Shared Role for Differentially Methylated Domains of Imprinted Genes

Bonnie Reinhart,¹ Mariam Eljanne,^{2†} and J. Richard Chaillet^{3*}

Department of Biological Sciences,¹ Department of Human Genetics,² and Department of Pediatrics,³
University of Pittsburgh, Pittsburgh, Pennsylvania 15213

Received 26 September 2001/Returned for modification 2 November 2001/Accepted 4 January 2002

For most imprinted genes, a difference in expression between the maternal and paternal alleles is associated with a corresponding difference in DNA methylation that is localized to a differentially methylated domain (DMD). Removal of a gene's DMD leads to a loss of imprinting. These observations suggest that DMDs have a determinative role in genomic imprinting. To examine this possibility, we introduced sequences from the DMDs of the imprinted *Igf2r*, *H19*, and *Snrpn* genes into a nonimprinted derivative of the normally imprinted *RSVlgmyc* transgene, created by excising its own DMD. Hybrid transgenes with sequences from the *Igf2r* DMD2 were consistently imprinted, with the maternal allele being more methylated than the paternal allele. Only the repeated sequences within DMD2 were required for imprinting these transgenes. Hybrid transgenes containing *H19* and *Snrpn* DMD sequences and ones containing sequences from the long terminal repeat of a murine intracisternal A particle retrotransposon were not imprinted. The *Igf2r* hybrid transgenes are comprised entirely of mouse genomic DNA and behave as endogenous imprinted genes in inbred wild-type and mutant mouse strains. These types of hybrid transgenes can be used to elucidate the functions of DMD sequences in genomic imprinting.

At imprinted loci in mammalian species, the maternal and paternal alleles of a gene are distinguished from one another by different epigenetic modifications, genomic imprints, which are established during oogenesis and spermatogenesis. The transmission of these imprints is essential for normal embryonic development and leads to monoallelic gene expression in the embryo and adult (2, 11). The epigenetic modification that distinguishes the maternal and paternal alleles of most, if not all, imprinted genes is DNA cytosine methylation (10, 11, 23). The parental alleles have different levels of DNA methylation, usually concentrated in a single location within or surrounding the gene. A genomic region with this epigenetic feature is generally 1 to 5 kb in size and is called a differentially methylated domain (DMD). Within a DMD, one parental allele is highly methylated on the majority of CpG dinucleotides, and the opposite parental allele is unmethylated or methylated on a small percentage of CpG dinucleotides.

The methylation patterns of several DMDs have been extensively studied, including DMD2 of the insulin-like growth factor type 2 receptor (*Igf2r*) gene, the DMD of the *Snrpn* gene, and the DMD of the *H19* gene. DMD2 is found in the second intron of *Igf2r*, is approximately 3 kb in size, and contains 28 CpG dinucleotides (27). The DMD2 CpGs are methylated on the maternal allele and unmethylated on the paternal allele. The DMD of *Snrpn* includes promoter sequences, the entire first exon, and the first intron of the gene and is approximately 6 kb in size (6, 24). Like DMD2 of *Igf2r*, the DMD of *Snrpn* is highly methylated on the maternal allele and unmethylated on the paternal allele (6, 24). These differences in meth-

ylation are established during gametogenesis; the DMDs of *Igf2r* and *Snrpn* are highly methylated in oocytes and unmethylated in sperm (7, 24). In contrast, the DMD of *H19* is approximately 2 kb in size, is found 5' of promoter sequences, and acquires extensive CpG methylation exclusively during spermatogenesis (30). Following fusion of the gametes at conception, the maternal *Igf2r* and *Snrpn* methylation patterns and the paternal *H19* methylation pattern are maintained in the embryo. Many other imprinted genes have strict parent-of-origin differences in methylation, concentrated in small DMDs (2, 22), suggesting that there is an essential role for the DMDs in genomic imprinting.

Deletion of DMD sequences from an endogenous imprinted locus removes all evidence of allelic differences between the maternal and paternal alleles (4, 29, 33, 34). A 300-kb transgene containing the entire 93-kb *Igf2r* locus is imprinted if DMD2 is present, but not imprinted if it is removed (32). However, smaller transgenes comprised only of DMD2 sequences are not imprinted. Likewise, 130-kb transgenes, which contain both the *Igf2* and *H19* genes, are imprinted (1). However, small transgenes containing the entire *H19* DMD plus immediately surrounding sequences are not consistently imprinted (construct XXRsdBam) (12). There are no reports of transgenes made solely of *H19* DMD sequences. These observations indicate that sequences outside of DMDs are also required for gene imprinting. Therefore, the elements required for imprinting these transgenes, the imprint control elements (ICEs), most likely consist of the DMD sequences and unidentified sequence elements that are found at a distance from the DMD. The mechanism by which the ICEs function to imprint the gene is not known.

RSVlgmyc is an imprinted mouse transgene of approximately 17 kb, comprised of sequences from nonimprinted sources, that is imprinted at all chromosomal integration sites.

* Corresponding author. Mailing address: Department of Pediatrics, University of Pittsburgh, Pittsburgh, PA 15213. Phone: (412) 692-7993. Fax: (412) 692-6096. E-mail: Chaillet@pitt.edu.

† Present address: Cytoc Corporation, Boxborough, MA 01719.

