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A 5′ Differentially Methylated Sequence and the 3′-Flanking Region Are Necessary for *H19* Transgene Imprinting

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The mouse *H19* gene is expressed exclusively from the maternal allele. The imprinted expression of the endogenous gene can be recapitulated in mice by using a 14-kb transgene encompassing 4 kb of 5′-flanking sequence, 8 kb of 3′-flanking sequence, which includes the two endoderm-specific enhancers, and an internally deleted structural gene. We have generated multiple transgenic lines with this 14-kb transgene and found that high-copy-number transgenes most closely follow the imprinted expression of the endogenous gene. To determine which sequences are important for imprinted expression, deletions were introduced into the transgene. Deletion of the 5′ region, where a differentially methylated sequence proposed to be important in determining parental-specific expression is located, resulted in transgenes that were expressed and hypomethylated, regardless of parental origin. A 6-kb transgene, which contains most of the differentially methylated sequence but lacks the 8-kb 3′ region, was not expressed and also not methylated. These results indicate that expression of either the *H19* transgene or a 3′ DNA sequence is key to establishing the differential methylation pattern observed at the endogenous locus. Finally, methylation analysis of transgenic sperm DNA from the lines that are not imprinted reveals that the transgenes are not capable of establishing and maintaining the paternal methylation pattern observed for imprinted transgenes and the endogenous paternal allele. Thus, the imprinting of the *H19* gene requires a complex set of elements including the region of differential methylation and the 3′-flanking sequence.

Genomic imprinting is the differential expression of the alleles of a gene depending on parental origin (13, 30). This unusual form of gene expression renders the maternal and paternal pronuclei of mammals functionally inequivalent and is the most probable cause of the developmental failure associated with uniparental mammalian embryos. It has long been held that the identification of genes that are imprinted not only would lead to a better understanding of why uniparental embryos fail early in development but also would facilitate the investigation of how imprinting is controlled. However, the first endogenous imprinted genes were not identified until a few years ago. Prior to the identification of these genes, transgenes were used as molecular tools to study the process of imprinting (9, 33). These transgenes exhibited differential expression and methylation patterns depending on the sex of the transmitting parent (11, 14, 17, 26, 28, 29, 34, 36).

The covalent addition of a methyl group to the cytosine residue of CpG dinucleotides is an attractive candidate for serving as the mechanism that distinguishes, as well as silences, the parental alleles of imprinted genes and transgenes (25). Imprinted transgenes provided the initial evidence that methylation might be involved in the differential expression of genes. It was observed that for two imprinted transgenes, the RSVIgmyc and hepatitis B virus surface antigen genes, tissue-specific expression was inversely correlated with methylation levels (17, 34). Differentially methylated transgenes also provided insight as to when the imprint might be established. The analysis of two independent transgenes revealed that the differential methylation was initiated during gametogenesis (11, 36). In both cases, the maternal-specific methylation pattern was fully acquired during oogenesis.

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It is not entirely clear why a subset of transgenic lines exhibit imprinted behavior. In most cases, the transgene was designed to study a biological process other than imprinting, and parental-specific expression or methylation was observed when the progeny were analyzed. One possibility for this behavior is that imprinted transgenes have inserted into or adjacent to regions that harbor imprinted genes (12). While there is presently no such example, it has been hypothesized that these transgenes could provide a molecular tag to isolate the genomic loci of imprinted genes. Alternatively, imprinted transgenes could result from the artificial combination of sequences and lead to a novel imprinting signal. This is likely to be the case for the imprinted RSVIgmyc transgenic lines (10). Finally, sequences present in imprinted transgenes could reflect the imprinting of their endogenous counterparts. This behavior has been observed for the maternally expressed *H19* gene (3).

Of the 15 endogenous imprinted genes which have been identified in mice and humans (2), the *H19* gene is presently the only one for which a transgene that mimics the endogenous expression has been generated (3). This 14-kb transgene contains 4 kb of 5′-flanking sequence, 8 kb of 3′-flanking sequence, which includes the two endoderm-specific enhancers, and an internally deleted structural gene (8). In two independent lines generated with the 14-kb construct, the transgene was expressed in progeny who inherited the transgene from the mother and repressed in progeny who inherited the transgene from the father. Interestingly, the *H19* transgene expression patterns were opposite to previously reported imprinted transgenes in that the other transgenes were exclusively expressed when transmitted by the father. Thus, it appears that the *H19* transgene imprinting reflects the endogenous *H19* gene expression pattern and can be used to study the imprinting of this gene.

The methylation of the *H19* transgenes is also similar to the methylation observed for the endogenous *H19* gene. Previous

analysis of the methylation status of the endogenous *H19* gene led to the identification of a 7- to 9-kb region of paternal-specific methylation (3, 6, 16). A subset of these differentially methylated CpG dinucleotides located from −2 kb to −4 kb relative to the start of transcription are also differentially methylated in the gametes and preimplantation embryos, leading to the suggestion that, similar to what has been found for imprinted transgenes, methylation is likely to play an essential role in determining the imprinted expression of the *H19* gene (35). When the methylation pattern of one of the imprinted transgenic lines was analyzed, it was demonstrated that the repressed, paternally derived transgenes were hypermethylated relative to the expressed, maternally derived transgenes (3).

In this study, we have extended the original findings by generating additional mouse lines with the 14-kb transgene and correlating the expression of the transgene with methylation. Furthermore, we have engineered deletions of the 5′ differentially methylated region and demonstrated that this region, while not necessary for the appropriate expression pattern, is essential to the imprinting of the transgenes. Additionally, we have shown that the 3′ region harboring the *H19* enhancers is essential to the expression and differential methylation of the transgene.

MATERIALS AND METHODS

Preparation and microinjection of transgene DNA. Double-cesium-banded plasmid DNA containing the transgenic fragment was digested to completion with the appropriate restriction enzyme to liberate the insert from vector DNA. The DNA was isolated in a 1% agarose gel run in 1× TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). The gel was stained with ethidium bromide, the appropriate band was visualized and excised, and the DNA was recovered by electroelution in dialysis tubing (Spectra/Por; molecular weight cutoff, 6,000 to 8,000) and subsequent ethanol precipitation. The recovered DNA was passed through an ion-exchange column (Schleicher and Schuell Elutip-d column) according to the manufacturer's instructions and ethanol precipitated. The pellet was resuspended in injection buffer (10 mM Tris, 0.1 mM EDTA [pH 7.5]) quantified, and diluted to approximately 2 to 4 ng/μl with injection buffer.

