A silencer element identified in *Drosophila* is required for imprinting of *H19* reporter transgenes in mice

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ABSTRACT   The *H19* gene is subject to genomic imprinting because it is methylated and repressed after paternal inheritance and is unmethylated and expressed after maternal inheritance. We recently identified a 1.1-kb control element in the upstream region of the *H19* gene that functions as a cis-acting silencer element in *Drosophila*. Here we investigate the function of this element in mice. We demonstrate that both *H19–lacZ* and *H19–PLAP* reporter transgenes can undergo imprinting with repression and hypermethylation after paternal transmission at many integration sites. However, transgenes that were deleted for the 1.1-kb silencer element showed loss of paternal repression, but they did not show marked changes in the paternal methylation of the remaining upstream region. This study demonstrates that the 1.1-kb control element identified in *Drosophila* is required to silence paternally transmitted *H19* minitransgenes in mice.

Genomic imprinting confers different functions on the two parental genomes during development by silencing one allele of each imprinted gene in a parent-of-origin-dependent manner (1–4). This transcriptional repression is brought about by epigenetic modifications that are thought to be erased and reestablished during germ cell development. Methylation of the dinucleotide CpG has been extensively studied as a candidate for the imprinting mark, because it is both heritable and associated with transcriptional repression. In support of this model, the inactive paternal *H19* allele is hypermethylated with a compacted chromatin structure over an 8-kb region encompassing the body of the gene and −4 kb of upstream sequence (5–9). By contrast, the active maternal allele is unmethylated with an open chromatin configuration. Methylation of the paternal allele is initiated early in the germ line and fully established in sperm (6, 7, 10). This is most pronounced in a region between −2 kb and −4 kb (the differentially methylated domain, henceforth called the DMD), which is resistant to the genome-wide demethylation that occurs during pre-implantation development (11–13). Futhermore, in embryos with the null mutation for the DNA methyltransferase gene (*Dnmt1*), there was a loss of DNA methylation at the locus and consequent biallelic expression of *H19* (14).

A number of studies have used mouse transgenes as a stringent test for the presence of control elements. *H19* transgenes with −3.8 kb of upstream sequence (and therefore containing the DMD) were subject to imprinting when present as multicopy loci at random integration sites. This was evidenced by transcriptional repression and hypermethylation after inheritance through the male germ line and demethylation and expression after maternal transmission (7, 15, 16). A larger 130-kb yeast artificial chromosome (YAC) transgene with both *Igf2* and *H19* genes reliably underwent imprinting as one- to two-copy loci (17). Although

imprinting of the smaller *H19* transgenes was variable, it nevertheless indicated the presence of a cis-control element within the −3.8-kb region, particularly because transgenes with only −1.8 kb of upstream flank (and no DMD) did not show repression (16). However, separate transgene modifications, by replacement of the *H19* gene with the luciferase *(luc)* reporter gene or the deletion of 701 bp in the 5′ most portion of the first exon, also abrogated repression despite the presence of the −3.8-kb region (15). It was therefore inferred that the *H19* transcript or genomic sequence were required for *H19* imprinting. However, "knock-in" targeting of endogenous *H19* with neomycin *(neo)* or *luc* resulted in appropriate imprinting of the replacement prokaryotic gene (18, 19). The reasons for these contradictory results are unclear.

We recently introduced an *H19–lacZ* reporter transgene into *Drosophila* to test for conserved function of the −3.8-kb upstream region in an organism that lacks DNA methylation or genomic imprinting. Using a genetic screen, we identified a 1.1-kb region lying between −2.9 and −1.8 kb from the transcriptional start site that acted as a silencer element (20). The location of this silencer region shows a striking overlap with the DMD described above. A similar silencer element was also detected in the Prader–Willi/ Angelman syndrome locus (21). These data suggested the intriguing possibility of a mechanistic link between gene silencing in *Drosophila* and imprinting in mice.

As a first step toward elucidating this relationship, we examined the imprinting of *H19* reporter constructs in mice together with the effects of deleting the 1.1-kb control element. This study demonstrates that the 1.1-kb cis element is required for silencing the paternally inherited *H19* reporter transgenes but is not required for intiating or maintaining parent-of-origin-specific methylation in the remaining upstream region.

MATERIALS AND METHODS

Transgenes. Genomic fragments were obtained from a λ2001 mouse 129/Sv library (a gift of A. Smith, Univ. of Edinburgh) and from the cosmid cAH (8). Subclones encompassing −0.25, −3.8, and −10.5 kb of upstream sequence were cloned into pBluescript KS+ (Stratagene) together with the *H19* promoter (22). The plasmid pZ (a gift of P. Kastner, College de France), which contains a β-galactosidase *(lacZ)* reporter with a nuclear localization signal was further modified by the insertion of flanking *loxP* sites. The resulting reporter fragment was then inserted at a *Sma*I site just beyond the TATA box in the *H19* promoter. HGF20 had a polymorphic *H19* genomic fragment (+2041, *Acc*I → *Sac*I) inserted distal to the 3′ *loxP* site. A 2.2-kb

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Abbreviations: DMD, differentially methylated domain; PLAP, placental alkaline phosphatase; YAC, yeast artificial chromosome.