All independently derived transgenic lines have a highly methylated maternal allele and an undermethylated paternal allele (9). Like endogenous imprinted genes, *RSVlgmyc* undergoes allele-specific changes in DNA methylation during development and expresses a gene from only one parental allele (10, 28). The absence of a single required *RSVlgmyc* sequence element for its imprinting suggests a functional redundancy for the ICEs within the *RSVlgmyc* transgene (16). We show here that the DMD of *RSVlgmyc* is the region that contains *pBR322* and Rous sarcoma virus (RSV) sequences. Like the DMDs of the larger *Igf2r* transgenes, the DMD of *RSVlgmyc* alone is not imprinted, and removal of DMD sequences from the *RSVlgmyc* transgene results in a nonimprinted transgene. To examine the possibility that DMDs from different imprinted genes have a shared imprinting function, we introduced DMD sequences from these genes into the nonimprinted version of *RSVlgmyc*. Transgenes containing sequences from the *Igf2r* DMD2 were imprinted, with the maternal allele being more methylated than the paternal allele. Importantly, imprinted *Igf2r* transgenes containing only the direct repeats of DMD2 exhibit the same behavior as endogenous imprinted genes in different inbred and mutant mouse strains.

MATERIALS AND METHODS

DNA isolation and Southern blotting. DNA was isolated from the tail of adult mice or entire embryos by proteinase K (Roche) digestion, followed by phenol-chloroform extraction and ethanol precipitation. DNA samples were resuspended in TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). DNA was digested at the appropriate temperature with restriction endonucleases (10 U/ μ g of DNA), electrophoresed on an agarose gel, and transferred to GeneScreen nylon filters (NEN Research Products, Boston, Mass.) by the method of Southern (26). Filters were hybridized with one of four probes at 42°C in 40% formamide and washed in a mixture containing 0.1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate at 65°C. The α probe is a 1.75-kb *EcoRI-XbaI* fragment of α . Probe a is a 0.6-kb *PstI-BamHI* fragment from the 3' region of *c-myc*, and probe b is a 1.3-kb *PvuII-XhoI* fragment of *c-myc* exon 3. The RSV probe is a 0.44-kb fragment from the long terminal repeat (LTR) region of RSV (9).

Mice. All transgenic mice were created in an inbred FVB/N genetic background by pronuclear injection (21). Transgenic lines were established from these founders, and imprinting characteristics of the lines were evaluated by passage through the maternal and paternal germ lines.

Transgene DNA constructs. DNA fragments injected into mouse zygotes for the creation of transgenic mice were derivatives of the *RSVlgmyc* construct (28). All constructs were created by placing plasmid vector sequences (pKS+; Stratagene) into the unique *KpnI* site of *RSVlgmyc* or into a unique *NotI* site created by inserting a *NotI* linker at the *KpnI* site. DMD and intracisternal A particle (IAP) sequences were inserted at the unique *EcoRI* site generated by the removal of the *pBR322/RSV* region. The inserted sequences for *IAPlgmyc*, *Igf2rIgmyc*, *H19SIgmyc*, *SnrpnIgmyc*, *TR1Igmyc*, and *TR2+3Igmyc* transgenes were derived by PCR amplification of genomic DNA with oligonucleotide primers designed to introduce flanking *EcoRI* sites. The *H19LIgmyc* construct was created with an *H19 HindIII* fragment (2,232 bp, accession no. U19619; nucleotides [nt] 942 to 3174); the *HindIII* and *EcoRI* sites were filled in and destroyed to subclone the *H19* fragment into *Igmyc*. The constructs were linearized prior to pronuclear injection by removal of all pKS+ sequences by *KpnI* or *NotI* digestion and gel isolation.

PCR amplification. PCR amplification of each transgene insert was conducted under the following reaction conditions: 0.5 μ g of mouse genomic DNA (except IAP, which used 10 ng of a plasmid containing the 5' LTR from the *A^{iap}* allele) (18), 1 $\times$ PCR buffer (Gibco/BRL), 3 mM $MgCl_2$ (Gibco/BRL), 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.4 μ M each primer, and 1.25 U of *Taq* DNA polymerase (Gibco/BRL) in a 50- μ l reaction mixture. The cycling conditions were as follows: 94°C for 3 min; 30 cycles (25 cycles for IAP) of 94°C for 1 min, 65°C for 30 s, and 72°C for 1.5 min; and a final extension at 72°C for 10 min.

PCR primers. The following PCR primers were used in this study: *Igf2rIgmyc*, IGR2R-C (TCCGAATTCCTAGTGGGCACCTTCATTGTCATG) and

IGF2R-D (CGTGAATTCGATTTAGCACAACTCCAATTGTGTC); *H19SIgmyc*, H19-C (GCAGAATTCGCAAGGAGACCATGCCTATTCTTGG) and H19-D (GCAGAATTCCTCATGAAGCCCATGACTATGGG); *SnrpnIgmyc*, SNRPN-A (TATGGAATTCGATATAGCCTAGAAACCAG) and SNRPN-B (AAAAT TCCAAATCTAGAAATGTTTGGTC); *IAPlgmyc*, U5IAP (CGAGAATTCCTG TTATTCGACGCGTTCTCACG) and U3IAP (GTGGGAATTCGCCCCAC ATT); *TR1Igmyc*, IGF2R-C and TR1-R (AGGGAATTCGTGATCAGGGCCAA CGC); and *TR2+3Igmyc*, IGF2R-D and TR2+3-F (CCTGAATTCAGAACCC TTCGAATCC).

RESULTS

Transgenes derived from *RSVlgmyc*. The experiments described here examine the parent-specific methylation of a number of mouse transgenes. All were derived from the imprinted *RSVlgmyc* transgene. The *RSVlgmyc* plasmid was made by combining two *EcoRI* DNA fragments: one was a 15-kb mouse genomic fragment from a murine translocation breakpoint (*Ig/myc*), and the other was a 2.5-kb fragment containing *pBR322* and RSV LTR sequences (*pBR/RSV*) (9). *Ig/myc* and *pBR/RSV* mouse transgenic lines were created and studied. As well, a number of mouse transgenes were made by combining *Ig/myc* sequences with other mouse genomic sequences, either from the DMDs of endogenous imprinted genes or from an IAP LTR. The imprinting of each transgene was studied by comparing DNA methylation patterns of the maternal and paternal alleles. Allelic differences in expression were not examined, because in the absence of RSV sequences, most of the hybrid transgenes would not be transcriptionally active (9, 16).

