Germ-line passage is required for establishment of methylation and expression patterns of imprinted but not of nonimprinted genes

Kerry Lee Tucker,^{1,2} Caroline Beard,¹ Jessica Dausman,¹ Laurie Jackson-Grusby,¹ Peter W. Laird,^{1,4} Hong Lei,³ En Li,³ and Rudolf Jaenisch^{1,2,5}

¹Whitehead Institute for Biomedical Research, ²Massachusetts Institute of Technology, Department of Biology, Cambridge, Massachusetts 02142 USA; ³Massachusetts General Hospital East, Cardiovascular Research Center, Charlestown, Massachusetts 02129 USA

Embryonic stem (ES) cells homozygous for a disruption of the DNA (cytosine-5)-methyltransferase gene (*Dnmt*) proliferate normally with their DNA highly demethylated but die upon differentiation. Expression of the wild-type *Dnmt* cDNA in mutant male ES cells caused an increase in methylation of bulk DNA and of the *Xist* and *Igf2* genes to normal levels, but did not restore the methylation of the imprinted genes *H19* and *Igf2r*. These cells differentiated normally in vitro and contributed substantially to adult chimeras. While the *Xist* gene was not expressed in the remethylated male ES cells, no restoration of the normal expression profile was seen for *H19*, *Igf2r*, or *Igf2*. This indicates that ES cells can faithfully reestablish normal methylation and expression patterns of nonimprinted genes but lack the ability to restore those of imprinted genes. Full restoration of monoallelic methylation and expression was imposed on *H19*, *Igf2*, and *Igf2r* upon germ-line transmission. These results are consistent with the presence of distinct de novo DNA methyltransferase activities during oogenesis and spermatogenesis, which specifically recognize imprinted genes but are absent in the postimplantation embryo and in ES cells.

[Key Words: DNA methylation; genomic imprinting; DNA methyltransferase; *Igf2*; *H19*; *Igf2r*; *Xist*]

Received January 16, 1996; revised version accepted March 1, 1996.

Much evidence indicates that in vertebrates the methylation of DNA at cytosine residues correlates with gene inactivity (Yeivin and Razin 1993). Methylation patterns are generally stably maintained in somatic cells of the animal but change dramatically during early development when the genome of the mammalian embryo undergoes consecutive waves of demethylation and de novo methylation (Razin and Shemer 1995). The methylation present in the DNA of the zygote is erased during cleavage so that the genome of the blastocyst is almost completely unmethylated (Monk et al. 1987; Kafri et al. 1992). After implantation, the embryo undergoes a wave of global de novo methylation that restores the genomic methylation levels of the gastrulating embryo to that seen in the adult (Jähner et al. 1982; Monk et al. 1987). During gametogenesis, the unmethylated DNA of the primordial germ cells becomes de novo methylated (Monk et al. 1987; Kafri et al. 1992), a process that ultimately leads to the high methylation levels of the zy-

gote. De novo methylation of transduced retroviral genomes is also seen in pregastrulation embryos (Jähner et al. 1982) and in embryonic carcinoma (EC) and stem (ES) cells (Stewart et al. 1982; H. Lei, S.P. Oh, R. Jüttermann, R. Jaenisch, K.A. Goss, and E. Li, in prep.), consistent with the hypothesis that a de novo DNA methyltransferase activity is expressed in early stages of development.

The crucial importance of DNA methylation in development was demonstrated by the targeted mutation of the single known mammalian DNA methyltransferase gene (*Dnmt*) in ES cells (Li et al. 1992). *Dnmt* is believed to encode a maintenance methyltransferase, because in vitro the enzyme (MTase) shows a substrate preference for hemimethylated DNA (Gruenbaum et al. 1982; Bestor and Ingram 1983). Three mutant alleles of the *Dnmt* gene have been generated (Li et al. 1992, 1993; H. Lei, S.P. Oh, R. Jüttermann, R. Jaenisch, K.A. Goss, and E. Li, in prep.). A partially inactivated allele (*Dnmtⁿ*) causes lethality of homozygous mutant embryos at the 25 somite stage, whereas embryos homozygous for completely inactivated alleles (*Dnmt^s* and *Dnmt^c*) die at the 5 somite stage. ES cells homozygous for any of the alleles

⁴Present address: Norris Comprehensive Cancer Center, Los Angeles, CA 90033 USA.

