bp were found to be methylated on the wild-type paternal chromosome, although the methylation at each individual residue was variable (Table 2) as has been reported earlier (Tremblay et al. 1997). However, hypermethylation was missing on the paternal chromosomes from which the DMR was deleted either in the germ line ($DMR^{\Delta G}$) or at the zygotic stage ($DMR^{\Delta Z}$). In $DMR^{\Delta S}$ mutants, hypermethylation on the mutated paternal chromosome was very similar to that of the wild-type paternal chromosome. Thus, a paternal DMR is required at least in the early embryo to direct CpG methylation of the *H19* promoter region. However, once established, this promoter methylation is not dependent on the continued presence of the DMR. Because the DMR deletion in $DMR^{\Delta S}$ mutants occurs in terminally differentiated cells, our results do not address the stability of the promoter methylation on multiple cell divisions in the absence of the DMR.

We note that all four mutations DMR^{flox}, $DMR^{\Delta G}$, $DMR^{\Delta Z}$, and $DMR^{\Delta S}$, when present on the maternal chromosome, did not have any effect on monoallelic expression of *H19*, which continues to be solely maternal

(data not shown). This result was expected on the basis of the *cis*-acting nature of the DMR (Thorvaldsen et al. 1998), (C. Kaffer and M. Srivastava, in prep.).

Analysis of allele-specific Igf2 *expression*

Allele-specific expression of *Igf2* was also investigated subsequent to deletion of the DMR at different stages using protocols similar to those described above for *H19*. Deletion of the DMR from the maternal chromosome either in the germ line, $DMR^{\Delta G}$, or in the zygote, $DMR^{\Delta Z}$, led to biallelic expression of *Igf2* (Fig. 3h,i; Table 1). When the DMR was deleted in differentiated cells using an MCK-promoter driven cre recombinase (DMRΔS), the excision efficiency in skeletal muscle and heart was >50% as described earlier. In contrast to what we observed for *H19*, excision of the DMR in differentiated cells results in a loss of imprinting of *Igf2*. The mutant maternal $DMR^{\Delta S}$ allele expressed *Igf2* (Fig. 3j; Table 1). These results demonstrate that the DMR is continually required on the maternal chromosome to keep *Igf2* silenced. Its deletion at any stage leads to biallelic expression of *Igf2*.

The presence of any of the four mutations, DMR^{flox}, $DMR^{\Delta G}$, $DMR^{\Delta Z}$, and $DMR^{\Delta S}$, on the paternal allele did not interfere with monoallelic paternal expression of *Igf2* (data not shown) consistent with the idea that it is a *cis*-acting element (Thorvaldsen et al. 1998), (C. Kaffer and M. Srivastava, in prep.).