The DMD of *RSVlgmyc*. Assuming *RSVlgmyc* and endogenous imprinted genes share a common imprinting mechanism, the DMD of *RSVlgmyc* should be a required imprinting element. To define *RSVlgmyc*'s DMD, a series of Southern blots were performed with DNA from carriers of an *RSVlgmyc* transgene. Details of the structure of *RSVlgmyc*, including the location of methylation-sensitive restriction sites and hybridization probes used in Southern blots, are shown in Fig. 1A. Using double digests of *EcoRI* plus a methylation-sensitive restriction endonuclease, we showed that CpG dinucleotides within *HpaII*, *HhaI*, and *BstUI* sites (recognition sequences CCGG, GCGC, and CGCG, respectively) of *pBR/RSV* are methylated on the maternal *RSVlgmyc* allele, but unmethylated on the paternal allele (Fig. 1B). In contrast, the extent of methylation difference between the maternal and paternal alleles within the adjacent *IgA* and *c-myc* sequences is negligible. *IgA* sequences (defined by an *EcoRI-BglII* digest) are heavily methylated on both the maternal and paternal alleles (Fig. 1C). The CpG island sequences of *c-myc*, located within intron 1, have previously been shown by a *HpaII* digest to be unmethylated on both parental alleles (16). *HhaI* and *BstUI* sites in the CpG island (between the *BglII* and *XbaI* sites) were also unmethylated on both the maternal and paternal alleles (data not shown). From this analysis, *pBR/RSV*, clearly delineated by *EcoRI* sites, is the only element within *RSVlgmyc* in which one parental allele (maternal) is highly methylated and the other allele (paternal) is unmethylated. Therefore, *pBR/RSV* is the DMD of *RSVlgmyc*.

Requirement of the *RSVlgmyc* DMD for transgene imprinting. Removal of DMD sequences from imprinted *Igf2r* transgenes abolishes their imprinting (32). Correspondingly, removal of *pBR/RSV* from *RSVlgmyc* should negate its imprinting.

FIG. 1. The DMD of *RSVIgmyc*. (A) Schematic of the *RSVIgmyc* transgene linearized at the unique *KpnI* restriction site. $C\alpha$ and RSV hybridization probes for Southern blots are shown as thick black lines. *MspI*, *HhaI*, and *BstUI* methylation-sensitive restriction sites are shown as vertical lines. The boxed numbers 1, 2, and 3 designate the exons of the *c-myc* gene, and 3' myc indicates the noncoding genomic sequence of *c-myc*. pBR/RSV refers to *pBR322* vector sequences and RSV LTR sequences. *IgA* indicates coding sequences and switch recombination sequences of *IgA*. E, *EcoRI*; B, *BglII*; X, *XbaI*. (B) Southern blot of DNA samples from maternal (M) and paternal (P) hemizygous carriers hybridized with the RSV probe. DNA samples were digested with *EcoRI* to isolate the pBR/RSV region, followed by digestion with either *HpaII*, *HhaI*, *BstUI*, or *MspI*. A 2.5-kb hybridization band indicates methylation of all of the relevant methylation-sensitive restriction sites. (C) Southern blot of DNA samples from maternal (M) and paternal (P) hemizygous carriers hybridized with the $C\alpha$ probe. DNAs were digested with *EcoRI* and *BglII* to isolate the *IgA* region immediately adjacent to the pBR/RSV region, followed by digestion with *HpaII*, *HhaI*, *BstUI*, or *MspI*. The $C\alpha$ hybridization probe recognizes a methylated 3.3-kb *EcoRI*-*BglII* transgene fragment and an 8-kb endogenous band. All DNA samples were obtained from tail biopsies performed at the time of weaning (3 to 4 weeks).

However, it was previously shown that removal of *pBR322* alone or removal of *RSV* alone from *RSVIgmyc* does not disrupt its imprinting (9). To address the requirement of the entire DMD (*pBR/RSV*) for *RSVIgmyc* imprinting, we created three *Ig/myc* transgenic lines by using a transgene from which the *pBR/RSV* segment of *RSVIgmyc* was excised (Fig. 2A). For each line, the maternal allele's methylation pattern was compared to that of the paternal allele. The results for one line are shown in Fig. 2B to D. Genomic DNAs from two hemizygous transgenic carriers, one with a maternal *Ig/myc* allele and one with a paternal allele, were digested with the methylation-sensitive enzyme *HpaII*, *HhaI*, or *BstUI*. A Southern blot was made from the digested DNAs and hybridized with a probe to the 3' *c-myc* region of the transgene (Fig. 2B). For each re-

striction enzyme, the patterns of hybridizing bands on the two DNA samples are identical, consistent with the presence of equal, nonimprinted methylation patterns on the two parental alleles. Methylation patterns within other portions of the transgene were determined with the same DNA samples and restriction enzyme digests, but using hybridization probes to the *IgA* and 5' *c-myc* regions of the transgene (Fig. 2C and D, respectively). There were no observed differences between Southern blots made from DNA of a maternal *Ig/myc* carrier and those made from DNA of a paternal *Ig/myc* carrier. Identical maternal and paternal methylation patterns were observed for three other independent *Ig/myc* transgenic lines (Table 1). The consistent similarity between maternal and paternal *Ig/myc* methylation patterns, in comparison to the con-