⁵Corresponding author.

proliferate normally with their genomic DNA highly demethylated but die upon induction of differentiation. ES cells homozygous for any disruption, however, retain the ability to de novo methylate transduced DNA, supporting the notion of an independently encoded de novo DNA methyltransferase being expressed in embryonic cells (H. Lei, S.P. Oh, R. Jüttermann, R. Jaenisch, K.A. Goss, and E. Li, in prep.).

Imprinted genes display parent-specific monoallelic expression (Surani 1994; Barlow 1995), although often not at all developmental stages (Szabo and Mann 1995). Genes that are predominantly expressed from the maternal allele include *Igf2r* (Barlow et al. 1991), *H19* (Bartolomei et al. 1991), *Mash2* (Guillemot et al. 1995) and *p57^{KIP2}* (Hatada and Mukai 1995), whereas *Igf2* (DeChiara et al. 1991), *Mas* (Villar and Pedersen 1994), *Snrpn* (Leff et al. 1992), *U2afbp-rs* (Hayashizaki et al. 1994), *ins1/ins2* (Giddings et al. 1994), *IPW* (Wevrick et al. 1994), and *Peg1/Mest* (Kaneko-Ishino et al. 1995) are expressed paternally. DNA methylation has been proposed to constitute the molecular mark that distinguishes the two alleles of imprinted genes. Evidence for DNA methylation constituting the imprinting mark includes differential methylation of imprinted transgenes (Reik et al. 1987; Sapienza et al. 1987; Swain et al. 1987; Chaillet et al. 1991) and imprinted endogenous genes. The acquisition and developmental propagation of the methylation mark has been described in detail for the two imprinted genes *H19* and *Igf2r*, and for *Xist*, a gene encoded at the center for X chromosome inactivation that shows imprinted expression only in preimplantation embryos and extraembryonic tissues (Kay et al. 1993). The *H19* gene acquires paternal-specific methylation in a region upstream of its promoter during spermatogenesis (Tremblay et al. 1995), whereas *Igf2r* (Stöger et al. 1993) and *Xist* (Ariel et al. 1995; Zuccotti and Monk 1995) acquire a maternal-specific methylation mark during oogenesis. Importantly, the allele-specific methylation differences in these "imprinting boxes" are resistant to the wave of global demethylation during cleavage and the subsequent wave of global de novo methylation before gastrulation (Bartolomei et al. 1993; Brandeis et al. 1993; Stöger et al. 1993; Tremblay et al. 1995). This assures that the methylation mark imposed on the two alleles of imprinted genes during gametogenesis remains unaltered during development and, therefore, distinguishes the two alleles in each cell of the organism. In contrast to *H19* and *Igf2r*, the methylation imprint of the maternal *Xist* allele is erased after implantation in cells of the embryonic lineage, and either the paternal or the maternal allele becomes methylated at the time of X inactivation (Norris et al. 1994).

Strong evidence for methylation being crucial for the maintenance of monoallelic expression of imprinted genes was provided by the analysis of *Dnmt* mutant embryos (Li et al. 1993; Beard et al. 1995). Hypomethylation of the DNA in mutant embryos or ES cells resulted in loss of monoallelic expression, either in the activation of the inactive *H19* and *Xist* alleles or in the inactivation of the active *Igf2r* and *Igf2* alleles. These experiments did

not, however, provide insights into the role of methylation in the establishment of genomic imprinting during gametogenesis.

In this paper we address the process by which the allele-specific methylation of imprinted genes is acquired. We have expressed the wild-type *Dnmt* cDNA in homozygous *Dnmt* mutant ES cells to assess whether this would restore the low methylation levels of the genomic DNA to normal. Although normal methylation levels of nonimprinted DNA were reestablished, imprinted genes were neither remethylated nor appropriately expressed. However, passage of the remethylated ES cells through the germ line restored normal methylation and expression to all imprinted genes analyzed.