Microinjection was performed by Jean Richa at the University of Pennsylvania Core Transgenic Facility. DNA was injected into one of the pronuclei of fertilized one-cell mouse eggs derived from (C57BL/6 × SJL)F_1 intercrosses. Founder animals were identified by Southern blot analysis of tail DNA and outbred to DBA/2J mice, purchased from The Jackson Laboratory, to maintain transgenic lines.

Preparation of genomic DNA and total RNA. Liver or tail tissue samples were incubated overnight at 55°C in 500 μl of digestion buffer (50 mM Tris-HCl [pH 8.0], 100 mM EDTA, 0.5% sodium dodecyl sulfate) with 0.25 μg of proteinase K (Boehringer Mannheim). NaCl was added to a final concentration of 150 mM, and the mixture was twice extracted with phenol-chloroform-isoamyl alcohol (25:24:1). DNA was ethanol precipitated and resuspended in 70 μl of TE (10 mM Tris-HCl [pH 7.6], 1 mM EDTA).

RNA from 5-day-old mice was prepared by the lithium chloride method as previously described (1).

PCR to determine transgenic animals. The oligonucleotide primers used to differentiate transgenic from nontransgenic progeny flanked the *Bam*HI deletion in the transgene. Their sequences were 5′-CCTTGGAGACAGTGGCAG-3′ (5′) and 5′-GACATGAGCTGGGTAGCAC-3′ (3′). Amplification at the transgenic locus would produce a 165-bp fragment, while the endogenous locus would produce a 1,145-bp fragment. Using 25 ng of genomic DNA, we amplified each sample in a 25-μl reaction mixture containing 12.5 ng of each primer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μM each deoxynucleoside triphosphate, and 1 U of AmpliTaq DNA polymerase (Perkin-Elmer). Amplification was carried out in a Perkin-Elmer DNA Thermal Cycler 480, using 32 cycles of 94°C denaturation for 1 min (except for the first two cycles, which had 4-min denaturation steps), 55°C annealing for 2 min, and 72°C extension for 2 min. PCR products were run on 7% acrylamide gels and visualized by ethidium bromide staining.

S1 nuclease protection assay. Fragments for DNA probes (Fig. 1) were isolated on agarose gels, electroeluted in dialysis tubing or spin eluted through polyester fiber, precipitated, and quantified via ethidium bromide staining. One-half to 1 μg of DNA was labeled at 37°C for 30 min in a 30-μl reaction, using 5 U of Klenow fragment (New England Biolabs) and 40 μCi each of [α-^{32}P]dCTP and [α-^{32}P]dGTP. Radiolabeled probe was purified with NucTrap columns (Stratagene). The assay was performed as previously described (22), with the following modifications. Prior to hybridization, samples were denatured at 75°C (PP probe) or 95°C (EB probe). Hybridization was carried out at 55°C. Samples were electrophoresed (between 11.5 and 13 V/cm) on a 1.0-mm-thick denaturing acrylamide gel containing 7 M urea. Gels were fixed for 15 min in a solution containing 10% (vol/vol) methanol and 10% (vol/vol) acetic acid, dried, and exposed to Kodak XAR-5 X-ray film or PhosphorImager (Molecular Dynamics) screens.

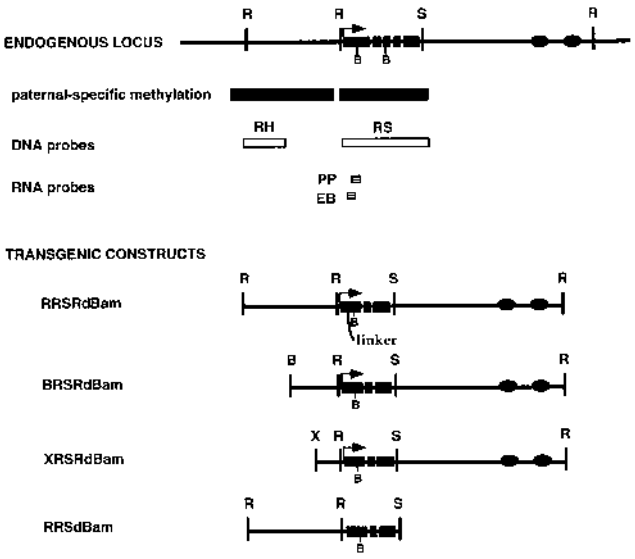

FIG. 1. Endogenous *H19* gene locus and transgenes. The top line corresponds to approximately 20 kb of the endogenous *H19* gene locus. The five-exon endogenous *H19* structural gene, spanning ~2.8 kb, is represented by the five black boxes, and the 3′ endoderm-specific enhancers (38), situated at +8 and +9.5 kb relative to the transcription initiation site (arrow), are indicated by black ovals. The restriction sites, whose positions are used to designate the various constructs, are *Bam*HI (B), *Eco*RI (R), *Sal*I (S), and *Xba*I (X). All transgenic constructs contain a 0.9-kb deletion (referred to as dBam) spanning the 3′ half of exon 1, all of exon 2, and the 5′ half of exon 3, as well as the intervening introns. The RRSRdBam construct carries a linker, inserted into a *Sac*I site at +580 bp, which contains two *Xba*I sites (8). The RRSRdBam, BRSRdBam, and XRSRdBam constructs have 8 kb of 3′ sequence flanking the gene body and 4, 2, and 0.8 kb, respectively, of 5′-flanking sequence. RRSdBam contains 4 kb upstream of the gene body and a truncated gene body with no sequences 3′ to the polyadenylation signal. The black bars under the diagram of the endogenous locus represent the regions of endogenous *H19* that display methylation preferentially on the paternally inherited allele. The gray boxes represent genomic fragments used for probing DNA, and the striped boxes represent the locations of probes used for S1 nuclease analysis (see Materials and Methods).

Methylation analysis. To assess methylation status at the 5′ end of the transgene, each sample of liver DNA was digested twice: once with *Sal*I and *Bam*HI to liberate the 5′ 2.0 kb of the transgene (*Sal*I is located 5′ to the *Eco*RI site in the polylinker) and once with these two enzymes plus *Hpa*II. To assess methylation within the gene body, DNA was digested once with *Pvu*II, to release a 3.4-kb fragment encompassing the *H19* coding region, and once with *Pvu*II and *Hpa*II. Ten micrograms of each DNA sample was digested and run on a 1% agarose gel with 1× TBE (0.089 M Tris-HCl, 0.089 M boric acid, 0.004 M EDTA). DNA was transferred to nitrocellulose via Southern blotting (31) and hybridized to a 1.8-kb *Eco*RI-*Hin*dIII probe from the 5′ end of the transgene or a 3.0-kb gene body probe (RH and RS, respectively [Fig. 1]). After washing (37), filters were exposed to Kodak XAR-5 X-ray film and to PhosphorImager (Molecular Dynamics) screens overnight and scanned.