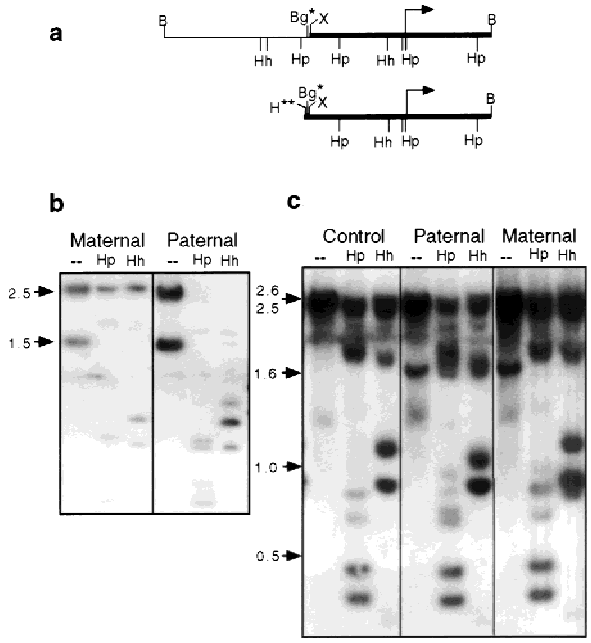

Figure 4. CpG methylation in the *H19* promoter region as assayed by digestion with methylation-sensitive enzymes. (*a*) Restriction map of the region. Wild-type and DMR^{flox} alleles are distinguished by the presence of a *Bgl*II site at –0.8-kb (*top*). Cre recombinase mediated excision of the DMR keeps the *Bgl*II site and also brings in juxtaposition a *Hin*dIII site (*bottom*). (Thick lines) The probes used for hybridization; (*) *Bgl*II site present only on the DMR^{flox} and DMR^{Δ} alleles; (**) *Hin*dIII site present only on DMR^{Δ} allele; (arrow) transcription start site for *H19*. (*b*) Maternal and paternal inheritance of $DMR^{\Delta G}$. DNA prepared from skeletal muscle of neonates inheriting $DMR^{\Delta G}$ either maternally or paternally was digested with *Bam*HI and *Bgl*II. Subsequent hybridization with a 1.4-kb probe (see *a*) reveals 2.5- and 1.5-kb bands representing the wild-type and mutant chromosomes, respectively. Additional digestion with *Hpa*II and *Hha*I was performed to monitor methylation. (*c*) Deletion of the DMR mediated by MCK-cre recombinase. DNA was prepared from neonates in which the $DMR^{\Delta S}$ mutation was generated on the maternal or paternal chromosome. Control DNA is from $+/DMR^{flox}$ mutants not carrying the MCK-*cre* transgene. Digestion with *Bam*HI and *Hin*dIII and subsequent hybridization as above reveals three bands of 2.6, 2.5, and 1.6 kb representing DMR^{flox}, wild-type, and $DMR^{\Delta S}$ alleles, respectively. Portions of the enzyme digest were also digested with *Hpa*II and *Hha*I and methylation of the $DMR^{\Delta S}$ allele was monitored by the disappearance of the 1.6-kb band. (B) *Bam*HI; (Bg) *Bgl*II; (H) *Hin*dIII; (Hh) *Hha*I; (Hp) *Hpa*II, and (X) *Xba*I.

Table 2. *Status of CpG methylation around the* H19 *promoter on paternal chromosome in DMR mutants*

CpG	wild type	$DMR^{\Delta G}$	$DMR^{\Delta Z}$	$DMR^{\Delta S}$
–170	5/8	0/7	0/5	11/14
–164	6/8	0/7	0/5	13/14
–147	6/8	0/7	1/5	13/14
–143	5/8	0/7	0/5	10/14
–139	6/8	0/7	0/5	13/14
–131	3/8	0/7	0/5	9/14
–106	3/8	0/7	0/5	12/14
–97	5/8	0/7	0/5	11/14
–94	6/8	0/7	0/5	12/14
–59	7/8	0/7	0/5	13/14
–46	8/8	0/7	0/5	13/14
–20	3/8	0/7	0/5	6/14
–6	3/8	0/7	0/5	8/14
+3	6/8	0/7	0/5	9/14
+43	0/8	0/7	1/5	13/14
+81	3/8	0/7	0/5	11/14
+90	8/8	0/7	2/5	12/14
+102	3/8	0/7	1/5	12/14
+167	8/8	2/7	1/5	14/14

Position of the CpG residues are in relation to the transcriptional start site of *H19*. Fraction of clones found to be methylated is shown.

Discussion

Imprinting of *H19* and *Igf2* is regulated by a *cis*-acting element present upstream of *H19* (Thorvaldsen et al. 1998). In the absence of this element, normally silent paternal *H19* and maternal *Igf2* alleles are transcribed. Such a loss of monoallelic expression will be manifest if the region is responsible for setting up the imprint during gametogenesis, maintaining the imprint in cells during development, or providing appropriate signals to the transcriptional machinery for monoallelic transcription. To understand the role of DMR in these distinct processes, we deleted the DMR during different stages that

could be important for imprinted expression. The DMR was deleted in the germ line, in the zygote, and in terminally differentiated muscle cells. Analysis of the deleted mutants revealed that maternal *Igf2* and paternal *H19* are silenced by two distinct mechanisms, suggesting the presence of two regulatory elements in the deleted DMR (Fig. 5). Whether the two regulatory elements are structurally distinct or share any sequences remains to be determined.