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*Spe*I–*Bgl*II fragment containing the downstream enhancers (23) was then inserted immediately 3′ to the poly(A) sequence of the reporter or the *H19* sequence.

The *H19*–*PLAP* transgene was built in a manner identical to the corresponding −10.5-kb *H19*–*lacZ* construct, except that a full-length genomic clone of human placental alkaline phosphatase (*PLAP,* also known as *ALPP*) was used as a reporter. This contained introns but not *loxP* sites and was excised as a 5-kb *Hin*dIII–*Eco*RI fragment from pSV2Apap (24). This was blunt ligated into the *Dra*III site at +5 bp in exon 1 of *H19* and therefore did not disrupt the promoter sequence beyond the TATA box. In addition a 4.2-kb *Sph*I–*Eco*RV fragment from cAH was inserted into the −10.5-kb flank because the *lacZ* transgene did not include a 136-bp *Eco*RI–*Eco*RI fragment that lies at −4 kb (11).

The silencer deletion for the DEL–*H19* transgene was created by *Bsp*EI–*Bam*HI digestion and blunt ligation of a plasmid containing the *Sph*I–*Eco*RV fragment, which was then inserted into *H19*–*PLAP* to make DEL–*H19*. This procedure resulted in a 1177-bp deletion with a 5′ boundary at −2999 bp that was only 81 bp larger than that identified in *Drosophila*. The *Bam*HI site at the 3′ boundary was preserved.

Generation of Transgenic Mice. DNA for pronuclear injection was purified away from vector sequences by *Not*I or *Not*I/*Xho*I digestion and agarose gel electrophoresis by using either elution from DE81 paper or dialysis and concentration with Elutip-D columns (Schleicher & Schüll). Fragments were filtered and dialyzed against injection buffer (25) with spin columns or drop dialysis (Millipore). DNA concentration was estimated by agarose gel electrophoresis after serial dilution and comparison to λ *Hin*dIII standards (New England Biolabs). Injections were carried out at 1 to 2 ng/ml. Pronuclear injection was performed as described into B6/CBA F₂ eggs (25). For the HGF20 lines, similarly purified DNA was co-electroporated with the *Pgkneo* fragment p770 (a gift of A. Smith) into R1 ES cells (26). G418 selection was started at 24 hr post-electroporation and clones were selected at day 6 and 7. Two low copy number clones were chosen for blastocyst injection (27).

Mouse Strains. Lines from founders derived by pronuclear injection were maintained on a mixed F₁ (C57BL/6 × CBA) background. The ES cell-derived lines were maintained on an inbred 129/Sv background.

Embryo Collection and Reporter Gene Staining. Embryos were collected at days 11.5 and 13.5 (counting the morning of the vaginal plug as day 0.5) from the uterus and placed in PBS. Whole mount staining with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) was carried out for day 11.5 embryos as described (17). Day 13.5 embryos were fixed for 1 hr and then cut sagitally on a −20°C cold stage. Fixation was continued for one further hour followed by staining as before. *H19*–*PLAP* embryos were fixed for 15–30 min in 4% paraformaldehyde and washed twice in PBS. Endogenous placental alkaline phosphatase (PLAP) was inactivated by incubating the embryos at 72°C for 1 hr. The embryos were allowed to cool, agitated in BM purple alkaline phosphatase substrate (Boehringer Mannheim) at 4°C overnight and postfixed in 4% paraformaldehyde for 24 hours. All embryos were then washed in PBS and stored in 70% ethanol.

Dehydration, clearing, and embedding were carried out as described (28). Clearing with oil of wintergreen or rapid chloroform extraction was used to prevent decolorization of *H19*–*lacZ* embryos. Sections were cut at 8 μm and mounted on polylysine-coated slides. PLAP staining was performed directly on the slide after mounting with BM purple alkaline phosphatase substrate. Sections were counterstained with nuclear fast red or eosin. Embryos and sections were photographed with Ektachrome 160T or Fujichrome 64T film.

Genomic DNA Analysis. DNA extraction, Southern blotting, and transgene-copy-number characterization were carried out as described (17). Genotyping of offspring and embryos was carried out by PCR (29) after lines had been characterized by Southern analysis. The primers HGFPRO1 (5′-GGCCATGTACTGATT-

GGTTGACA-3′), HGFPRO2 (5′-GAATTCCGGAAAACTT-TATCCAT-3′), H19PLAP1 (5′-TTGGTTGACAGAG-TAGGGGC-3′), and H19PLAP2 (5′-GAGCAAAGATCAG-GTCAGCC-3′) were used to amplify products spanning the *H19* promoter and the 5′ portion of the *lacZ* (HGFPRO1 + HGF-PRO2) or *PLAP* (H19PLAP1 + H19PLAP2) reporters. Methylation analysis was performed on tissue from hemisected or decapitated day 11.5 and day 13.5 embryos as previously described (17).