Quantification of Southern blots and S1 nuclease assays. Fixed and dried gels (S1 nuclease assays) and hybridized filters (Southern blots) were exposed to storage phosphor screens and scanned on a PhosphorImager 445 SI (Molecular Dynamics). Gels were analyzed, and relative band intensities were calculated by using IP Lab Gel H, 1.5e (Signal Analytics). After pseudocolor enhancement of the image, bands of interest were traced by using the freehand drawing tool. The average pixel values of individual segment boundaries were used as the background values. For S1 nuclease assays, the volume of the band representing transgene expression for each sample was normalized to the volume of the band representing endogenous expression. For Southern blots, the intensities of bands digested with a methylation-sensitive enzyme were compared directly to the intensities of undigested bands to determine the percentage of DNA molecules that were fully methylated. Note that the PhosphorImager is sensitive through 5 orders of magnitude.

TABLE 1. Imprinting status of mouse *H19* transgenes

Construct	Line	Copy no.	Expression	Imprinted[a]
RRSRdBam	64-21	2	−	No[c]
	77-5	12	+	Yes
	77-30	29	+	Yes
	382[b]	35	+	Yes
	699[b]	62	+	Yes
XRSRdBam	88-29	6	+	No
	88-25	14	+	No
	88-4	37	+	No
BRSRdBam	89-20	1	+	No
	89-2	7	+	No
	89-36	14	+	No
	89-4	23	+	No
RRSdBam	81-11	1	−	ND[d]
	81-7	5	−	No[c]
	81-1	10	−	No[c]
	81-18	39	−	No[c]

[a] Except where noted (RRSdBam), imprinted refers to the exclusive expression of the maternally derived transgenes.
[b] Previously described by Bartolomei et al. (3).
[c] Imprinting is assayed by determining whether the transgene undergoes parental-specific methylation.
[d] ND, not determined.

Determination of transgene copy number. Ten micrograms of genomic tail or liver DNA was digested to completion with the appropriate enzyme to liberate a fragment which distinguishes the endogenous and transgenic copies (*Xba*I for RRSRdBam lines, *Hin*dIII for RRSdBam lines, and *Pvu*II for BRSRdBam and XRSRdBam lines). The DNA was analyzed as described above except that the 3.0-kb RS probe was used for hybridization.

RESULTS

Imprinted expression of the RRSRdBam transgene. *H19* transgenes were initially derived to study the regulation of the *H19* gene (8). One of these transgenes, RRSRdBam (referred to as RRSRΔBam by Brunkow and Tilghman [8]), is a 14-kb transgene comprised of 4 kb of 5′ upstream sequence, the internally deleted structural gene, and 8 kb of 3′-flanking sequence harboring the two endoderm-specific enhancers (Fig. 1). In the course of these studies, it was determined that two of the RRSRdBam transgenic lines demonstrated a differential expression pattern that was similar to that of the endogenous, imprinted *H19* gene (3). That is, the transgene was expressed when transmitted to the progeny by the mother and repressed when transmitted by the father.

To investigate this original observation further, three additional lines containing approximately 2, 12, and 29 copies of the RRSRdBam transgene were generated, bred to DBA/2J mice, and tested for differential expression and methylation (Table 1). Line 64-21, which has two intact copies, did not express the transgene (data not shown). The absence of expression of this line is most likely a result of the site of integration, since other related low copy transgenes are normally expressed (Table 1).

The two higher-copy-number transgenic lines (77-5 and 77-30) expressed and imprinted their transgenes similarly to the previously described lines (382 and 699 [3]). These transgenic lines were imprinted through at least five successive backcrosses to DBA/2J mice. Figure 2A shows a representative example of the S1 nuclease assay of RNA from hemizygous transgenic progeny of line 77-30, which has 29 copies of the transgene. In this experiment, the analysis of two successive generations of the transgene revealed that the transgene is highly expressed when transmitted to the progeny by the mother and repressed when transmitted to the progeny by the father. By quantifying the endogenous and transgenic S1 products, it was determined that the progeny of N_4 females expressed each transgene copy at the same relative level as the maternal allele of the endogenous gene (Fig. 2A, lanes 1 to 3, and data not shown). In contrast, while the differential expression of this line is obvious in the progeny of the earlier N_3 generation (Fig. 2A, lanes 7 to 10), the maternally derived transgenes appear to be expressed at a lower level. This difference in the relative levels of expression in sequential generations was also observed in line 699 (data not shown) and could result from the inability of high-copy-number lines derived from the RRSRdBam transgene to transcribe each copy of the transgene.

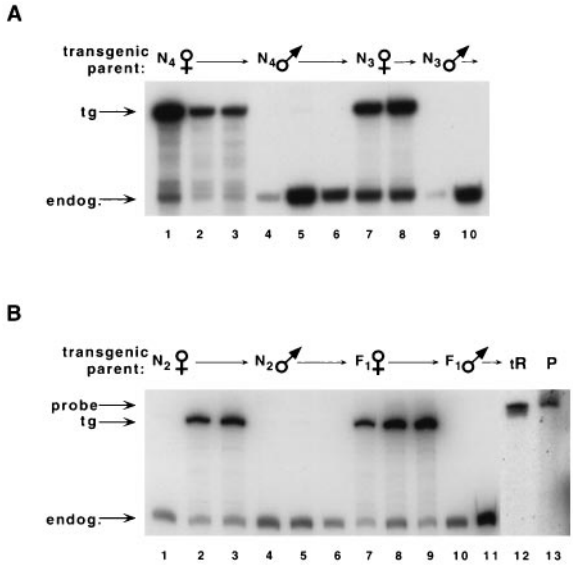

FIG. 2. RRSRdBam transgenic lines display imprinted gene expression. Total liver RNA, isolated from 4- to 7-day-old mice, was analyzed by S1 nuclease assay using the EB probe (Fig. 1). The transgenic progeny of male and female (as indicated above lanes) transgenic mice from lines 77-30 (A) and 77-5 (B) were analyzed for endogenous and transgenic *H19* expression. Endogenous expression (endog.) is used as a loading control, and the level of transgenic expression (tg) was quantified relative to this level (see Materials and Methods for details). The protected transgenic fragment migrates at a position corresponding to 504 bp; the endogenous fragment migrates at 379 bp. Self-hybridized probe (DNA-DNA hybrids) migrates at 561 bp but is largely undetectable under our hybridization conditions unless the probe is incubated without RNA or with tRNA alone (B, lane 12). Lane 13 shows diluted unhybridized probe. (A) Ratios of transgene expression to endogenous gene expression in line 77-30 for progeny of the N_4 female are 24:1 (lane 1), 12:1 (lane 2), 9.4:1 (lane 3), and 28:1, 27:1, and 22:1 (siblings for which data are not shown). Ratios for the progeny of the N_3 female are 3.3:1 (lane 7), 4.5:1 (lane 8), and 4.9:1 (data not shown). (B) The ratio of transgene expression to endogenous gene expression in line 77-5 for the progeny of female transgenic mice is approximately 10:1 (lanes 2, 3, 7, and 8 and data not shown) with the exception of the sample in lane 9, in which the ratio is 31:1.