Activation of the paternal *H19* was observed when the DMR was deleted in the paternal germ line as was expected on the basis of earlier studies (Thorvaldsen et al. 1998). When setting up of the imprint was normal and the DMR was deleted only in the zygote, activation of paternal *H19* was still observed, showing a requirement of this region post-fertilization. However, when the DMR was deleted in the terminally differentiated cells, *H19* expression remained solely maternal just as in the wild-type mice. Because the DMR is required subsequent to fertilization but not continually required to prevent transcription from the *H19* promoter, it must direct certain epigenetic modifications of the region during development that lead to the silencing of the *H19* promoter. Once silencing has been achieved, it is stable despite the removal of DMR, indicating that the DMR and associated proteins do not interact directly with the transcriptional machinery to prevent transcription from the *H19* promoter. Whether DMR-mediated changes are stable during mitosis was not addressed by these experiments.

Additionally, the *H19* promoter was found to be methylated when silent (wild-type and DMRΔS) and nonmethylated when actively transcribing ($DMR^{\Delta Z}$ and $DMR^{\Delta G}$). It is known that whereas the DMR is methylated in the sperm, a region encompassing the *H19* promoter on the paternal chromosome acquires methylation only during early embryogenesis (Brandeis et al. 1993; Tremblay et al. 1995, 1997). Although methylation of the *H19* promoter correlates well with its silencing, there might be other epigenetic modifications required (Brenton et al. 1999). The methylated state of the DMR itself does seem to be critical, as demonstrated by biallelic *H19* expression in embryos homozygous for the deletion of DNA methyltransferase gene, *dnmt* (Li et al. 1993). It appears that DMR in its methylated state, as on the paternal chromosome, has the ability to unidirectionally silence the adjacent genes, perhaps by acting as a nucleation center for spread of methylation and hypoacetylation or specifically recruiting enzymes that cause such modifications at the *H19* promoter. The nature of such enzymes is presently unknown. Recently identified de novo methylases *dnmt3a* and *dnmt3b*, which are required during development (Okano et al. 1999), may have a role to play in this process. Other enzymes that either methylate DNA (*dnmt*) or recognize methylated DNA (MeCP2 and MBD2) have all been shown to have histone deacetylase activity, demonstrating a strong functional link between methylation, histone deacetylation, and silencing (Nan et al. 1998; Ng et al. 1999; Wade et al. 1999; Fuks et al. 2000). Whether *H19* silencing involves any of these processes, remains to be investigated. Interestingly, a 1.1-kb domain (–2.9 to –1.8 kb upstream of *H19*) within the DMR has been shown to act as a bidirectional silencer in *Drosophila* (Lyko et al. 1997), an organism that lacks DNA methylation. In mice, transgenes of the *H19* region that are devoid of this silencing domain do not exhibit silencing of *H19* when paternally inherited (Brenton et al. 1999). The element is an active silencer only in its methylated state (as on the paternal chromosome) and is inactive on the nonmethylated maternal chromosome. The relationship of the 1.1-kb element to act as a bidirectional silencer in *Drosophila* in an unmethylated state and to act as a unidirectional silencer in mice in a methylated state is presently intriguing.

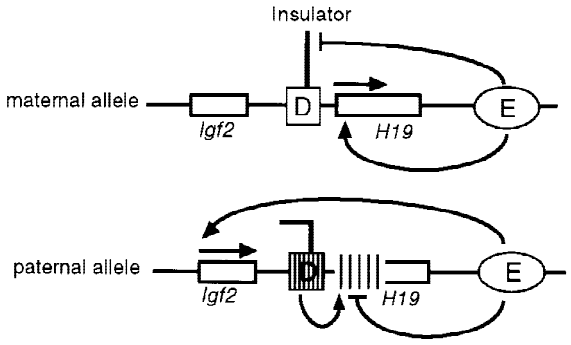

Figure 5. A model to explain *H19* and *Igf2* imprinting. Hypermethylation and other associated changes (close vertical bars) at the DMR on the paternal chromosome accomplish two functions. They inactivate the insulator function residing in the DMR to allow *Igf2* promoter-enhancer interaction and also direct epigenetic modification (sparse vertical bars) of the *H19* promoter region leading to its transcriptional silencing. The shared endodermal enhancers for *H19* and *Igf2* expression have been mapped from the +7- to +9-kb region downstream of *H19* (Leighton et al. 1995). The enhancers for mesodermal expression of *H19* are located downstream to +12 kb of *H19* and on the basis of identical expression patterns of the two genes, are presumably shared by *Igf2*. (D) DMR; (E) enhancer.