RESULTS

Transgenes containing reporters allow comprehensive analysis of the extent of expression within individual tissues and cells in a large number of embryos. We used the *lacZ* reporter because it has proved an excellent marker of transgene expression. However, its high CpG content could influence epigenetic modifications on the transgenes (30). Therefore, we also used the mammalian gene *PLAP* (24, 31). *H19*–*lacZ* transgenes with −10.5 kb, −3.8 kb and −2.5 kb of flanking sequence were constructed to test the function of the upstream region in imprinting (Fig. 1). The transgene HGF22 with −3.8 kb, of upstream sequence had been previously introduced into *Drosophila* (20). Twenty-one transgenic lines were obtained, but 9 of the 16 *H19*–*lacZ* lines were excluded from the analysis (3 lines failed to transmit the transgene and the remaining 6 lines showed no expression) (Table 1). In addition, four *DEL*–*H19* lines were generated that had a 1.2-kb deletion of the silencer region identified in *Drosophila* (Fig. 1 and Table 1).

Expression Pattern of the *H19* Reporter Transgenes. The *H19*–*lacZ* and *H19*–*PLAP* reporter transgenes contained two previously characterized downstream enhancers (22, 32). Their expression was first examined after maternal transmission in day 11.5 and 13.5 embryos. The overall pattern of expression between lines was highly consistent and was unaffected by the size of the upstream flank or by the choice of the reporter. At day 11.5, strong expression was seen in the liver and endothelium of the gut and blood vessels (Fig. 2). Expression in mesodermal and ectodermal tissues was also demonstrated by staining throughout the sclerotome and in the eye and periventricular tissues of the forebrain. The expression in brain parenchyma was unexpected (33), but *in situ* hybridization analysis on wild-type day 11.5 and day 13.5 embryos confirmed low-level expression of the endogenous gene in the lens epithelium and forebrain (data not shown). Low levels of expression were detected in the meninges but not in the choroid plexus. At day 13.5, endodermal expression was present throughout the gut, liver, pancreas, kidney, and gonad. Cartilage, which is derived from migrated sclerotome, was strongly stained. No expression was seen in skeletal muscle and only low-level expression was seen in the heart and base of the tongue. *lacZ* reporter transgenes containing the *Igf2* P3 promoter and the identical downstream enhancer fragment showed similar patterns of expression (W. M. Rideout III and J.D.B., unpublished observations). These data confirmed that the two downstream enhancers are not solely endoderm specific, but have additional expression in the mesodermal tissues. Expression analysis from the *Igf2*–*H19* YAC transgene suggests that those for skeletal muscle must lie within its 130-kb domain (17).

H19 Reporter Transgenes Are Repressed on Paternal Transmission. To ask whether the transgenes showed evidence of imprinting, we compared the expression of *H19*–*lacZ* or *H19*–*PLAP* in transgenic embryos after male and female transmission. Embryos from eight lines with −10.5 kb and one line with −3.8 kb of upstream flank were examined at day 11.5 after whole mount staining. Seven of these transgenic lines showed marked repression of the reporters after paternal inheritance (Fig. 2*B* and Table 1). Indeed, one line, FL16, showed imprinting similar to the endogenous gene with complete repression after paternal inheritance and high-level expression after maternal inheritance (Fig. 2*A*). However, two lines (FL8 and FL15) also showed repression on maternal inheritance.
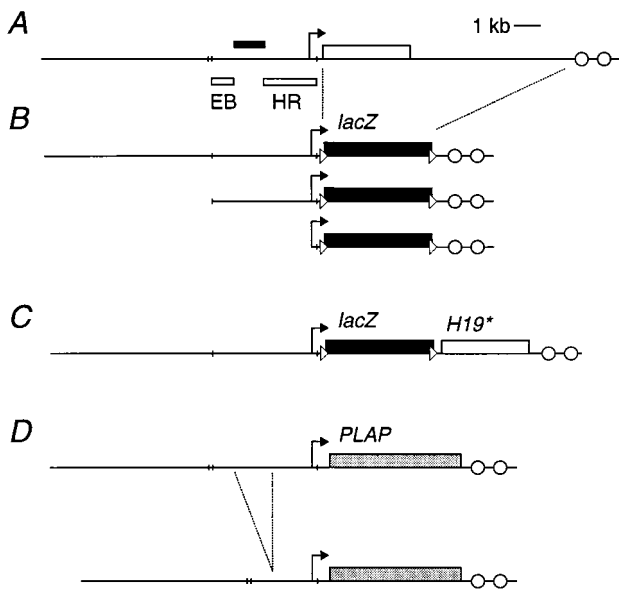
FIG. 1. Structure of the *H19* genomic locus and reporter transgenes. (*A*) The *H19* gene is depicted by the open rectangle with the arrow indicating the promoter (internal exon/intron boundaries not shown). The downstream enhancers are represented by open circles. The filled rectangle above the horizontal line indicates the position of the silencer region identified in *Drosophila* (20). The open rectangles below the line show the probes EB and HE that were used for methylation analysis. The short vertical lines indicate *Eco*RI sites. (*B*) *H19–lacZ* transgenes with −10.5 kb, −3.8 kb, and −0.25 kb of upstream region (HGF23, HGF22, and HGF21, respectively). The filled rectangle represents the bacterial *lacZ* gene and the dashed lines show the region of the wild-type locus that has been replaced by the reporter. The *loxP* sites are depicted by triangles. (*C*) HGF20 transgenes were identical to HGF23 except that a 4-kb *H19* genomic fragment containing an artificial polymorphism was inserted downstream of the *lacZ* poly(A) and 3′ *loxP* site. (*D*) *H19–PLAP* and DEL–*H19* transgenes with and without the silencer region. The gray rectangles depict the human *PLAP* genomic reporter (internal exon/intron boundaries not shown). The dashed lines show the 1.2-kb *Bsp*EI–*Bam*HI deletion and the resulting DEL–*H19* transgene has −9.3 kb of remaining upstream region.