Line 77-5, which has 12 copies of RRSRdBam, also imprints its transgene, but its behavior is not as consistent as that observed for line 77-30 and the previously defined transgenic lines (3). Figure 2B shows the expression of maternally and paternally derived transgenes for two sequential generations of crosses to DBA/2J mice. In all progeny, the transgene was repressed when inherited from the father (Fig. 2B, lanes 4 to 6, 10, and 11), but the expression of the maternally derived transgene was variable. In some cases, the transgene was expressed and other cases it was not expressed (Fig. 2B, lanes 1 to 3, and data not shown). This behavior was also observed in later generations. While it is not clear why the imprinting of line 77-5 is more variable than that of line 77-30 and the other

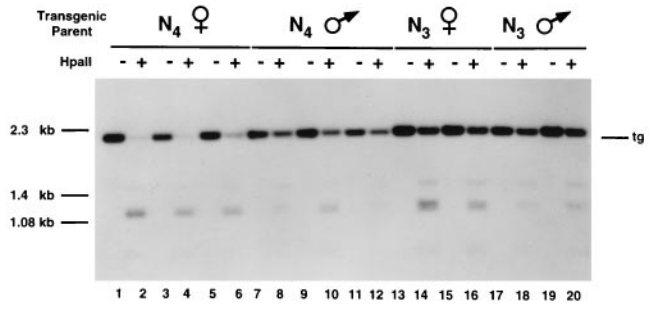

FIG. 3. RRSRdBam transgenic lines are differentially methylated. Genomic DNA from the livers of neonatal mice from line 77-30 was subjected to Southern analysis (31). The DNA was digested with *Sal*I (which digests in the 5′ polylinker of the transgene) and *Bam*HI to liberate a 2-kb 5′ fragment, and even-numbered samples were also digested with the methylation-sensitive restriction enzyme *Hpa*II to assay methylation status of the fragment. The blot was probed with the RH fragment described in Fig. 1. Each transgenic parent's sex and generation are indicated at the top, and molecular sizes are shown at the left. The full-length transgenic band (tg) is indicated at the right. Due to the high copy number of this transgenic line, the endogenous fragment is not visible at this exposure. The relative level of methylation of the transgenes was determined by comparing the intensities of the 2-kb fragment with and without *Hpa*II within each sample. Within a given generation, the maternally transmitted transgenes were always less methylated than the paternally transmitted transgenes.

previously characterized lines 699 and 382, it is possible that the higher copy number of these three lines stabilizes the maternal-specific expression signal.

Methylation analysis of the imprinted RRSRdBam transgenic lines. We analyzed the methylation status of RRSRdBam transgenes to determine if the differentially expressed transgenes exhibited differential methylation, as had been observed for the endogenous *H19* gene (3). Consistent with the endogenous paternal-specific hypermethylation pattern, we previously demonstrated that the transgene expression of line 699 correlated with methylation in that hypermethylation of paternally derived transgenes was linked to decreased expression (3). Figure 3 shows the DNA methylation pattern of the line 77-30 transgenic progeny analyzed for expression in Fig. 2A. While this experiment tests the methylation status of three methylation-sensitive *Hpa*II sites within a 2-kb fragment located at the 5′ end of the transgene, other experiments analyzing *Hpa*II sites within the gene body and upstream *Hha*I sites have shown that the relative levels of methylation are consistent between the 5′-flanking sequence and the gene body (data not shown). The relative amount of *Hpa*II digestion of the 2-kb *Sal*I-*Bam*HI fragment was calculated for each sample shown in Fig. 3. Comparison of the DNA from maternally and paternally derived transgenes (Fig. 3, lanes 1 to 6 and 7 to 12, respectively) revealed that the maternally derived transgenes were less highly methylated and consequently more highly digested by *Hpa*II. Additionally, quantitative analysis of the progeny of N_3 and N_4 females revealed that transgenes from progeny of the N_4 mice (Fig. 3, lanes 1 to 6) were less methylated than those from the earlier generation (Fig. 3, lanes 13 to 16). This result complements the expression pattern observed in Fig. 2A, in which progeny of N_3 females expressed the transgene at a lower level than those from the subsequent generation, and supports the argument that expression and methylation are highly related for the *H19* transgenes and the endogenous gene. Furthermore, there is a greater proportion of completely methylated 2.0-kb *Sal*I-*Bam*HI fragments in the transgenic DNA from the progeny of N_3 females than that from N_4 females. Since hypermethylated transgenic DNA is not likely to be expressed, the reduced expression observed in the progeny of N_3 females probably results from the subset of unmethylated transgenes.

Transgenic progeny from line 77-5 also exhibited a methylation pattern that paralleled their expression pattern (data not shown). *Hpa*II and *Hha*I restriction endonuclease sites from the gene body and upstream region were highly methylated in progeny that did not express the transgene and less methylated in progeny that expressed the transgene. That is, the methylation of the maternally transmitted transgenes was lower than that of the paternally derived transgenes. However, the DNA was also hypermethylated in nonexpressed maternally derived progeny.

The methylation pattern of the nonexpressed line 64-21 was also tested. While lack of expression was surprising, it was still possible that the transgene was differentially methylated because it harbored most of the paternally methylated sequences thought to be essential for imprinting of the endogenous *H19* gene (3). When the DNA from transgenic progeny derived from males and females was tested for methylation differences, none were observed (data not shown). In fact, the transgene exhibited a level of methylation that was intermediate to that observed for the endogenous alleles of *H19*.

5′ deletion analysis of *H19* transgenes. We have previously demonstrated that a 7- to 9-kb region of the endogenous *H19* gene, including the transcription unit and at least 4 kb of 5′ flanking sequence, is methylated on the inactive paternal allele (3). The 5′ portion of this region is differentially methylated in blastocysts and gametes and is a strong candidate for the mark that distinguishes the parental alleles of the *H19* gene (35). Because the RRSRdBam transgene harbors most of these differentially methylated sequences, we generated 5′ deletions in RRSRdBam and derived transgenic lines to test the hypothesis that these sequences are critical for imprinting (Fig. 1). The deletions should only test imprinting and not affect expression potential, since only 250 bp of sequence 5′ to the start of transcription is necessary for liver-specific transcription (38).