A

B

C

D

E

FIG. 2. Requirement of the *RSVIgmyc* DMD for transgene imprinting. (A) Schematic of transgenes derived from *RSVIgmyc*. Black lines indicate the probes used in the Southern blots below. *Ig/myc* retains all of the *RSVIgmyc* transgene sequence, except the *pBR/RSV* region between the two *EcoRI* (E) sites. *pBR/RSV* is composed entirely of the sequence delineated by the two *EcoRI* sites. (B to D) Southern blots of DNAs from maternal (M) or paternal (P) hemizygous carriers of the *Ig/myc* transgene digested with either *HpaII*, *HhaI*, *BstUI*, or *MspI* and hybridized with probes to various regions of the transgene (B, probe a, 3' *c-myc* region; C, α probe, *IgA* region; D, probe b, exon 3 region of *c-myc*). (E) The Southern blot was performed as described above and hybridized with a probe to the RSV region of the transgene. All DNA samples were obtained from tail biopsies performed at the time of weaning (3 to 4 weeks).

sistent differential methylation of *RSVIgmyc* (9), shows that removal of the *pBR/RSV* region from the *RSVIgmyc* transgene eliminates its imprinting. This observation suggests that the *pBR322* region and the RSV region of the *RSVIgmyc* transgene have redundant functions; individually, they function to maintain imprinting, but removal of both abolishes imprinting.

Because transgenes composed entirely of *Igf2r* DMD2 sequences are not imprinted (32), *pBR/RSV* (DMD) sequences were tested for their ability to generate imprinted transgenes. Previously, two *pBR/RSV* transgenic lines were created, and the methylation patterns of maternal and paternal transgene

alleles were compared (9). In these two lines, the *pBR/RSV* transgene was highly methylated on both the maternal and paternal alleles and, therefore, was not imprinted. To extend these findings, the *EcoRI* *pBR/RSV* fragment from the *RSVIgmyc* transgene was used to create two new transgenic lines, and DNAs from transgenic carriers of these additional lines were digested with *HpaII*, *HhaI*, or *BstUI*. For each of the three enzymes, the Southern blots of the maternal and paternal alleles showed identical patterns of intensely hybridizing, high-molecular-weight bands, indicating that both maternal and paternal alleles are equally methylated (Table 1 and Fig. 2E). The

TABLE 1. Features of *RSVlgmyc*-derived transgenic lines

Transgenic line ^a	No. of transgene copies ^b	Methylation ^c
<i>pBR/RSVA</i>	10	Maternal = paternal
<i>pBR/RSVC</i>	10	Maternal = paternal
<i>Ig/mycA</i>	12	Maternal = paternal
<i>Ig/mycB</i>	10	Maternal = paternal
<i>Ig/mycC</i>	10	Maternal = paternal
<i>Ig/mycD</i>	15	Maternal = paternal
<i>Igf2rIgmycA</i>	5	Maternal > paternal
<i>Igf2rIgmycB</i>	3	Maternal > paternal
<i>Igf2rIgmycC</i>	10	Maternal > paternal
<i>H19SIgmycA</i>	10	Maternal = paternal
<i>H19SIgmycB</i>	10	Maternal = paternal
<i>H19LIgmycA</i>	8	Maternal = paternal
<i>H19LIgmycB</i>	10	Maternal = paternal
<i>H19LIgmycC</i>	12	Maternal = paternal
<i>SnrpnIgmycA</i>	20	Maternal = paternal
<i>SnrpnIgmycB</i>	10	Maternal = paternal
<i>IAPlgmycA</i>	3	Maternal = paternal
<i>IAPlgmycD</i>	15	Maternal = paternal
<i>IAPlgmycE</i>	15	Maternal = paternal
<i>IAPlgmycF</i>	10	Maternal = paternal
<i>TR2+3Igmyc</i>	20	Maternal > paternal
<i>TR1Igmyc</i>	10	Maternal = paternal

^a Two additional nonimprinted *pBR/RSV* lines were previously analyzed (9).

^b The number of transgene copies per diploid genome was estimated by comparing the intensity of the transgene hybridization band to the intensity of the hybridization band from the endogenous immunoglobulin heavy-chain locus on a Southern blot hybridized with the α probe.

^c Transgene methylation was determined by comparison of the methylation patterns of maternal and paternal alleles from Southern blots. Results are described as the difference or equivalence between maternal and paternal methylation.

data presented above show that the *pBR/RSV* region of *RSVlgmyc* (the DMD) alone is not imprinted.

Restoration of transgene imprinting. The imprinted *RSVlgmyc* transgene was generated by addition of nonimprinted

pBR/RSV sequences to the nonimprinted *Ig/myc* genomic fragment. We tested the ability of other sequences, particularly those found in the DMDs of imprinted genes, to imprint *Ig/myc*. The *Igf2rIgmyc* transgene was created by completely replacing the *pBR/RSV* region of the *RSVlgmyc* transgene with a 668-bp fragment from the center of the DMD2 region of the *Igf2r* gene (Fig. 3 and 4A). Three *Igf2rIgmyc* transgenic lines were created, and maternal and paternal methylation patterns were compared by using Southern blot analysis of *HpaII*-digested DNA hybridized with a probe to the *IgA* region of the transgene. As shown in Fig. 4B, the maternal *Igf2rIgmyc* allele is highly methylated, whereas the paternal allele is relatively undermethylated, consistent with an imprinted *Igf2rIgmyc* locus. Assuming *c-myc* CpG island sequences are unmethylated on both parental *Igf2rIgmyc* alleles, as seen with *RSVlgmyc* transgenes (16), these Southern blots indicate maternal *Igf2r* DMD2 sequences are methylated and paternal DMD2 sequences are unmethylated. Similar results were obtained from two additional *Igf2rIgmyc* lines (Table 1). These data demonstrate that *Igf2r* DMD2 sequences are able to restore imprinting to the *Ig/myc* transgene.