The degree of repression upon paternal inheritance was, however, variable between embryo littermates, with a minority of

embryos apparently escaping transgene repression altogether. This variability was not unexpected because it has been reported previously by others who used *H19* minitransgenes (7, 15, 16). Five lines (MLK16.1, FL5, FL8, FL15, and FL16) were studied in some detail for expression after sequential paternal and maternal transmission. The paternal repression was relieved after transmission through the female germ line, which is consistent with the effect of erasure on the imprinting signal in the paternal germ line. In the two lines where repression was still seen on maternal transmission, FL8 showed repression in 3 of 7 embryos, compared with 21 of 25 embryos after maternal inheritance, whereas in line FL15, 13 of 14 maternally transmitted embryos and 27 of 29 paternal embryos were repressed.

In contrast, no repression was seen in three lines containing only −0.25 kb of upstream flank (thus lacking the DMD and the 1.1-kb region). In the small number of embryos examined, high-level expression after paternal and maternal transmission was seen. Taken together, these data further confirmed that sequences between −3.8 and −0.25 kb contain a key control element and that transgenes containing this region are prone to repression after paternal inheritance but not after maternal inheritance.

DEL–*H19* Reporter Transgenes Are Not Repressed on Paternal Transmission. Having established that the *H19* reporter transgenes showed evidence of imprinted expression, we went on to test the function of the 1.1-kb upstream silencer element previously identified in *Drosophila*. We deleted a 1.2-kb *Bsp*EI–*Bam*HI fragment from the −10.5-kb flank of the *H19–PLAP* reporter transgene, resulting in a partial deletion of the DMD with 840 bp remaining in −9.3 kb of upstream flanking sequence.

Embryos from the four transgenic lines were analyzed on day 11.5 after maternal and paternal transmission. The most striking difference we found was the absence of silencing of the paternally inherited transgene in the DEL–*H19* lines compared with those that contained the intact flanking region (Fig. 2*B*). Only 4 of 90 embryos showed any repression of the reporter. In line DEL7, repression was detected in 2 of 22 embryos after paternal inheritance; repression was also detected in line DEL16 for 1 of 14 paternally transmitted and 1 of 9 maternally transmitted transgenes.

DNA Methylation Analysis of Transgenes. We then examined methylation of the DMD (−1.8 to −3.8 kb) of the *H19* transgenes to ask whether the changes in expression were accompanied by appropriate parent-of-origin methylation changes (Fig. 3). This region shows differential methylation within the *H19* locus and

Table 1. Summary of *H19–lacZ*, *H19–PLAP*, and DEL–*H19* transgenic lines

Upstream region, kb	Transgene	Line	Copy no.	Transgenic embryos, total no.	Imprinting	Differential methylation
−10.5	*H19–PLAP*	FL3	1	26	No	No
		FL5	7	56	Yes	Yes
		FL8	9	32	Yes*	ND
		FL15	1	43	No	Yes
		FL16	14	47	Yes	Yes
	HGF20	MLK16.1	4	22	Yes	Yes
	HGF23	3	2	34	Yes	ND
		16b	1	41	Yes	ND
−3.7	HGF22	3.7RF3y	6	14	Yes	Yes
−0.2	HGF21	SCB01	5	3	No	NA
		SCB02	>50	10	No	NA
		SCB10	10	5	No	NA
−9.3	DEL–*H19*	DEL7	1	35	No*	Yes
		DEL16	10	23	No*	Yes
		DEL21	5	11	No	Yes
		DEL24	4	21	No	Yes