Thus, whereas the details of the actual molecular events leading to silencing of *H19* remain elusive, there are two questions pertaining to *H19* silencing. First, how does the DMR direct modifications of the *H19* locus during cell differentiation? And second, how does the transcriptional machinery recognize the modified *H19* promoter region to be distinct from the unmodified maternal *H19* promoter leading to a differential transcriptional response? Our analysis of conditional mutants suggests that these two questions can be temporally divided. Further, our results demonstrate that the original imprint can lead to establishment of new distinctions between the paternal and maternal alleles and that it is these new distinctions that are actually pertinent for allele-specific activation by the transcription machinery of the cells.

Deletion of the DMR on the maternal chromosome led to the activation of maternal *Igf2*, irrespective of whether the deletion was effected in the germ line during establishment of the imprint, post-fertilization, or in

differentiated tissue. Its constant requirement to keep *Igf2* silenced is in consonance with the idea that the region harbors an insulating element that prevents the interaction of the upstream *Igf2* promoter with downstream enhancers (Schmidt et al. 1999). The observation that deletion of the DMR led to expression of both *Igf2* and *H19* from the same chromosome (this work; C. Kaffer and M. Srivastava, in prep.) and that insertion of DMR sequences between the *H19* promoter and its skeletal muscle enhancers abolished *H19* expression in skeletal muscle (C. Kaffer and M. Srivastava, in prep.) strongly supports this idea. Such an insulation prevents the transcription of maternal *Igf2*. When methylated, as on the paternal chromosome, the insulating function is abrogated, leading to paternal expression of *Igf2*. The important role of methylation is again evident as homozygous deletion mutants of the DNA methyltransferase gene exhibited complete loss of *Igf2* expression (Li et al. 1993).

Presently, however, the role of other epigenetic modifications cannot be ruled out. Also, no *trans*-acting factors are currently known that work with this insulator to bring about silencing of *Igf2*. Insulating elements have been reported to regulate gene expression in other systems (Kellum and Schedl 1992; Chung et al. 1993; Geyer 1997). The ability of epigenetic modifications, either directly or in concert with other *trans*-acting factors, to control the insulating activity in a parent-of-origin-dependent manner appears to be the special feature of the *Igf2* locus.

The conclusion that monoallelic expression of the *H19* and *Igf2* genes occurs via distinct mechanisms is supported by several molecular and biochemical analyses in addition to the genetic studies described here. Early experiments examining nuclease sensitivity at the two genes' promoters suggested that differential regulation might be related to the changes at the *H19* promoter (Sasaki et al. 1992; Bartolomei et al. 1993), but that both the maternal and paternal *Igf2* promoters were available for active transcription (Sasaki et al. 1992). Further, monoallelic expression of *H19* and *Igf2* in cell lines is differentially sensitive to drugs that affect histone acetylation and methylation. Maternal *Igf2* repression could be alleviated by using inhibitors of histone deacetylase, but alleviation of paternal *H19* repression required both an inhibition of histone deacetylation and an inhibition of DNA methylation (Pedone et al. 1999), suggesting that different mechanisms were being used to achieve silencing of these genes.

Our results suggest that imprinted expression of genes may be achieved by taking advantage of mechanisms that are normally used for temporal and spatial regulation of genes as long as the imprint can interfere with this mechanism. Given the variabilty in the mechanisms of gene regulation, the mechanisms that accomplish allele-specific regulation may be very diverse. Association of hypermethylation in some genes with the expressed allele and in others with the nonexpressed allele (Caspary et al. 1998), supports the idea that the distinction of the two alleles is relevant rather than the modification itself. The distinction may be either the original gametic imprint or the changes directed by that imprint. In the case of the DMR, the original state of differential methylation is retained. However, it is possible that for some other locus, the original imprint is lost during development once it has successfully directed certain epigenetic modifications important for allele-specific expression.