The DMD2 fragment in *Igf2rIgmyc* contains a series of repetitive sequences, organized as three short tandem repeats, each approximately 30 bp in length, and approximately three longer tandem repeats, each approximately 175 bp in length (Fig. 4A). The short repeat is designated TR1, and the long repeat is designated TR2+3 (20). To determine if the TR1 repeats alone or the TR2+3 repeats alone could restore imprinting the *TR1Igmyc* and *TR2+3Igmyc* transgenes were constructed, which completely replace the *pBR/RSV* region of *RSVlgmyc* with TR1 and TR2+3 sequences, respectively. *TR1Igmyc* and *TR2+3Igmyc* transgenic lines were created, and methylation patterns were analyzed by Southern blot analysis of transgenic carrier DNA (Fig. 4B). The maternal allele of

FIG. 3. *RSVlgmyc* and derivative transgenes. (A) Transgenes created by replacement of the *pBR322* and *RSV* region (*pBR/RSV*) of the *RSVlgmyc* transgene, bordered by *EcoRI* sites (E), with fragments containing DMDs from endogenous imprinted genes or the LTR of an IAP element (solid boxes). (B) Sources of DNA fragments (solid boxes) used to replace *pBR/RSV*. Arrows indicate start sites of transcription, and open boxes define DMDs. The drawing is not to scale. The following accession numbers and sequences were used: *H19SIgmyc* and *H19LIgmyc*, U19619 (30), nt 1434 to 1726 (292 nt) and nt 942 to 3174 (2,232 nt), respectively; *Igf2rIgmyc*, L06446 (27), nt 741 to 1408 (667 nt); *SnrpnIgmyc*, AF130843 (13), nt 3237 to 3745 (508 nt); and *IAPlgmyc*, reference 18 (Fig. 2B).

FIG. 4. Restoration of transgene imprinting. (A) Sequence of the *Igf2r* DMD2 region used to replace *pBR/RSV*. The entire depicted sequence (nt 741 to 1408) was used to construct *Igf2rIgmyc*. The nt 741 to 950, including the TR1 repeats (gray boxes), were used to construct *TR1Igmyc*. The nt 950 to 1408, composed of TR2+3 repeats (underlined sequences), were used to create the *TR2+3Igmyc* transgene. Sequences are from accession no. L06446 (27). (B) Southern blot of DNAs from maternal (M) or paternal (P) hemizygous carriers of the *Igf2rIgmyc*, *TR1Igmyc*, or *TR2+3Igmyc* transgenes, digested with *HpaII* or *MspI*, and probed with the α probe to the *IgA* region of the transgene. All DNA samples were obtained from tail biopsies performed at the time of weaning (3 to 4 weeks). (C) *Igf2rIgmyc* transgenic mice were crossed to C57BL/6 mice for three generations, and Southern blots were performed as described above.

TR2+3Igmyc was highly methylated, and the paternal allele was undermethylated. Conversely, both parental alleles of the *TR1Igmyc* transgene were highly methylated. Thus, the *TR2+3Igmyc* transgene retained the imprinted phenotype of the *Igf2rIgmyc* transgene, whereas the *TR1Igmyc* transgene did not.

Influence of genetic background on transgene imprinting.

All of the transgenes described above were analyzed in an inbred FVB/N background. In previous studies, we showed that the *RSVlgmyc* transgene is imprinted in an FVB/N background. However, when it was analyzed in an inbred C57BL/6 background, its imprinting was lost because the paternal allele became heavily methylated in the embryo, obtaining a level of methylation similar to that of the maternal allele. This embryonic methylation is most likely due to de novo methylation of *RSVlgmyc*'s DMD in C57BL/6 embryos (9). To test whether the *Igf2rIgmyc* transgene was similarly affected, carriers of the transgene were crossed for three generations to inbred C57BL/6 mice, and the methylation patterns of maternal and paternal *Igf2rIgmyc* alleles were compared on a Southern blot of *HpaII*-digested transgenic carrier DNA. In a C57BL/6 background, the *Igf2rIgmyc* transgene maintains its imprinting; the

maternal allele has a high level of methylation, and the paternal allele has a low level of methylation (Fig. 4C). Importantly, the paternal *Igf2rIgmyc* allele's methylation in a C57BL/6 background is indistinguishable from that of the paternal allele in an inbred FVB/N background. A similar effect has been observed at endogenous imprinted loci, where imprinting is not dependent on strain background. Thus, in contrast to *RSVlgmyc*'s DMD sequences, the *Igf2r* DMD2 sequences of *Igf2rIgmyc* do not become methylated in the embryo.