The first transgene, BRSRdBam, deletes 2 kb of the 5′ portion of RRSRdBam, leaving 1.8 kb of sequence 5′ to the transcription initiation site. While BRSRdBam still contains 4 kb of DNA shown to be differentially methylated at the endogenous *H19* gene locus, this transgene deletes the differentially methylated sequences most likely to serve as the imprinting mark (35). A second transgene, XRSRdBam, deletes an additional 1 kb of 5′ sequence, including a 460-bp G-rich repeat located between −1745 and −1285 bp relative to the start of transcription (35).

The transgenic lines derived from BRSRdBam and XRSRdBam were backcrossed to DBA/2J, and expression of hemizygous progeny was tested (Table 1). S1 nuclease analysis revealed that founder lines from both deletion transgenes were expressed at a level proportionate to the copy number, indicating that their expression potential was not compromised by the deletions (Fig. 4). The imprinting of the transgenic lines was tested in multiple generations of transgenic progeny. Figures 4A and B show representative samples of the S1 nuclease assay of neonatal liver RNA from six independent lines generated from BRSRdBam and XRSRdBam transgenes, respectively. In all cases tested, the transgene was expressed regardless of whether it was maternally or paternally inherited, indicating that at least part of the signal essential to imprint these transgenes was missing.

The methylation status of the transgenes was also determined. As expected from the high level of expression, both maternally and paternally transmitted BRSRdBam and XRSRdBam transgenes exhibited a low level of methylation that was characteristic of the expressed and hypomethylated

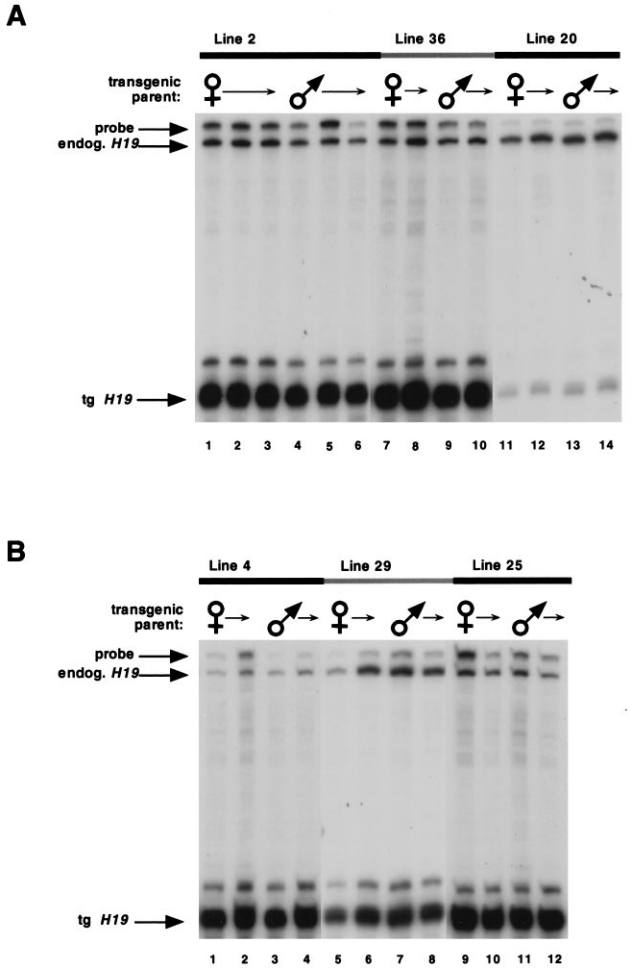

FIG. 4. BRSRdBam and XRSRdBam transgenic lines are expressed regardless of parental origin. Total liver RNA, isolated from 4- to 7-day-old mice, was analyzed by S1 nuclease assay using the PP probe (Fig. 1). The transgenic progeny of male and female (as indicated above the lanes) N_2 generation transgenic mice from three BRSRdBam (A) and XRSRdBam (B) lines were analyzed for endogenous and transgenic *H19* expression. The same expression pattern was observed for progeny of N_1 through N_3 transgenic animals. Endogenous expression (endog.) is used as a loading control and migrates at a position corresponding to 343 bp. "probe" indicates a self-hybridized probe (DNA-DNA hybrids) and migrates at 397 bp. The transgenic (tg) band migrates at 119 bp.

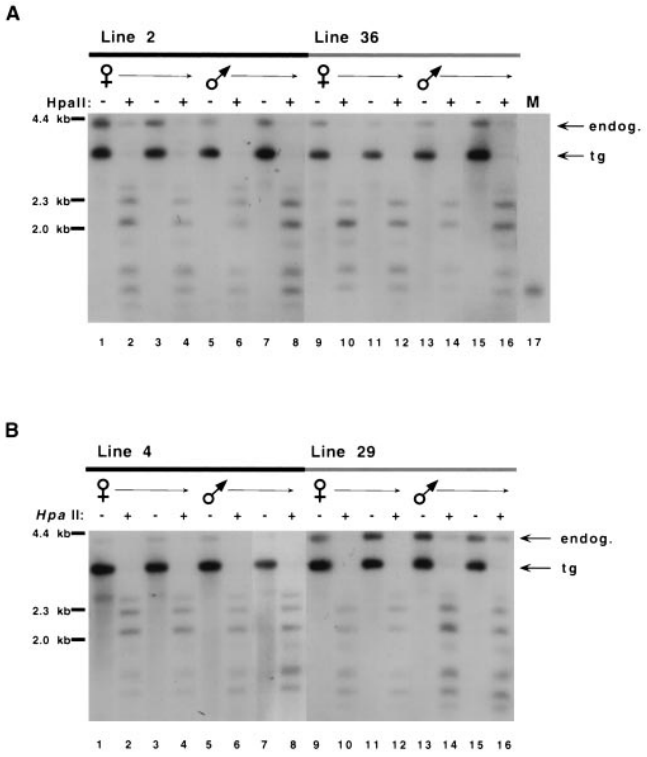

FIG. 5. Maternally and paternally transmitted BRSRdBam and XRSRdBam transgenes demonstrate low but not differential levels of methylation. Genomic DNA, isolated from the livers of 4- to 7-day-old mice, was analyzed by Southern blotting for methylation within the gene body (31). The transgenic progeny of male and female (as indicated above the lanes) transgenic animals from various BRSRdBam (A) and XRSRdBam (B) lines were examined. The progeny of N_2 transgenic mice were assayed for all lines except BRSRdBam line 36, in which the progeny were derived from N_3 transgenic mice. Each DNA sample was digested with *Pvu*II alone (odd-numbered lanes except lane 17 in panel A) or *Pvu*II and *Hpa*II (even-numbered lanes). Lane 17 in panel A shows a complete digestion with *Pvu*II and *Msp*I (M). The uppermost, endogenous bands (endog.) are used as a loading control and as a comparison to determine copy number in the transgenic lines; "tg" indicates location of the *Pvu*II-digested transgenic product. Molecular size markers are indicated at the left. The blot was probed with RS (Fig. 1).

endogenous maternal allele of the *H19* gene (Fig. 5). Thus, it appears that the 1.8- and 2.8-kb deletions eliminated a signal important for conferring the paternal-specific pattern of methylation and expression.