The copy number, total number of transgenic embryos examined, imprinting as judged by repression on paternal transmission, and differential methylation of the DMD region are indicated for each line. Nine *H19–lacZ* lines were excluded from the analysis because of failure of transmission or expression. Transgenes from line HGF20-23 each contain the *lacZ* reporter, but differ in the amount of upstream region. Asterisks in "Imprinting" column mark lines that showed some anomalous repression as described in the text. ND, not done. NA, not applicable.
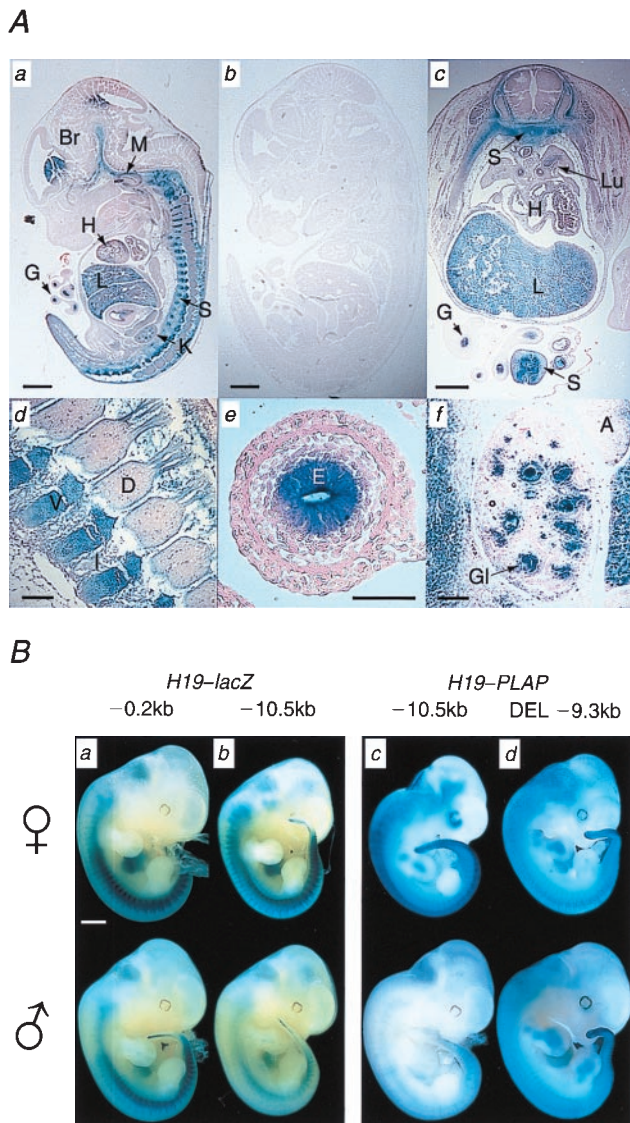
FIG. 2. (*A*) *PLAP* reporter gene expression in FL16 day 13.5 embryo after maternal and paternal transmission. (*a*) Midsagittal section after maternal transmission shows strong staining in liver, sclerotome, brain, meninges, and gut endoderm. Low levels of expression are seen in the heart. (*b*) Midsagittal section after paternal transmission shows complete repression of PLAP activity. (*c*) Transverse section through lower thoracic region after maternal transmission. The PLAP staining clearly shows the developing sclerotome, which is migrating dorsally to form the vertebral bodies and ventrally to form the cartilage and bones of the thoracic ribs. There is no expression in the spinal cord or dermatomyotome. Extracoloemic gut shows strong endothelial staining and some expression is also seen in the heart and bronchial epithelium of the lung. (*d*) Parasagittal view of upper thoracic vertebral column showing the formation of the vertebral bodies and intervertebral discs. (*e*) Sagittal section through extracoloemic gut showing high-level expression in the endoderm-derived endothelium. (*f*) Sagittal section through kidney showing PLAP expression in the developing glomeruli, but not in the tubules. Scale bar indicates 1 mm in *a, b,* and *c* and 0.1 mm in *d, e,* and *f*. A, adrenal gland; Br, brain; D, dorsal root ganglion; E, gut endothelium; G, gut; Gl, glomerulus; H, heart; I, intervertebral disk; L, liver; Lu, lung; K, kidney; M, meninges; S, sclerotome; V, vertebral body. (*B*) Repression of *H19–lacZ* and *H19–PLAP* transgenes requires the silencer region. Representative day-11.5 transgenic embryos stained as whole mount specimens for lacZ or PLAP activity after maternal (*Upper*) or paternal (*Lower*) transmission. The size of upstream region in the transgenic line is shown above the panel. (Scale bar indicates 1 mm.) The level of expression of the reporter gene was quantitated by using SCION IMAGE software (Scion, Frederick, MD). The mean density values for the embryos depicted were normalized against an