It is logical to think that *cis* elements involved in transcriptional regulation will either be required to establish an open/closed promoter structure at a specific stage and are redundant thereafter, or will be continually required to provide signal to the transcriptional machinery to transcribe/not transcribe a given gene. The DMR between *H19* and *Igf2* genes happens to contain both kinds of elements. The role in the epigenetic silencing of *H19* in a temporal fashion appears to be a unique characteristic of the deleted DMR region. It may represent a new class of mammalian silencers, which are only required to establish a closed promoter structure at a specific stage and are redundant thereafter as has been reported in yeast (Holmes and Broach 1996). Such silencers must have a very different mechanism of action than those that are continually required to keep the promoter shut off. Having dissected the temporal requirements of the DMR in regulating silencing of *H19* and *Igf2*, it is now possible to look for *trans*-acting factors and details of molecular mechanisms involved in silencing achieved by these two kinds of elements.

To our knowledge, no *cis*-acting elements involved in transcription, enhancers, insulators, silencers, or LCRs have been dissected in a temporal manner in the mammalian system. We believe that the *loxP*/*cre* mediated approach will prove useful for studies investigating the mechanism of such elements. How would enhancer function, for example, be affected in such an experiment? Our studies provide a new perspective on the temporal requirements that may aid the understanding of mechanisms underlying the action of *cis* elements on genes both within and outside of the realm of the imprinting.

Materials and methods

Generation of conditional mutants of DMR

The targeting vector was constructed by use of DNA derived from 129/SvJ mice. The vector contained sequences extending from –11-kb *Eco*RI to +6.5-kb *Xba*I relative to the *H19* transcriptional start site. Additionally, it carried a neomycin-resistance gene flanked by *loxP* sites inserted at the –7.0-kb *Hin*dIII site, another *loxP* site inserted at the –0.8-kb *Xba*I site, and a diptheria toxin gene as a negative marker. The orientation of all three *loxP* sites was the same and the insertion of the *loxP* site at the –0.8-kb *Xba*I position was engineered to also create a new *Bgl*II site at this position. The targeting vector was linearized with *Not*I and electroporated into mouse R1 embryonic stem cells. Correctly targeted clones were confirmed by Southern hybridization (Fig. 1). Positive clones were electroporated with plasmid pBS185 (GIBCO BRL) directly expressing cre recombinase to excise the Neo^r gene. These DMR^{flox} alleles were iden-

tified by using a PCR-based strategy to detect the presence of *loxP* sites on either side of the DMR by amplifying the region around the –7.0-kb *Hin*dIII site with primers PrA (5′-CAGGCCTGTCCTCACCTGAAC-3′) and PrB (5′-GCCAGCTTGCCTTGGCAACCCCTT-3′), and around the –0.8-kb *Xba*I site with primers PrC (5′-CCACTGCTGAGTGGTCATG-3′) and PrD (5′-CGTGCGTGCGTATACCATTGCTC-3′). The amplification products were confirmed by sequencing. Clones carrying the DMR^{flox} allele were introduced into C57/BL6-J blastocysts to generate chimeric founder mice. $DMR^{\Delta G}$, $DMR^{\Delta Z}$, and $DMR^{\Delta S}$ alleles were obtained by the action of cre recombinase on DMR^{flox} alleles in vivo and screened for the excision by PCR using primers PrA and PrD. The efficiency of the deletion in the muscle and heart was assayed by Southern blotting.

SNuPE assay for allele-specific expression analysis

Liver, heart, and skeletal muscle RNA samples were DNase treated and amplified for *H19* and *Igf2* by RT–PCR using the Superscript Preamplification System (GIBCO BRL). Absence of any DNA contamination in the RNA samples was evidenced by absence of any amplification from parallel RT minus controls for each RT–PCR reaction. The amplification primers for *H19* were 5′-GCACTAAGTCGATTGCACTGG-3′ and 5′-GCCTCAAGCACACGGCCACA-3′. Those for *Igf2* were 5′-CCATCAATCTGTGACCTCCTCTTG-3′ and 5′-TGTTGTTCTCAGCCAGCTTTACAC-3′. The PCR products (164 bp for *H19* and 574 bp for *Igf2*) were purified twice using the High Pure PCR Purification Kit (Roche) to eliminate free dNTPs. Approximately 5 ng of the PCR product was used as a template in SNuPE assays for incorporation of dATP or dGTP essentially as described (Kuppuswamy et al. 1991). The primers used for extension were 5′-CGTATGAATGTATACAGCAAGTGTGTAA-3′ for *H19* and 5′-ACACCATCGGGCAAGGGGATCTCAGCA-3′ for *Igf2*. Extended primers were analyzed on an 18% polyacrylamide–6 M urea gel. Incorporation was quantified using the Molecular Dynamics Storm PhosphorImaging System. For each experiment, PCR products amplified from the DNA of *castaneus* × *domesticus* F_1 mice were used as controls in the SNuPE reaction. Incorporation of dATP/dGTP in F_1 DNA was used as a correction factor (F). F × dGTP incorporation was taken as the corrected dGTP incorporation and used to calculate the relative contribution of the paternal *H19* allele to total *H19* expression [A/(A+G)] and of the maternal *Igf2* allele to total *Igf2* expression ([G/(A+G)] shown in Table 1.