Methylation analysis of lines derived from an enhancerless *H19* transgene. To determine whether the differentially methylated sequences alone are sufficient to confer a parental-specific methylation pattern, we constructed a transgene harboring most of the differentially methylated region. This transgene (RRSdBam [Fig. 1]) includes the 4-kb 5′-flanking sequence and the 2-kb internally deleted structural gene but does not contain the 8-kb 3′-flanking *Sal*I-*Eco*RI fragment where the two endoderm-specific enhancers are located (38). Most importantly, the 2-kb region characterized as serving as the strongest candidate for the imprinting mark is included in this transgene (35). Because RRSdBam lacks the enhancers and is not likely to be expressed, this transgene could also assess whether expression of *H19* is necessary for differential methylation. If the expression of the transgene is not required in *cis* and there are no other sequences important to conferring the imprint in the 3′ *Sal*I-*Eco*RI fragment, then the transgenic RRSdBam lines should exhibit parental-specific methylation of the transgene.

Four independent RRSdBam transgenic lines were tested for expression and differential methylation (Table 1). As expected from the absence of the enhancers, the transgene was not expressed in any of the lines (data not shown). Three of the lines (81-7, 81-1, and 81-18) were also tested for parental-specific methylation differences, and none were observed. The results for one of the lines, 81-7, is presented in Fig. 6. The methylation state of the 5′ transgenic DNA from neonatal livers was tested by digestion with *Sal*I and *Bam*HI and the methylation-sensitive enzyme *Hpa*II (Fig. 6, even-numbered lanes). Compared to DNA that was digested with *Sal*I, *Bam*HI, and the methylation-insensitive enzyme *Msp*I (which digests at the same recognition sequence as *Hpa*II, regardless of methylation), both maternally and paternally transmitted transgenes were similarly hypomethylated. This surprising methylation pattern is similar to that of the expressed maternal endogenous *H19* allele and indicates that DNA sequences that are impor-

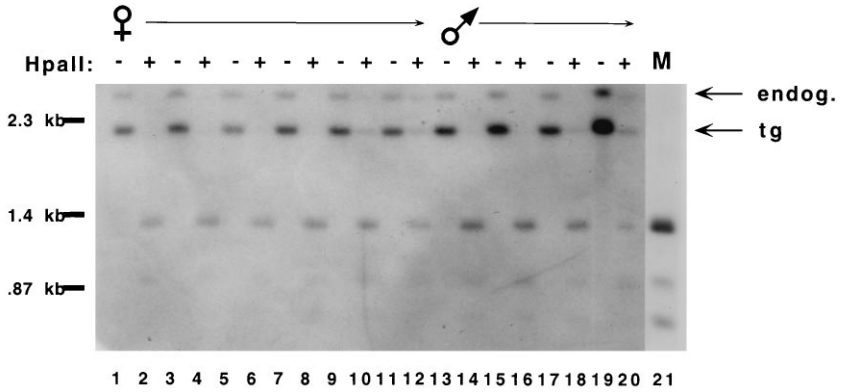

FIG. 6. RRSdBam transgenic lines display low levels of transgene methylation, regardless of parental origin. Genomic DNA, isolated from the livers of 4- to 7-day-old mice, was analyzed by Southern blotting for methylation at the 5′ end of the transgene (31). Transgenic progeny of male and female N_3 transgenic mice from line 81-7 were examined. Each DNA sample was digested with *Sal*I and *Bam*HI alone (odd-numbered lanes except lane 21) or with *Sal*I, *Bam*HI, and *Hpa*II (even-numbered lanes). Lane 21 shows a complete digestion with *Sal*I, *Bam*HI, and *Msp*I (M). The uppermost, endogenous band (endog.) is used as a loading control; "tg" indicates the location of the transgenic *Sal*I-*Bam*HI fragment. Molecular size markers are indicated at the left. The blot was probed with RH (Fig. 1).

tant for the typical endogenous paternal hypermethylated pattern are missing from the RRSdBam transgene. Hence, expression of either the *H19* gene itself or an element in the 8-kb 3′ DNA fragment (or both) is required for the differential as well as the paternal-specific methylation pattern. Furthermore, the hypomethylation of the nonexpressed RRSdBam transgene is contrary to the widely held belief that methylation is the consequence of gene inactivity and that a gene that is not expressed will be hypermethylated. This result may indicate that in the case of the *H19* gene, methylation is the cause, and not the consequence, of the repression of the paternal allele.

Methylation analysis of the transgenes in sperm and testes. The hypermethylation of the endogenous paternal allele of *H19* that is observed in somatic tissues is largely derived from the male gametes (3, 35). To determine whether *H19* transgenic DNA is methylated during male gametogenesis and whether the methylation reflects the subsequent pattern of transgene expression, we analyzed sperm and testis samples from a representative sample of the transgenic lines. As shown in Fig. 7, the sperm and testis transgenic DNA is hypermethylated. However, the sperm DNA from the imprinted RRSRdBam line 77-30 is more highly methylated than the sperm DNA from RRSdBam lines in which the paternally derived transgene is neither expressed nor hypermethylated in somatic tissues (Fig. 7A; compare lane 3 to lanes 1 and 2). Furthermore, while the sperm DNA from XRSRdBam and BRSRdBam is more highly methylated than the corresponding liver samples from paternally derived transgenes, the transgene is not completely methylated (Fig. 7B). Thus, unlike the endogenous *H19* gene and the imprinted transgenic lines, the sequences present in the RRSdBam, BRSRdBam, and XRSRdBam transgenes can lead to only partial methylation. The partial methylation is apparently not maintained since the DNA from paternally derived neonatal mice is demethylated. These experiments lead to the conclusion that the unimprinted transgenes can neither fully establish nor maintain the paternal allelic mark.