has been proposed to confer the parent-of-origin-dependent mark that is required for imprinting (11, 12). DNA was obtained from day-13.5 embryos after maternal and paternal transmission. The methylation status of *Cfo*I sites within the DMD of the *H19–PLAP* lines was examined by using the probe EB and digestion with *Sph*I, which gave a transgene-specific band of 4477 bp (Fig. 3*A*). Because the *H19–lacZ* lines did not have a suitable polymorphism within the DMD, the intensity of the −1.8- to −3.8-kb region was compared after maternal and paternal transmission by using a full-length probe and digestion with *Eco*RI, *Bam*HI, and *Cfo*I. Six lines (MLK16.1, FL3, FL5, FL8, FL15, and FL16) with −10.5 kb of upstream flank were examined. The single-copy line FL3, which did not exhibit paternal repression, showed no methylation for either maternal and paternal transmission (Fig. 3*B*). The remaining lines showed relative hypermethylation of transgenes after paternal inheritance when compared with the hypomethylation seen after maternal inheritance (Fig. 3*B* and data not shown). Although this represented a switch in the epigenetic state of the transgene, it was not as complete as that demonstrated at the locus or on the *Igf2–H19* YAC transgene. Incomplete differential methylation has been observed in other *H19* mini transgenes (7, 15, 16) and appears to become more complete with successive generations.

We next asked whether the apparent lack of repression of the transgene in DEL–*H19* lines after paternal inheritance was accompanied by changes in the degree of methylation of the upstream region. The DEL–*H19* transgenes contained a polymorphic 840-bp fragment within the DMD that was not removed by the silencer deletion. Using probe EB, we examined the methylation of the two remaining *Cfo*I sites (Fig. 3*A*). Strikingly, this partial DMD sequence showed appropriate paternal methylation and was predominantly unmethylated on maternal transmission (Fig. 3*C*). Partial maternal methylation was seen in lines DEL7 and DEL21 (Fig. 3*C*). This apparent gain of methylation was unexpected and it is unclear whether it was an effect of the silencer deletion or an aberrant methylation imprint as has been shown by other nondeleted *H19* minitransgenes (7).

Methylation analysis was also carried out for the promoter-proximal region of the transgenes. It is important to note that this region shows considerable variability in the degree of methylation even at the wild-type locus, and it is therefore less informative than the distal region (11, 12). We first examined methylation proximal to the transgene *H19* promoter by using day-13.5 embryonic material from the same six lines with −10.5 kb and one line with −3.8 kb of upstream flank. The *H19–lacZ* lines contained a 2090-bp *Hin*dIII transgene-specific fragment that was probed with HE. For the *H19–PLAP* lines, the analysis was based on the comparison of intensity differences within the −1.8-kb region after *Eco*RI, *Bam*HI, and *Cfo*I digestion. As shown in Fig. 3 *D* and *E* (and data not shown), digestion with the methylation-sensitive enzyme *Cfo*I showed relative hypermethylation of transgenes after paternal inheritance compared with that shown after maternal inheritance, although there was variation among individuals in the degree of methylation.

The four DEL–*H19* lines were then analyzed. Methylation analysis of the proximal *H19* region was carried out as done previously for the *H19–PLAP* lines. Differential methylation was observed in only one line (DEL16) with the remainder of