Results

Expression of functional MTase in Dnmt mutant ES cells leads to restoration of genomic methylation

Previous work had demonstrated that the DNA of homozygous mutant *Dnmt* ES cells was highly hypomethylated (Li et al. 1992; H. Lei, S.P. Oh, R. Jüttermann, R. Jaenisch, K.A. Goss, and E. Li, in prep.). To assess whether expression of the wild type MTase would restore genomic methylation levels and rescue the lethal phenotype of differentiated mutant cells, we introduced the *Dnmt* cDNA into mutant ES and embryonic germ (EG) cells. The two cell types displayed identical molecular characteristics and functional capacity before and after expression of the *Dnmt* cDNA. For the sake of simplicity, they are collectively referred to as ES cells in the text. To assure correct quantitative and developmental expression of the construct, the cDNA was inserted by homologous recombination into the cognate *Dnmt* locus upstream of the mutation, using a strategy we refer to as CHIP (cDNA homologous insertion protocol; see Materials and methods). The vector (Fig. 1A) fused the *Dnmt* cDNA 3' to a 4.8-kb piece of genomic *Dnmt* sequence that ended in the second known exon (Rouleau et al. 1992). Homologous insertion of this hybrid vector was expected to leave all upstream control elements of the gene intact, leading to restoration of normally regulated MTase expression. *Dnmt* mutant cells were electroporated with the linearized construct and selected for puromycin resistance. All puromycin-resistant colonies were expanded and analyzed by Southern blot analysis for vector integration and levels of genomic methylation. Genomic DNA was digested with *ScaI* and Southern blots were hybridized with probe HV. This yielded a 17.5-kb fragment in wild-type J1 and *Dnmt* mutant cell lines (Fig. 1B, lanes 1,2), but a 22- and a 7.4-kb fragment for the targeted allele of an homologous integrant (lanes 3,4). By this analysis, 73 of 182 puromycin-resistant clones had undergone homologous recombination events in which a single copy of the plasmid integrated at a single allele (data not shown). Northern blot analysis was used to assess the level of *Dnmt* transcription. The size of the endogenous mRNA is 5.2 kb (Fig. 1C, lane 1). Virtually no mRNA of this size was observed in homozygous mu-

Figure 1. Targeting of the *Dnmt* cDNA to its cognate locus results in normal expression levels of *Dnmt* mRNA. (A) Diagram representing the *Dnmt*-targeting construct pMWT-PURO and its method of integration into the *Dnmt* locus. pMWT-PURO is represented schematically. A 4.8-kb *Dnmt* genomic sequence that is shared between pMWT-PURO and the *Dnmt* locus is indicated by a broken line and two dark boxes corresponding to the first two exons, drawn to scale. This was fused to the remainder of the *Dnmt* cDNA (hatched box) in the second exon at a *NaeI* (N) site, 20 bp upstream of the initial ATG. A *Pgk-I*-derived polyadenylation sequence (*Pgk*-PolyA) was inserted at the end of the cDNA, and the plasmid also contained a puromycin-selectable marker (*Pgk*-Puro) and pGEM-11zf(+) bacterial plasmid sequences (*Amp^r*). The *HindIII* linearization site is indicated (H). The *Dnmt* locus targeted with this construct is represented beneath by a broken line upstream of the same two exons depicted in pMWT-PURO. The genomic probe (HV) for Southern blot analysis and the *ScaI* (S) digestion product expected from the locus are also indicated above. *HindIII* (H) and *NaeI* (N) sites are as indicated above. Within the vertical arrow is a closeup, drawn to scale, of the crossover event in which pMWT-PURO was targeted to the *Dnmt* locus. The plasmid was cut at the unique *HindIII* (H) site indicated, and after electroporation the entire vector homologously inserted into the endogenous gene, yielding the genomic structure shown beneath. pMWT-PURO shares 4.1 and 0.7 kb of sequence identity with the target locus 5' and 3', respectively, of the *HindIII* linearization site. The bottom schematic illustrates the duplication of the 4.8-kb *Dnmt* genomic sequence expected upon integration, seen with the exons (dark boxes) drawn to scale. It also displays the two products expected from a *ScaI* (S) digestion, using the HV probe. The scale is interrupted by several breaks in the genomic sequence, indicated by slashes through the line. The integrated pMWT-PURO plasmid is indicated by a solid line. (B) Southern blot analysis of *Dnmt*-targeted clones. *ScaI*-digested genomic DNA displayed a single 17.5-kb band from both untargeted *Dnmt* alleles in wild-type J1 (lane 1) and homozygous *Dnmt* mutant (lane 2) cells. Homologous insertion of a single copy of the pMWT-PURO plasmid at the *Dnmt* locus resulted in production of 21- and 7.4-kb bands (lanes 3,4). The untargeted allele is also visible at 17.5 kb. *HindIII*-digested λ DNA size standards are indicated to the left. (C) Northern blot analysis of *Dnmt* expression in ES cell lines stably transfected with pMWT-PURO. Total RNA was prepared from the homologous integrants shown in B (lanes 4,5), and the *Dnmt* cDNA was used as a probe for the Northern blot (top). The following cells are shown for comparison: wild-type J1 (lane 1), a heterozygous (lane 2), and homozygous (lane 3) *Dnmt* mutant cell line. (Bottom) The same blot rehybridized with an α -tubulin cDNA, to control for amount of RNA loaded. The positions of the 28S and 18S rRNA species are indicated at left.