Bisulphite-based DNA methylation analysis

DNA from wild-type, $DMR^{\Delta G}$, and $DMR^{\Delta Z}$ mutants was digested with *Bam*HI and treated with sodium bisulphite using the CpGenome DNA modification kit (Intergen Company). The region around the *H19* promoter (–266 to +338) was amplified by use of a nested PCR strategy. The primer sequences used for this purpose were chosen such that they are completely devoid of CpG residues and were designed such that they would anneal to the modified DNA in which the cytosine residues have been changed to thymidine. The primers for the first PCR were 5′-GTTTTAGATAGGGTTTTAGTAGGTTA-3′ and 5′-CTACTACCAACTATACCTTCACTACC-3′, and those for the nested PCR were 5′-TTAAGGGAGATATTTGGGGATAATGTTA-3′ and 5′-AACTATACCTTCACTACCCAAATCTAAA-3′. DNA from at least four PCR reactions was cloned and sequenced. Paternal clones were identified on the basis of a polymorphism at +167 between *castaneus* and *domesticus* DNA. Because $DMR^{\Delta S}$ DNA has a mix of maternal, undeleted paternal (DMR^{flox}), and deleted paternal $DMR^{\Delta S}$ alleles, primers were chosen to selectively amplify only the deleted paternal allele. The primers for the first PCR were 5′-TGGAATTGATGGTGGTGTTTGTATTT-3′ and 5′-CTACTACCAACTATACCTTCACTACC-3′, whereas those for the nested PCR were 5′-TTAAGGGAGATATTTGGGGATAATGTTA-3′ and 5′-AACTATACCTTCACTACCCAAATCTAAA-3′. In this case, bisulphite conversion was carried out in agarose beads (Olek et al. 1996) carrying 400 ng of DNA per bead. The PCR products were cloned and sequenced. The sequencing primer for all sequencing reactions was 5′-CTCCCCATTCCTCTCCAACCCTAACTC-3′.

Acknowledgments

We thank Ronald Kahn for providing the mice with MCK-*cre* transgene and Pierre Vassalli for providing the plasmid pCAGGS-*cre*. We also thank Paul Love and members of the Pfeifer laboratory for their help with the manuscript.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Araki, K., M. Araki, J. Miyazaki, and P. Vassalli. 1995. Site-specific recombination of a transgene in fertilized-eggs by transient expression of cre recombinase. *Proc. Natl. Acad. Sci.* **92:** 160–164.

Bartolomei, M.S. and S.M. Tilghman. 1997. Genomic imprinting in mammals. *Annu. Rev. Genet.* **31:** 493–525.

Bartolomei, M.S., S. Zemel, and S.M. Tilghman. 1991. Parental imprinting of the mouse H19 gene. *Nature* **351:** 153–155.

Bartolomei, M.S., A.L. Webber, M.E. Brunkow, and S.M. Tilghman. 1993. Epigenetic mechanisms underlying the imprinting of the mouse H19 gene. *Genes & Dev.* **7:** 1663–1673.

Brandeis, M., T. Kafri, M. Ariel, J.R. Chaillet, J. McCarrey, A. Razin, and H. Cedar. 1993. The ontogeny of allele-specific methylation associated with imprinted genes in the mouse. *EMBO J.* **12:** 3669–3677.

Brannan, C.I. and M.S. Bartolomei. 1999. Mechanisms of genomic imprinting. *Curr. Opin. Genet. Dev.* **9:** 164–170.