The *Dnmt1* ^{Δ^{10}} mutation eliminates expression of the oocyte-specific form of the Dnmt1 methyltransferase and results in loss of methylation from one-half of normally imprinted alleles in offspring of homozygous mutant females (15). These experiments examined the methylation of a number of imprinted genes; however, *Igf2r* was not among them. If one-half of *Igf2r* maternal alleles also lose their methylation in the absence of the Dnmt1o protein, we would expect that some of *Igf2rIgmyc*'s normally methylated maternal alleles would be poorly methylated in offspring that inherited *Igf2rIgmyc* from homozygous *Dnmt1* ^{Δ^{10}} mutant females. To test this hypothesis, inbred FVB/N females homozygous for the *Dnmt1* ^{Δ^{10}} allele and car-

FIG. 5. Loss of transgene imprinting on a *Dnmt1* $\Delta 10$ -deficient background. *Dnmt1* $\Delta 10$ females carrying the *Igf2rIgmcyd* transgene in an FVB/N background (left panel) or wild-type females carrying the *Igf2rIgmcyd* transgene in an FVB/N background (right panel) were mated to FVB/N males. DNA was extracted from entire transgenic D10.5 embryos and digested with *Hpa*II, and Southern blots were performed with the C α probe to the IgA region of the transgene.

rying the *Igf2rIgmcyd* transgene were mated to wild-type FVB/N males and *Igf2rIgmcyd* methylation patterns examined in offspring at day 10.5 of embryogenesis (D10.5). As shown in Fig. 5, the methylation patterns were quite variable among the transgenic embryos. Some transgenic embryos had methylation patterns that were a combination of the heavily methylated maternal pattern and the poorly methylated paternal pattern found on *Igf2rIgmcyd* alleles that were maintained on a wild-type *Dnmt1* background. A few embryos had a heavily methylated maternal pattern, and a few others had a poorly methylated paternal pattern. Methylation patterns of control D10.5 transgenic embryos obtained from a mating between an *Igf2rIgmcyd* female and a wild-type FVB/N male are also shown. Except for one embryo, the maternal transgenes among control D10.5 embryos are all heavily methylated. The exceptional embryo had a poorly methylated paternal-like pattern. We conclude from this analysis that much of the maternally acquired methylation of the *Igf2rIgmcyd* transgene is lost when the transgene is inherited from a homozygous *Dnmt1* $\Delta 10$ female. This could be due to a requirement of the *Dnmt1*o protein for establishing *Igf2rIgmcyd* methylation in the oocyte or for maintaining oocyte-specific methylation during preimplantation development.

Other hybrid transgenes. The creation of an imprinted transgene by addition of *Igf2r* DMD2 sequences to *Ig/mcyd* would suggest that different imprinted transgenes might be created by the combination of *Ig/mcyd* and other DMD sequences. This was tested with sequences from the DMDs of the imprinted *H19* and *Snrpn* genes. Three different transgene constructs were made: *H19SIgmcyd*, *H19LIgmcyd*, and *SnrpnIgmcyd* (Fig. 3). *H19SIgmcyd* contains 292 bp of *H19* DMD sequences, including a single CTCF binding site located approximately 4 kb 5' of the site of transcription initiation (3, 14). *H19LIgmcyd* contains 2,232 bp of the *H19* DMD, located approximately 2 to 4 kb 5' of the transcription start site. *SnrpnIgmcyd* contains 529 bp of the *Snrpn* DMD, including the entire first exon, but excluding any direct repeats found in the first intron (6, 13, 24). Several transgenic lines of each construct were generated (Ta-

ble 1). For each line of a particular transgene construct, the maternal and paternal methylation patterns were identical (Fig. 6). As well, all independently derived lines of a particular construct showed nearly identical levels of methylation (data not shown). In the case of the *H19SIgmcyd* and the *H19LIgmcyd* transgenes, both the maternal and paternal alleles had a high level of methylation. In contrast, both the maternal and paternal alleles of the *SnrpnIgmcyd* transgene showed a low level of methylation. From this analysis, we conclude that not all DMD sequences are able to restore *Ig/mcyd* transgene imprinting, either because these DMD sequences are unable to establish gametic methylation patterns or because they do not maintain their gametic methylation patterns during embryogenesis.

In addition to the generation of a variety of transgenes containing DMD and *Ig/mcyd* sequences, we also produced a

FIG. 6. Other hybrid transgenes. Representative Southern blots of DNAs from maternal (M) or paternal (P) hemizygous carriers of the *H19SIgmcyd*, *H19LIgmcyd*, *SnrpnIgmcyd*, and *LAPgmcyd* transgenes. DNA was digested with *Hpa*II, and Southern blots were hybridized with the C α probe. All DNA samples were obtained from tail biopsies performed at the time of weaning (3 to 4 weeks).

containing transgenes contrasts with the absence or low frequency of imprinting among many of the transgenes primarily derived from endogenous *Igf2r* sequences. For instance, all transgenes of less than or equal to 14 kb that contain the *Igf2r* DMD2 and contiguous surrounding genomic sequences are not imprinted (32). Moreover, hybrid *Igf2r* transgenes that incorporate DMD2 and *Appt* gene sequences are infrequently imprinted (25). Only much larger *Igf2r* transgenes, on the order of 300 kb in size and containing DMD2, are consistently imprinted. Taken together, these observations suggest two possible explanations for the location of *cis*-acting imprinting signals. Besides DMD2 sequences, either a sequence remote from DMD2 is also needed for *Igf2r* imprinting, or a combination of genomic sequences neighboring DMD2 is also needed. Studies of the *RSVlgmyc* transgene favor the latter explanation; there is no single element within *Ig/myc* that is absolutely required for *RSVlgmyc* imprinting, and many different combinations of sequence elements within *Ig/myc* support *RSVlgmyc* imprinting (9, 16).

Common structural features of DMDs. Different genomic DNA sequence features are associated with imprinted genes, including CpG islands and clustered direct repeats of short unit lengths (11, 13, 20). In the case of the imprinted *Igf2r* and *Snrpn* genes, these two features coincide with the genes' DMDs (Fig. 7A). For each gene, direct repeats of a unit length between 30 and 175 nt are interspersed with unique DNA of the DMD. Remarkably, although there are no apparent sequence similarities between the *Snrpn* and *Igf2r* DMDs, the numbers and relative positions of the repeats within the two DMDs are nearly identical (Fig. 7A). Either the RSV sequence element alone or the *pBR322* element alone can function as *RSVlgmyc*'s DMD (9). Notably, both of these elements contain copies of short tandem repeats (Fig. 7A).