tant *Dnmt* cells (lane 3). Puromycin-resistant cells that underwent homologous recombination (lanes 4,5) showed that the inserted cDNA was transcribed at a level and size comparable to that of the endogenous allele (lane 2).

All 73 homologous recombinants examined showed near-complete genomic remethylation (Fig. 2), as assayed using two repetitive sequence probes: a Moloney murine leukemia virus (MoMuLV) cDNA, which cross-hybridizes with endogenous C-type retroviruses scattered throughout the genome (Stuhlmann et al. 1981), and a

minor satellite centromeric repeat (Chapman et al. 1984). Both of these repeated sequences are heavily methylated in J1 cells (Fig. 2, lane 1), as seen by Southern blot analysis of genomic DNA digested with *HpaII*, an enzyme sensitive to the methylation of internal cytosines in its CCGG recognition sequence. DNA methylation in *Dnmt* mutant cells was highly reduced (lane 2), as seen by the increasing intensity of lower molecular weight bands in *Dnmt* mutant cell lines. The methylation level of both sequences was restored to normal in all clones with a homologously integrated construct

Figure 2. Normal genomic DNA methylation levels are restored upon expression of the *Dnmt* cDNA. Southern blot analysis of *HpaII*-digested genomic DNA, probed with either of two repetitive sequences: MoMuLV cDNA (*top*) or a centromeric minor satellite repeat (*bottom*). Wild-type J1 (lane 1) and homozygous *Dnmt* mutant ES cells (lane 2) are shown, followed by the rescued mutant cells seen in Fig. 1 (lanes 3,4). *HindIII*-digested λ DNA size standards are indicated to the left of each blot.

(lanes 3,4). No puromycin-resistant clones in which pMWT-PURO had integrated nonhomologously displayed either *Dnmt* expression or genomic remethylation (data not shown). We conclude that remethylation of bulk DNA in the genome of homozygous mutant *Dnmt* cells is achieved by expression of the *Dnmt* cDNA from its endogenous promoter. *Dnmt* mutant cells that express the cDNA shall henceforth be referred to as "rescued" cells.

MTase expression complements lethal phenotype of differentiating mutant Dnmt ES cells

Previous observations indicated that homozygous mutant *Dnmt* ES cells, although proliferating normally when undifferentiated, die upon induction of differentiation (Beard et al. 1995; H. Lei, S.P. Oh, R. Jüttermann, R. Jaenisch, K.A. Goss, and E. Li, in prep.) and do not contribute to adult chimeras after injection into wild-type blastocysts (see below). We were interested to test whether restoration of genomic DNA methylation levels

in mutant ES cells carrying the *Dnmt* cDNA would rescue the lethal mutant phenotype. To test in vitro differentiation, the rescued cells were induced to differentiate by plating on bacteriological petri dishes, in the absence of a fibroblast feeder layer and leukemia inhibitory factor (LIF). In contrast to the parental mutant cells (H. Lei, S.P. Oh, R. Jüttermann, R. Jaenisch, K.A. Goss, and E. Li, in prep.), the rescued cells differentiated and proliferated normally (data not shown). This indicates that in vitro differentiation potential was restored by genomic remethylation.