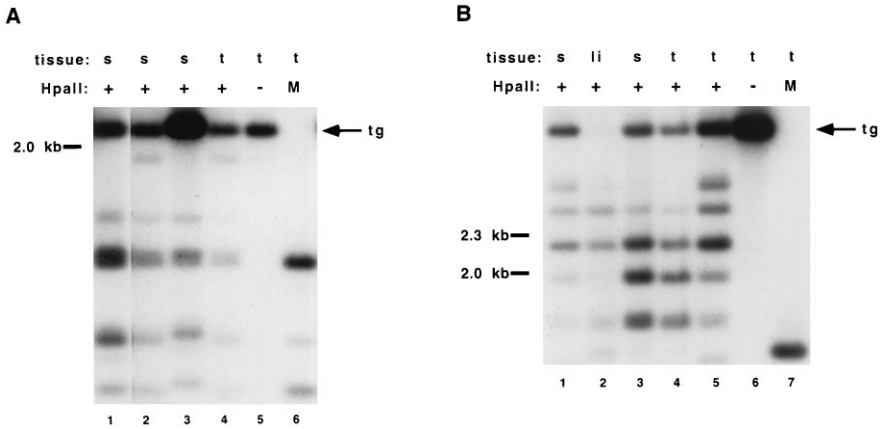

FIG. 7. Transgenic sperm DNA is hypermethylated. Genomic DNA was isolated from sperm and testes of adult males and analyzed by Southern blotting for methylation at the 5′ end (A) and within the transcription unit (B) (31). (A) For 5′ analysis, the DNA was digested with *Sal*I, *Bam*HI, and *Hpa*II (lanes 1 to 4), *Sal*I, *Bam*HI, and *Msp*I (M; lane 6), or *Sal*I and *Bam*HI (lane 5), and the blot was probed with RH (Fig. 1). The samples are as follows: sperm DNA (s) from RRSdBam 81-1 (F_1 mice; lane 1), RRSdBam 81-7 (N_3 mice; lane 2), and RRSRdBam 77-30 (N_5 mice; lane 3) and testis DNA (t) from RRSdBam 81-7 (N_3 mice; lanes 4 to 6). The gel was exposed half the length of time for lane 1 as for lanes 2 to 6. (B) For analysis of the transcription unit, the DNA was digested with *Pvu*II and *Hpa*II (lanes 1 to 5), *Pvu*II alone (lane 6), and *Pvu*II and *Msp*I (lane 7), and the blot was probed with RS (Fig. 1). The samples are as follows: sperm DNA from XRSRdBam line 88-4 (N_2 mice; lane 1) and BRSRdBam line 89-36 (N_3 mice; lane 3), liver (li) and testis DNA from a paternally derived BRSRdBam line 89-36 transgenic male (N_3 mice; lanes 2 and 4, respectively), and testis DNA from XRSRdBam line 88-4 (N_2 mice; lanes 5 to 7). Note that less than 10 μg of DNA is digested in lane 1. "tg" corresponds to the full-length transgenic band, and molecular size markers are indicated at the left.

DISCUSSION

The RRSRdBam transgene mimics endogenous *H19* gene expression. We have reported that the RRSRdBam transgene is imprinted similarly to the endogenous *H19* gene. This is the first example of a transgene mimicking the expression pattern of its corresponding endogenous gene (3). Therefore, this 14-kb transgene, which includes a 4-kb 5′ flanking region, a 2-kb internally deleted structural gene, and the 8-kb 3′ flanking region where the endoderm-specific enhancers are located, contains the minimal elements required for imprinting. These results are consistent with our expectations, since the transgene harbors most of the differentially methylated sequences proposed to be important for *H19* gene imprinting (3, 6, 16).

However, it is clear that the RRSRdBam transgene does not have all of the elements necessary to recapitulate precisely endogenous *H19* imprinting. For example, lines 77-30, 382, and 699, which all have more than 25 copies of the transgene, exhibit imprinted expression and methylation patterns, but the level of the maternal expression varies according to the generation. In contrast, line 77-5, which has approximately 12 copies of the transgene and is also imprinted, has occasional female-derived progeny that do not express the transgene. Repression of the transgene and hypermethylation were observed for the vast majority of paternally derived progeny in the four transgenic lines. Thus, it appears that sequences necessary to repress the transgene are present in RRSRdBam, whereas all of the sequences necessary to activate the transgene may not be present. It is possible that high-copy-number transgenic mice can activate some copies of the maternally derived transgene due to the presence of multiple copies of a subset of activating elements but more than one passage through the female germ line is required to activate each copy of the transgene. To test the hypothesis that positive regulatory elements are absent from the RRSRdBam transgene, we are presently generating transgenic mice by using a transgene containing an additional 2 kb of 5′-flanking sequence. This new transgene has all of the differentially methylated DNA proximal to the *H19* gene. If this new construct harbors the complete set of positive and negative elements that are crucial for imprinting, then we expect that all transgenic lines, regardless of copy number, will be appropriately imprinted.

The upstream differentially methylated region is required for imprinting the *H19* transgenes. The methylation of the cytosine residue in CpG dinucleotides has traditionally been the most attractive candidate for the signal that confers parental identity to the alleles of imprinted genes (25). The attractiveness of this epigenetic modification derives in part from the presumed ease by which methylation may be erased and reestablished in the gametes when the genes pass from one generation to the next, a key quality if methylation is to serve as the imprint. Furthermore, the association of hypermethylation with gene repression favors methylation as the imprint, since this modification could additionally serve as the repressor of transcription on the inactive allele. Importantly, in order for methylation to serve as the mark that designates parental identity, the methylation must be differential in gametes, as this is the only time when the parental alleles are in distinct compartments and can be independently modified, and the methylation must be maintained throughout development. Maintenance is especially critical during preimplantation development, since the embryo undergoes a period of generalized demethylation at this time (23, 27).

Although the exact role of methylation in imprinting remains to be established, all of the imprinted genes that have been examined exhibit a region of parental-specific methylation. The *H19* gene fulfills many of the criteria for methylation serving as the imprinting mark. We and others have defined a 7- to 9-kb region of hypermethylation encompassing the transcription unit and approximately 4 kb of 5′-flanking sequence on the inactive paternal allele (3, 6, 16). While the part of the gene encoding the transcript was not differentially methylated in preimplantation embryos (6), the 4-kb 5′-flanking region had a number of CpG dinucleotides that were differentially methylated (35). The 5′ 2-kb region, in particular, harbors several CpG dinucleotides which satisfy the criteria for methylation serving as the imprinting mark (35).