Brenton, J.D., R.A. Drewell, S. Viville, K.J. Hilton, S.C. Barton, J.F.X. Ainscough, and M.A. Surani. 1999. A silencer element identified in Drosophila is required for imprinting of H19 reporter transgenes in mice. *Proc. Natl. Acad. Sci.* **96:** 9242–9247.

Bruning, J.C., M.D. Michael, J.N. Winnay, T. Hayashi, D. Horsch, D. Accili, L.J. Goodyear, and C.R. Kahn. 1998. A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. *Mol. Cell* **2:** 559–569.

Caspary, T., M.A. Cleary, C.C. Baker, X.J. Guan, and S.M. Tilghman. 1998. Multiple mechanisms regulate imprinting of the distal chromosome 7 gene cluster. *Mol. Cell. Biol.* **18:** 3466–3478.

Chung, J.H., M. Whiteley, and G. Felsenfeld. 1993. A 5′ element of the chicken Beta-globin domain serves as an insulator in human erythroid-cells and protects against position effect in Drosophila. *Cell* **74:** 505–514.

DeChiara, T.M., E.J. Robertson, and A. Efstratiadis. 1991. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* **64:** 849–859.

Efstratiadis, A. 1994. Parental imprinting of autosomal mammalian genes. *Curr. Opin. Genet. Dev.* **4:** 265–280.

Elson, D.A. and M.S. Bartolomei. 1997. A 5′ differentially methylated sequence and the 3′-flanking region are necessary for H19 transgene imprinting. *Mol. Cell. Biol.* **17:** 309–317.

Ferguson-Smith, A.C., H. Sasaki, B.M. Cattanach, and M.A. Surani. 1993. Parental-origin-specific epigenetic modification of the mouse H19 gene. *Nature* **362:** 751–755.

Frommer, M., L.E. McDonald, D.S. Millar, C.M. Collis, F. Watt, G.W. Grigg, P.L. Molloy, and C.L. Paul. 1992. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci.* **USA 89:** 1827–1831.

Fuks, F., W.A. Burgers, A. Brehm, L. Hughes-Davies, and T. Kouzarides. 2000. DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nat. Genet.* **24:** 88–91.

Geyer, P.K. 1997. The role of insulator elements in defining domains of gene expression. *Curr. Opin. Genet. Dev.* **7:** 242–248.

Gu, H., Y.R. Zou, and K. Rajewsky. 1993. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell* **73:** 1155–1164.

Gu, H., J.D. Marth, P.C. Orban, H. Mossmann, and K. Rajewsky. 1994. Deletion of a DNA-polymerase-beta gene segment in T-cells using cell-type-specific gene targeting. *Science* **265:** 103–106.

Hark, A.T. and S.M. Tilghman. 1998. Chromatin conformation of the H19 epigenetic mark. *Hum. Mol. Genet.* **7:** 1979–1985.

Holmes, S.G. and J.R. Broach. 1996. Silencers are required for inheritance of the repressed state in yeast. *Genes & Dev.* **10:** 1021–1032.

Kellum, R. and P. Schedl. 1992. A group of scs elements function as domain boundaries in an enhancer-blocking assay. *Mol. Cell. Biol.* **12:** 2424–2431.

Khosla, S., A. Aitchison, R. Gregory, N.D. Allen, and R. Feil. 1999. Parental allele-specific chromatin configuration in a boundary-imprinting-control element upstream of the mouse H19 gene. *Mol. Cell. Biol.* **19:** 2556–2566.

Kuppuswamy, M.N., J.W. Hoffmann, C.K.Kasper, S.G. Spitzer, S.L. Groce, and S.P. Bajaj. 1991. Single nucleotide primer extension to detect genetic diseases: Experimental application to hemophilia B (factor IX) and cystic fibrosis. *Proc. Natl. Acad. Sci.* **88:** 1143–1147.

Lakso, M., J.G. Pichel, J.R. Gorman, B. Sauer, Y. Okamoto, E. Lee, F.W. Alt, and H. Westphal. 1996. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. *Proc. Natl. Acad. Sci.* **93:** 5860–5865.