To test the developmental potential of the remethylated cells more stringently, in vivo experiments were conducted. First, the cells were injected subcutaneously into syngeneic male host animals. Although the parental mutant cells failed to form palpable teratomas within 3–4 weeks ($n=0/24$), the rescued ES cells induced teratoma formation at a similar rate ($n=21/24$) as the wild-type J1 ES cells ($n=11/12$). Second, the rescued cells were injected into wild-type BALB/c blastocysts to test their ability to contribute to chimeras. The parental *Dnmt* mutant cells were completely incapable of contributing to adult chimeras ($n=0/64$ injected blastocysts). When rescued cells were used instead, 11 chimeras were generated with donation to coat color ranging from 10% to 70% ($n=11/92$ injected blastocysts). The animals appeared normal in size and appearance, and contribution to the germ line was observed in three chimeras derived from two rescued cell lines (see below).

The results described so far show that expression of functional MTase in *Dnmt* mutant ES cells restores normal methylation levels and results in the rescue of the lethal mutant phenotype.

Methylation and monoallelic expression of imprinted genes are not restored in rescued ES cells

The methylation levels and expression of the imprinted genes *H19*, *Igf2r*, *Igf2*, and *Xist* were assessed by Southern blot and by RNase protection analysis. The analyses were performed on undifferentiated and in vitro-differentiated ES cells. To investigate methylation and expression in terminally differentiated cells, fibroblasts were isolated from the chimeras described above. Primary fibroblast cultures were prepared from the carcasses of four chimeras. The primary cultures were immortalized with SV40 virus and then selected with puromycin to enrich for cells derived from the injected ES cells. Puromycin-selected populations were then subcloned at limiting dilution to prevent contamination from wild-type fibroblasts.

Methylation of *H19* In normal ES cells and mice a 3.8-kb region just upstream of the promoter displays a monoallelic methylation pattern (Bartolomei et al. 1993), in which the paternally derived allele is completely methylated (migrating at 3.8 kb, Fig. 3A, lanes

Figure 3. Methylation of the imprinted genes *H19* and *Igf2r* is not restored in rescued ES cells and their differentiated derivatives, but methylation of *Xist* and *Igf2* is restored to normal. Southern blot analysis of ES cells and fibroblast cultures derived from postnatal chimeras made with rescued ES cells. (A–D) Genomic DNA was isolated from undifferentiated (U) or differentiated (D) ES cells, cut with *SacI*, *PvuII*, *EcoRI*–*HindIII*, or *EcoRV* (A,B,C, and D, lanes 1, respectively), and probed with the gene sequence indicated beneath each respective blot. All other lanes were treated similarly but include the methylation-sensitive enzymes *HhaI* (A,D) or *HpaII* (B,C). Wild-type J1 (+/+), homozygous *Dnmt* mutant (–/–), and two independent rescued cell lines (–/– + cDNA) are shown. (A and C, lanes 10–12; B, lanes 6–8) SV40 virus-immortalized fibroblast cultures were examined. Fibroblasts were derived from an embryonic day 16.5 wild-type embryo (A and C, lanes 10; B, lane 6) and were compared with puromycin-resistant fibroblasts derived from two postnatal chimeras (A and C, lanes 11 and 12; B, lanes 7 and 8). The puromycin-resistant cells were shown to be derived exclusively from the injected rescued ES cells (data not shown). A map of the examined area is shown beneath each blot, with flanking *SacI* (S), *PvuII* (P), *EcoRI* (E), *HindIII* (H), and *EcoRV* (V) sites indicated. The probes used and the genomic regions examined are described in Results and Materials and methods. *HhaI* (A,D) and *HpaII* (B,C) sites are represented by small vertical lines; transcriptional start sites are shown by arrows in A and D. *HindIII*-digested λ DNA size standards are indicated to the left of each blot.

2,3), whereas the maternally derived allele is hypomethylated at the *HhaI* sites within the region (lower molecular weight bands, lanes 2,3). Within this region lie three CpG sites that become methylated in spermatogenesis and remain methylated exclusively on the paternally derived allele throughout development (Tremblay et al. 1995). This region becomes completely demethylated in *Dnmt* mutant ES cells (lanes 4,5), indicating a complete loss of methylation at both alleles. In the rescued ES cells (lanes 6,8) these sites remained hypomethylated at both alleles, and in vitro differentiation did not result in remethylation of this region (lanes 7,9). To see whether remethylation of this region occurred in terminally differentiated cell types in vivo, immortalized fibroblasts prepared from chimeras were examined. Puromycin-selected populations (lanes 11,12), which were derived

from the rescued ES cells, displayed no evidence of remethylation at either allele of *H19* [compare to fibroblasts derived from a wild-type mouse (lane 10), which display a fully methylated allele (migrating at 3.8 kb) and a demethylated allele (lower molecular weight bands)].