equivalent embryo with no staining. (*a*) Line SCB01 shows very slight repression after paternal expression. Maternal expression = 64.41. Paternal expression = 62.04. (*b*) Paternal transmission of line MLK16.1 shows marked repression of *lacZ*, most notable in the sclerotome. Maternal expression = 55.16. Paternal expression = 34.64. (*c*) Line FL8 shows strong repression of *PLAP* after paternal transmission. Some activity can be seen in the caudal sclerotome. Maternal expression = 98.90. Paternal expression = 22.19. (*d*) Line DEL24, which does not contain the 1.2-kb silencer, shows minor repression on paternal transmission. Maternal expression = 109.05. Paternal expression = 102.65.
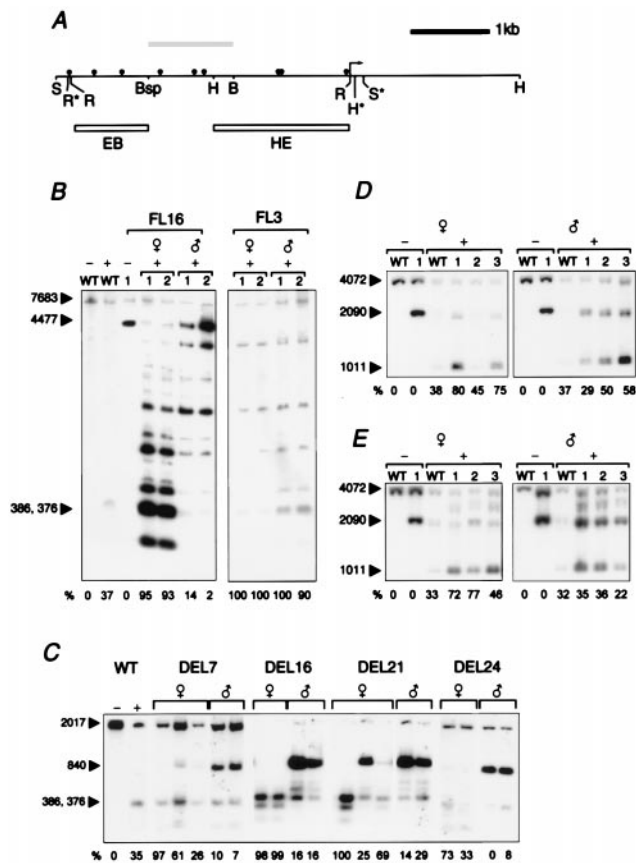
FIG. 3. Methylation of transgenes after maternal and paternal transmission. The relative degree of demethylation is quantiatated for all methylation analyses by measuring the intensity of the fully methylated transgene-specific and fully unmethylated bands with SCION IMAGE (except in wild-type control lanes, where the wild-type bands are compared). Values are corrected for copy number of the transgene and expressed as the percentage unmethylated DNA for each lane. (*A*) Methylation-sensitive *Cfo*I sites in the 4.0-kb region upstream of the *H19* gene. The arrow indicates the position of the *H19* promoter (gene body not shown). The lollipops above the line represent *Cfo*I sites. Polymorphic restriction sites within the transgenes are marked with an asterisk. The grey box above the line indicates the silencer region that is deleted in the DEL lines. The boxes below the line indicate the *Hin*dIII–*Eco*RI probe HE and the *Eco*RI–*Bsp*EI probe EB. [Restriction enzyme sites are labeled Bsp (*Bsp*EI), B (*Bam*HI), H (*Hin*dIII), R (*Eco*RI), and S (*Sph*I).] (*B*) Distal methylation in the region of the DMD for lines FL16 and FL3. WT indicates wild-type littermate; transgenic littermates are numbered. The plus sign (+) indicates the addition of *Cfo*I. Maternal and paternal transmission of the transgene is indicated by symbols above the lanes. Ten micrograms of DNA from day-13.5 embryos was digested sequentially with *Sph*I and *Cfo*I. The transgene-specific band of 4477 bp is indicated. Imprinted FL16 transgenes show low levels of methylation on maternal transmission and high levels of methylation on paternal transmission. The bi-allelic-expressing FL3 transgenes show the absence of methylation on maternal and paternal transmission. (*C*) Deletion of the silencer region does not alter parent-specific methylation. DNA from day-13.5 embryos from all four DEL lines was sequentially digested with *Bam*HI/*Eco*RI and *Cfo*I, and was then probed with EB. The transgene-specific band of 840 bp is indicated; it contains the 5′-most portion of the DMD. The transgenes were predominantly unmethylated on maternal transmission and methylated on paternal transmission. (*D*) Proximal methylation for line 3.7RF3y. DNA from day-13.5 embryos was digested sequentially with *Hin*dIII and the methylation-sensitive enzyme *Cfo*I, and was then probed with HE. The arrows indicate the wild-type *Hin*dIII fragment of 4072 bp and the transgene-specific band of 2090 bp. The transgene band is unmethylated on maternal transmission and partially methylated on paternal transmission. (*E*) Proximal methylation for line MLK16.1 containing −10.5 kb of upstream region. The digest was carried out as described before. The transgene-specific band shows predominate methylation on paternal transmission and less methylation after maternal transmission.

embryos examined showing partial methylation irrespective of parental origin (data not shown).

Taken together, these data showed that both *H19–lacZ* and *H19–PLAP* transgenes were predominantly paternally methylated and maternally unmethylated, confirming that reporter minitransgenes can undergo parental-specific methylation. Importantly, removal of the silencer element from the *H19–PLAP* transgenes abrogated imprinting as judged by transgene expression, but did not have a marked effect on the paternal-specific DNA methylation of the remaining upstream region.

DISCUSSION

Our results demonstrate that *H19* transgenes with either the *lacZ* or *PLAP* reporter genes can undergo appropriate imprinting at many integration sites. The *H19* gene was not required for imprinting to occur; these data differ from previous studies on *H19–luc* reporter transgenes, which did not show repression or parent-of-origin methylation changes (15). The reasons for this discrepancy are unclear but it is important to emphasize that our analysis allowed us to determine the degree of repression within all expressing tissues in developing embryos. Previous studies have restricted their analysis to expression in neonatal liver (7, 15, 16), which may be a disadvantage because expression from the downstream enhancers shows profound down-regulation shortly after birth (23). We also tested the function in mice of a 1.1-kb *cis* silencing element that had been independently identified in the DMD region with a genetic screen in *Drosophila* (20). Deletion of this element abrogated gene silencing previously seen after paternal inheritance of the *H19* transgenes. This finding is similar to previously published data that showed a lack of imprinting for *H19* transgenes truncated at −1.8 kb (and therefore not containing the 2-kb DMD) (16). Most suprisingly, however, our deletion did not alter the paternal hypermethylation for the remaining DMD, suggesting that the silencer element is not essential for the initiation of DNA methylation in this region.