It is important to note, however, that in some cases, such as the genes encoding the insulin-like growth factor type 2 and its receptor (*Igf2* and *Igf2r*, respectively), the active alleles are hypermethylated relative to the inactive alleles (6, 15, 32). In these cases, methylation could act by preventing the binding of a repressor on the active allele (32).

One of the most provocative experiments supporting the role of DNA methylation in imprinting is the expression analysis of imprinted genes in mice lacking DNA methyltransferase, an enzyme essential to the maintenance of cytosine methylation (4, 5). Absence of the gene encoding this enzyme causes death of mouse embryos at around 10 days of gestation (21). Prior to their death, allelic expression of *H19*, *Igf2*, and *Igf2r* was examined. *H19*, which is normally methylated on the inactive allele, was biallelically expressed, and *Igf2* and *Igf2r*, which are methylated on the active allele, were not expressed (20). While these experiments cannot distinguish between the role of methylation in setting versus maintaining the imprint, it is clear that methylation is essential to the monoallelic expression of these genes.

The RRSRdBam transgene characterized in this study as well as in a previous study (3) contains most of the region of differential methylation found at the endogenous locus, and consistent with expectations, this transgene was appropriately imprinted and differentially methylated. In the present analysis, we deleted the portion of the gene found to exhibit the most striking pattern of differential methylation. The 5′ portion of the BRSRdBam transgene contains only the proximal 2 kb of the 5′-flanking sequence. As predicted from the methylation analysis of the endogenous locus, BRSRdBam transgenic lines expressed the transgene regardless of parental origin. These transgenes also exhibited the maternal pattern of DNA hypomethylation. Thus, we conclude that a sequence important for conferring the paternal-specific pattern of repression is missing in this construct as well as in the XRSRdBam construct. We propose that the methylation of CpG dinucleotides located within the 2-kb deleted region is crucial to establishing and/or maintaining the *H19* transgenic imprint.

The 3′ region of *H19*, which harbors the endodermal enhancers, is required for *H19* transgene imprinting. This study demonstrates that removal of the 8 kb 3′-flanking region of the *H19* gene harboring the endodermal enhancers results in the loss of expression of the transgene, with a concomitant loss of imprinting and methylation. There are two possible reasons for these results. First, expression of the *H19* gene product is essential for imprinting, and second, DNA sequences in the 8-kb region act positively to confer the imprint. Although the second possibility is equally likely, there is presently no compelling evidence suggesting that imprinting sequences reside 3′ to the *H19* transcription unit. Methylation analysis of the 15 kb of sequence located 3′ of the polyadenylation signal revealed that this region was equally methylated on the maternal and paternal alleles (3). Furthermore, we and others analyzed the chromatin structure 3′ to the *H19* gene and identified no parental-specific DNA hypersensitivity (3, 16). To determine

whether any of these 3′-flanking sequences can confer imprinted expression, we are generating new transgenes harboring various portions of the 8-kb flanking region.

The alternative hypothesis suggests that the expression of the *H19* gene product is required for its own imprinting. The functional product of the *H19* gene is the RNA itself (7). While the mechanism of action of the RNA is not absolutely clear, absence of transcription of the *H19* gene at its endogenous locus caused the deregulation of the linked and oppositely imprinted *Igf2* gene (18). One possible interpretation of this experiment is that the *H19* RNA transcribed from the maternal allele acts in *cis* to prevent the transcription of the closely linked *Igf2* allele (18). Therefore, absence of maternally transcribed *H19* RNA led to the expression of the maternal allele of *Igf2*. It is also possible that transcription of the *H19* gene is essential to the establishment or maintenance of its own imprinting. Since the endogenous allele of *H19* was transcribed in all of the transgenic lines, the transgene product must be required in *cis*. It should be noted, however, that the RRSRdBam construct encodes an internally deleted transcript. Hence, a transgene lacking the part of the transcript encoded by the 3′ half of exon 1, exon 2, and the 5′ half of exon 3 was appropriately imprinted and, if required in *cis* for imprinting, was still active in that capacity.

Additional evidence supporting the role of the *H19* RNA in imprinting comes from studies using transgenes in which the *H19* gene is replaced with the luciferase gene (24). These transgenes contain the same 5′- and 3′-flanking sequences as RRSRdBam and exhibited the similar tissue and temporal expression patterns. However, the luciferase RNA was expressed regardless of parental origin, and the transgene was unmethylated. While the luciferase transgene experiments cannot distinguish between the possibilities that the loss of imprinting is caused by the absence of the RNA and by the absence of the DNA sequences encoding the RNA, these results, together with the RRSdBam results, strongly implicate the RNA as an important imprinting element. However, this hypothesis presents a potential problem in that the RNA is required in *cis* for silencing of the paternal allele to occur. Although we have yet to detect transcription, under normal conditions, of the paternal allele of the endogenous *H19* gene, it is possible that we have not looked at the critical time or with an appropriately sensitive assay. The role of the *H19* RNA in its own imprinting will be further assessed with the derivation of RRSR transgenes containing subtle mutations that disrupt transcription or mice harboring subtle mutations at the endogenous locus which perturb transcription.

Perhaps the strongest evidence indicating that the *H19* RNA may not be required for its own imprinting or differential methylation comes from experiments in which a 6.2-kb deletion including the two endoderm-specific enhancers was introduced at the endogenous *H19* gene locus (19). When the enhancers were deleted on the maternal allele, *H19* expression was absent in tissues of endodermal origin. However, the upstream sequences, which were not affected by the deletion, maintained their pattern of differential methylation (19). While these results suggest that the *H19* RNA is not important to confer differential methylation, it is difficult to compare the results at the endogenous locus with those of a transgene. For example, in the absence of the endoderm-specific enhancers, other uncharacterized control elements may be driving expression of *H19* at the time and place where the methylation imprint is conferred.

In conclusion, we have generated a transgenic mouse model which mimics the imprinting pattern of the endogenous *H19* gene. The requirement for multiple copies of the 14-kb RRSRdBam transgene may indicate that additional sequences are necessary for low-copy-number or single-copy lines to recapitulate the endogenous imprinting pattern. In the absence of these critical sequences, multiple copies of the sequences present in the 5′ part of the transgene may simulate the imprinting domain present at the endogenous locus. We have also demonstrated that two additional elements, the 5′ differentially methylated domain and the 3′-flanking region, are required for imprinting. The requirement for the 3′-flanking region could result from the necessity of the transgene to be expressed or a DNA sequence located in the region. Thus, the imprinting of the *H19* gene relies on a complex set of elements which will be further refined in future experiments using transgenic mice.

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