Only the TR2+3 repeat sequences within the *Igf2r* DMD2 are required to restore *Ig/myc* imprinting (Fig. 4 and 7A). These sequences are not contained within the *Air* promoter (17) and are distinct from the imprinting box defined by Birger et al. (5). In contrast, unique sequences within the 5' end of the *Snrpn* DMD and outside of the repeat-containing portion of the DMD are not able to restore *Ig/myc* imprinting (Fig. 6 and 7A), and when these sequences are deleted from the endogenous *Snrpn* locus, *Snrpn* remains imprinted (8). These observations strongly suggest that the DMD2 repeated sequences are required for imprinting the endogenous *Igf2r* gene. Moreover, these observations suggest that *Ig/myc* transgene imprinting may be restored by addition of *Snrpn* DMD repeated sequences and that these sequences may be important for imprinting the endogenous *Snrpn* gene.

At most, two copies of a repeat (from *Igf2r* or the RSV LTR) are required to restore transgene imprinting. If one or two unit copies of a repeat are the minimum requirement to restore *Ig/myc* imprinting, why does the number of repeats within the *Igf2r* DMD2 far exceed two? Perhaps the answer to this question lies in the analysis of *Igf2r*-containing hybrid transgenes. The efficiency of the establishment and/or maintenance of a maternal methylation imprint may rely on a number of features, including the length of a unit repeat, the number of repeats, and the organization of the repeats within the DMD. Three copies of the short *Igf2r* TR1 repeat may not have restored imprinting to *Ig/myc*, because the TR1 repeats do not

ensure the efficient establishment or maintenance of a maternal methylation imprint. Two complete copies of the longer TR2+3 repeat (in both *TR2+3lgmyc* and *Igf2rIgmyc* transgenic mice) can more efficiently establish and/or maintain a maternal methylation imprint. For example, only an occasional *Igf2rIgmyc* carrier fails to establish or maintain a maternal methylation pattern (Fig. 5). Therefore, the entire eight repeats of DMD2, interspersed with unique DMD2 sequences, may be necessary to ensure absolute imprinting of *Igf2r* in every oocyte.

The role of DMDs in genomic imprinting. DMDs are necessary, but not sufficient, *cis*-acting elements for imprinting a gene. Only when a DMD is placed in an appropriate genomic context is the absolute requirement of DMD sequences revealed (4, 29, 33). These observations suggest that there are two fundamental *cis*-acting requirements for imprinting a gene. One of these requirements is furnished by sequences within a DMD, and the other requirement is furnished by the surrounding genome. Because the DMD is the site of allele-specific methylation, a likely role for a DMD is to interact with the molecular machinery that establishes methylation patterns (Fig. 7C). This interaction could result in methylation of the DMD itself in both germ lines or, alternatively, could prevent its methylation in both germ lines. There is a precedent for tandem repeats from nonimprinted sources to become methylated; two copies of a mouse B1 element are much more efficient at attracting de novo methylation than a single copy (35). Repeats of DMDs may function similarly and become methylated by attracting de novo methylation.

Regardless of whether DMD sequences attract or prevent de novo methylation, the role of the neighboring genome would be to inhibit this effect in just one of the parental germ lines. In the case of *RSVlgmyc*, *Igf2rIgmyc*, *TR2+3lgmyc*, and many of the known imprinted genes, this mechanism would result in a higher level of methylation on the maternal allele. In the case of the *H19SIgmyc* and *H19LIgmyc* transgenes, which contain *H19* DMD sequences that are normally methylated only on the paternal allele, the high level of transgene methylation is not influenced by the *Ig/myc* sequences and the transgenes are not imprinted. This observation may indicate a fundamental difference in the mechanism of imprinting for those genes that acquire a heritable methylation pattern on DMD sequences during spermatogenesis.

ACKNOWLEDGMENTS

We thank J. Trasler and E. Michaud for their help and recommendations.

This work was supported by a grant from the NIH (J.R.C.).

REFERENCES

1. Ainscough, J. F., T. Koide, M. Tada, S. Barton, and M. A. Surani. 1997. Imprinting of *Igf2* and *H19* from a 130 kb YAC transgene. *Development* 124:3621–3632.
2. Bartolomei, M. S., and S. M. Tilghman. 1997. Genomic imprinting in mammals. *Annu. Rev. Genet.* 31:493–525.
3. Bell, A. C., and G. Felsenfeld. 2000. Methylation of a CTCF-dependent boundary controls imprinted expression of the *Igf2* gene. *Nature* 405:482–485.
4. Bielinska, B., S. M. Blaydes, K. Buiting, T. Yang, M. Krajewska-Walasek, B. Horsthemke, and C. I. Brannan. 2000. De novo deletions of SNRPN exon 1 in early human and mouse embryos result in a paternal to maternal imprint switch. *Nat. Genet.* 25:74–78.
5. Birger, Y., R. Shemer, J. Perk, and A. Razin. 1999. The imprinting box of the mouse *Igf2r* gene. *Nature* 397:84–88.