The imprinting of our *H19* minitransgenes was variable, although comparable to results reported previously (7, 15, 16). Our results may be explained in part by the mixed genetic background used in most of the matings; genetic background has been shown to be an important determinant of the basal level of *H19* expression (34). However, line MLK16.1 was maintained on the inbred 129/Sv strain and still showed variation. It is also possible that the substitution of the *H19* sequence with the reporter genes may have altered the persistence of the repressive signal, because targeted replacement of the *H19* gene with *luc* showed varying paternal repression (19). This imperfect imprinting differs greatly from that of the endogenous locus and the *Igf2–H19* transgene (17), which implies that the minitransgenes lack other *cis*-elements present within the 130-kb YAC that protect imprinting signals from position effects at the integration locus. However, this insulating effect may be simply a function of the larger transgene. It is interesting that the imprinting of minitransgenes seems to be shown best by multiple-copy loci and that one- to two-copy lines are either silenced or do not imprint. The YAC transgenes showed the opposite effect; they were imperfectly imprinted only when integrated in high copy numbers (although this result may have been related to the limiting effects of *trans* factors). One explanation for the improved imprinting of high-copy number minitransgenes may be augmentation of the repressive signal by the silencing effects of transgene arrays (35, 36).

How could the silencing of paternally inherited *H19* transgenes in mice and silencing in *Drosophila* be linked mechanistically? There is evidence for conservation of silencing mechanisms in eukaryotes, as exemplified by the conserved functions of the homologues of the Polycomb group proteins (37). However, there is no evidence yet to suggest that these proteins are responsible for the silencing/imprinting function in *Drosophila* and mice. The *Drosophila* and mouse data together suggest a model in which the initial repression in imprinting is mediated by protein–DNA interaction at the silencer element, which then acts as a template

for subsequent methylation that permanently stabilizes silencing (38). However, this model is not supported by our important finding that deletion of the 1.1-kb silencer did not abrogate methylation after paternal inheritance of the transgene. This finding implies that the two processes may function associatively rather than sequentially. To investigate this further and to exclude an artefactual methylation effect from the multicopy transgene arrays, we have deleted the silencer at the endogenous locus (R.A.D., J.D.B., J.F.-X.A., S.C.B., K.J.H., L. Dandolo, and M.A.S., unpublished work). From preliminary studies it is apparent that this mutation acts in a manner similar to the deletion in the transgenic DEL lines. Further studies are needed to identify the trans-acting factors in both *Drosophila* and mice so that the precise mechanism by which this cis control element functions can be explored.

It is possible that our experiments have identified only part of the control element needed for imprinting, albeit that portion with the most evolutionarily conserved epigenetic function. A partial deletion may explain why DNA methylation is unaffected and also why some repression was observed despite deletion of the silencer. It is very likely that other control elements may reside within the 8-kb locus or immediately upstream of the silencer region (9). Thorvaldsen *et al.* (39) have recently deleted the major portion of the DMD in mice and showed that the paternal copy of *H19* is no longer repressed. However, they also noted the loss of parent-specific methylation of the remaining DMD and the promoter proximal region. In contrast, our targeted deletion of the endogenous 1.1-kb region is in agreement with the methylation data reported in this reporter transgene study. The role of the 5' portion of the DMD in imprint initiation and maintenance requires further investigation.

We also noted a small but detectable, higher expression level after maternal inheritance of the DEL–*H19* and −0.25-kb *H19*–*lacZ* lines (which did not contain the DMD or the silencer), when compared with paternal inheritance. This effect has previously been described by using quantitative analysis of *Igf2*–*luc* transgenes in mice, which showed higher levels of expression on maternal inheritance (23). The analysis suggests the possibility of an important interaction between the *H19* silencer element and the downstream enhancers. The enhancers (or some other regulatory element within the downstream-3' region) may be required to overcome the activity of the silencer element after passage through the female germ line and could be a crucial step in the imprint switching of *H19*. This possibility may be tested by using other reporter transgenes that are entirely devoid of the 3' region.

These experiments demonstrate that a conserved silencing/imprinting function acts at the *H19* locus and also suggests that this may be independent of DNA methylation. Further approaches are required to show which cis-elements are sufficient for faithful imprinting and to characterize the trans effectors of the silencing mechanisms. Methods that use the targeted integration of single-copy transgenes into defined genomic loci (40) and modifications of large YAC or bacterial artificial chromosome inserts should improve our understanding of this complex locus.

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