Leighton, P.A., R.S. Ingram, J. Eggenschwiler, A. Efstratiadis, and S.M. Tilghman. 1995a. Disruption of imprinting caused by deletion of the H19 gene region in mice. *Nature* **375:** 34–39.

Leighton, P.A., J.R. Saam, R.S. Ingram, C.L. Stewart, and S.M. Tilghman. 1995b. An enhancer deletion affects both H19 and Igf2 expression. *Genes & Dev.* **9:** 2079–2089.

Li, E., C. Beard, and R. Jaenisch. 1993. Role For DNA methylation in genomic imprinting. *Nature* **366:** 362–365.

Lyko, F., J.D. Brenton, M.A. Surani, and R. Paro. 1997. An imprinting element from the mouse H19 locus functions as a silencer in Drosophila. *Nat. Genet.* **16:** 171–173.

Lyons, G.E., S. Muhlebach, A. Moser, R. Masood, B.M. Paterson, M.E. Buckingham, and J.C. Perriard. 1991. Developmental regulation of creatine-kinase gene-expression by myogenic factors in embryonic mouse and chick skeletal-muscle. *Development* **113:** 1017–1029.

Nan, X.S., H.H. Ng, C.A. Johnson, C.D. Laherty, B.M. Turner, R.N. Eisenman, and A. Bird. 1998. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* **393:** 386–389.

Ng, H.H., Y. Zhang, B. Hendrich, C.A. Johnson, B.M. Turner, H. Erdjument-Bromage, P. Tempst, D. Reinberg, and A. Bird. 1999. MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. *Nat. Genet.* **23:** 58–61.

Okano, M., D.W. Bell, D.A. Haber, and E. Li. 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* **99:** 247–257.

Olek, A., J. Oswald, and J. Walter. 1996. A modified and improved method for bisulphite based cytosine methylation analysis. *Nucleic Acids. Res.* **24:** 5064–5066.

Pedone, P.V., M.J. Pikaart, F. Cerrato, M. Vernucci, P. Ungaro, C.B. Bruni, and A. Riccio. 1999. Role of histone acetylation and DNA methylation in the maintenance of the imprinted expression of the H19 and Igf2 genes. *FEBS Lett.* **458:** 45–50.

Pfeifer, K., P.A. Leighton, and S.M. Tilghman. 1996. The structural H19 gene is required for transgene imprinting. *Proc. Natl. Acad. Sci.* **93:** 13876–13883.

Sasaki, H., P.A. Jones, J.R. Chaillet, A.C. Fergusonsmith, S.C. Barton, W. Reik, and M.A. Surani. 1992. Parental imprinting—potentially active chromatin of the repressed maternal allele of the mouse insulin-like growth factor-II (*Igf2*) gene. *Genes & Dev.* **6:** 1843–1856.

Schmidt, J.V., J.M. Levorse, and S.M. Tilghman. 1999. Enhancer competition between H19 and Igf2 does not mediate their imprinting. *Proc. Natl. Acad. Sci.* **96:** 9733–9738.

Sternberg, E.A., G. Spizz, W.M. Perry, D. Vizard, T. Weil, and E.N. Olson. 1988. Identification of upstream and intragenic regulatory elements that confer cell-type-restricted and differentiation-specific expression on the muscle creatine-kinase Gene. *Mol. Cell. Biol.* **8:** 2896–2909.

Thorvaldsen, J.L., K.L. Duran, and M.S. Bartolomei. 1998. Deletion of the *H19* differentially methylated domain results in loss of imprinted expression of *H19* and *Igf2. Genes & Dev.* **12:** 3693–3702.

Tremblay, K.D., J.R. Saam, R.S. Ingram, S.M. Tilghman, and M.S. Bartolomei. 1995. A paternal-specific methylation imprint marks the alleles of the mouse H19 gene. *Nat. Genet.* **9:** 407–413.

Tremblay, K.D., K.L. Duran, and M.S. Bartolomei. 1997. A 5′ 2-kilobase-pair region of the imprinted mouse H19 gene exhibits exclusive paternal methylation throughout development. *Mol. Cell. Biol.* **17:** 4322–4329.

Wade, P.A., A. Gegonne, P.L. Jones, E. Ballestar, F. Aubry, and A.P. Wolffe. 1999. Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. *Nat. Genet.* **23:** 62–66.