6. Blaydes, S. M., M. Elmore, T. Yang, and C. I. Brannan. 1999. Analysis of murine *Snrpn* and human SNRPN gene imprinting in transgenic mice. *Mamm. Genome* **10**:549–555.
7. 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.
8. Bressler, J., T. F. Tsai, M. Y. Wu, S. F. Tsai, M. A. Ramirez, D. Armstrong, and A. L. Beaudet. 2001. The SNRPN promoter is not required for genomic imprinting of the Prader-Willi/Angelman domain in mice. *Nat. Genet.* **28**:232–240.
9. Chaillet, J. R., D. S. Bader, and P. Leder. 1995. Regulation of genomic imprinting by gametic and embryonic processes. *Genes Dev.* **9**:1177–1187.
10. Chaillet, J. R., T. F. Vogt, D. R. Beier, and P. Leder. 1991. Parental-specific methylation of an imprinted transgene is established during gametogenesis and progressively changes during embryogenesis. *Cell* **66**:77–83.
11. Constancia, M., B. Pickard, G. Kelsey, and W. Reik. 1998. Imprinting mechanisms. *Genome Res.* **8**:881–900.
12. Cranston, M. J., T. L. Spinka, D. A. Elson, and M. S. Bartolomei. 2001. Elucidation of the minimal sequence required to imprint H19 transgenes. *Genomics* **73**:98–107.
13. Gabriel, J. M., T. A. Gray, L. Stubbs, S. Saitoh, T. Ohta, and R. D. Nicholls. 1998. Structure and function correlations at the imprinted mouse *Snrpn* locus. *Mamm. Genome* **9**:788–793.
14. Hark, A. T., C. J. Schoenherr, D. J. Katz, R. S. Ingram, J. M. Levarse, and S. M. Tilghman. 2000. CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature* **405**:486–489.
15. Howell, C. Y., T. H. Bestor, F. Ding, K. E. Latham, C. Mertineit, J. M. Trasler, and J. R. Chaillet. 2001. Genomic imprinting disrupted by a maternal effect mutation in the *Dnmt1* gene. *Cell* **104**:829–838.
16. Howell, C. Y., A. L. Steptoe, M. W. Miller, and J. R. Chaillet. 1998. *cis*-Acting signal for inheritance of imprinted DNA methylation patterns in the preimplantation mouse embryo. *Mol. Cell. Biol.* **18**:4149–4156.
17. Lyle, R., D. Watanabe, D. te Vrugte, W. Lerchner, O. W. Smrzka, A. Wutz, J. Schageman, L. Hahner, C. Davies, and D. P. Barlow. 2000. The imprinted antisense RNA at the *Igf2r* locus overlaps but does not imprint *Mas1*. *Nat. Genet.* **25**:19–21.
18. Michaud, E. J., M. J. van Vugt, S. J. Bultman, H. O. Sweet, M. T. Davisson, and R. P. Woychik. 1994. Differential expression of a new dominant agouti allele (*Aiapy*) is correlated with methylation state and is influenced by parental lineage. *Genes Dev.* **8**:1463–1472.
19. Morgan, H. D., H. G. Sutherland, D. I. Martin, and E. Whitelaw. 1999. Epigenetic inheritance at the agouti locus in the mouse. *Nat. Genet.* **23**:314–318.
20. Neumann, B., P. Kubicka, and D. P. Barlow. 1995. Characteristics of imprinted genes. *Nat. Genet.* **9**:12–13.
21. Palmiter, R. D., and R. L. Brinster. 1986. Germ-line transformation of mice. *Annu. Rev. Genet.* **20**:465–499.
22. Reik, W., and J. Walter. 2001. Evolution of imprinting mechanisms: the battle of the sexes begins in the zygote. *Nat. Genet.* **27**:255–256.
23. Sasaki, H., P. A. Jones, J. R. Chaillet, A. C. Ferguson-Smith, 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.
24. Shemer, R., Y. Birger, A. D. Riggs, and A. Razin. 1997. Structure of the imprinted mouse *Snrpn* gene and establishment of its parental-specific methylation pattern. *Proc. Natl. Acad. Sci. USA* **94**:10267–10272.
25. Sleutels, F., and D. P. Barlow. 2001. Investigation of elements sufficient to imprint the mouse *Air* promoter. *Mol. Cell. Biol.* **21**:5008–5017.
26. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503–517.
27. Stoger, R., P. Kubicka, C. G. Liu, T. Kafri, A. Razin, H. Cedar, and D. P. Barlow. 1993. Maternal-specific methylation of the imprinted mouse *Igf2r* locus identifies the expressed locus as carrying the imprinting signal. *Cell* **73**:61–71.
28. Swain, J. L., T. A. Stewart, and P. Leder. 1987. Parental legacy determines methylation and expression of an autosomal transgene: a molecular mechanism for parental imprinting. *Cell* **50**:719–727.
29. 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.
30. 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.
31. Walsh, C. P., J. R. Chaillet, and T. H. Bestor. 1998. Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. *Nat. Genet.* **20**:116–117.
32. Wutz, A., O. W. Smrzka, N. Schweifer, K. Schellander, E. F. Wagner, and D. P. Barlow. 1997. Imprinted expression of the *Igf2r* gene depends on an intronic CpG island. *Nature* **389**:745–749.
33. Wutz, A., H. C. Theussl, J. Dausman, R. Jaenisch, D. P. Barlow, and E. F. Wagner. 2001. Non-imprinted *Igf2r* expression decreases growth and rescues the *Tme* mutation in mice. *Development* **128**:1881–1887.
34. Yang, T., T. E. Adamson, J. L. Resnick, S. Leff, R. Wevrick, U. Francke, N. A. Jenkins, N. G. Copeland, and C. I. Brannan. 1998. A mouse model for Prader-Willi syndrome imprinting-centre mutations. *Nat. Genet.* **19**:25–31.
35. Yates, P. A., R. W. Burman, P. Mummaneni, S. Krussel, and M. S. Turker. 1999. Tandem B1 elements located in a mouse methylation center provide a target for de novo DNA methylation. *J. Biol. Chem.* **274**:36357–36361.