Methylation of *Igf2r* The methylation of the *Igf2r* gene was determined similarly. It has been shown that an intronic CpG-rich region (Stöger et al. 1993) displays a monoallelic methylation pattern in which the maternally derived allele is completely methylated (migrating at 2.9 kb, Fig. 3B, lane 2) while the paternally derived allele is hypomethylated at the *HpaII* sites within the region (lower molecular weight bands, lane 2). Both alleles were completely demethylated in *Dnmt* mutant ES cells (lane 3), indicating a complete loss of methylation

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at both alleles. In the rescued ES cells (lanes 4,5) these sites remained hypomethylated at both alleles. Neither in vitro differentiation of these cells nor 20 serial passages resulted in remethylation of this region (data not shown). The methylation pattern seen in puromycin-selected fibroblasts (lanes 7,8) was similar to that seen in rescued ES cells [compare fibroblasts derived from a wild-type mouse (lane 6), which display a monoallelic methylation pattern, with rescued fibroblasts, which are hypomethylated at both alleles].

Methylation of *Igf2* Unlike *H19* and *Igf2r*, *Igf2* does not possess a region that becomes differentially methylated in the gametes (Brandeis et al. 1993). However, in adult mice a 1.5-kb region upstream of the first *Igf2* promoter has been shown to display a differential methylation pattern (Sasaki et al. 1992; Brandeis et al. 1993; Feil et al. 1994). In contrast to *H19* and *Igf2r*, this region does not display absolute methylation differences, but the paternally derived allele is more methylated, on average. As reported before (Feil et al. 1994), examination of *HpaII*-digested genomic DNA from wild-type ES cells revealed a pattern similar to that seen in embryonic and adult tissues, with a fully methylated band (migrating at 1.5 kb, Fig. 3C, lane 2) and a ladder of fragments representing progressive demethylation, moving 3' to 5', of four *HpaII* sites (lower molecular weight bands, lane 2). This region became mostly demethylated in *Dnmt* mutant ES cells (lane 4), indicating a near-complete loss of methylation at both alleles. In the rescued ES cells (lanes 6,8), a methylation pattern very similar to wild-type ES cells was established, though the fraction of demethylated 3'-proximal *HpaII* sites remained higher than in wild-type cells. It is likely that this pattern reflected biallelic methylation. Upon in vitro differentiation, *Igf2* became slightly demethylated (lanes 7,9), whereas puromycin-selected fibroblasts (lanes 11,12) revealed a state of hypomethylation almost as extensive as that seen in *Dnmt* mutant ES cells (lanes 4,5), in striking contrast to the pattern seen in wild-type fibroblasts (lane 10).

Expression of *H19*, *Igf2r*, and *Igf2* Expression was analyzed by RNase protection assay in wild-type, *Dnmt* mutant, and rescued ES cells before and after induction of differentiation. All undifferentiated cells showed the same level of *Igf2r* expression and little or no expression of *H19* and *Igf2* (Fig. 4, B, A, and D, lanes 1, 3, and 5, respectively). Upon differentiation, however, the expression of *Igf2* increased significantly in wild-type ES cells (Fig. 4D, lane 2) with little or no increase seen in the mutant (lane 4) or rescued ES cells (lane 6). Although *Igf2r* expression appeared to increase only slightly upon differentiation of wild-type ES cells (Fig. 4B, lane 2), it showed a noticeable decrease in expression in the differentiated mutant (lane 4) and rescued ES cells (lane 6). Like *Igf2*, *H19* expression increased significantly in wild-type ES cells (Fig. 4A, lane 2) upon differentiation and further increased by at least a factor of two in mutant (lane 4) and rescued ES cells (lane 6). In *Dnmt* mutant embryos, an increase in *H19* expression relative to wild-

Figure 4. Normal expression of imprinted genes is not restored in rescued ES cells. (A,B,D,E) Total RNA isolated from undifferentiated (U) and in vitro-differentiated (D) ES cells was analyzed by RNase protection using antisense RNA probes for *H19* (A), *Igf2r* (B), *Igf2* (D), and *Xist* (E). Antisense RNA probes for cytoplasmic actin (A,B) or γ actin (D,E) were used as loading controls. Wild-type J1 (+/+), homozygous *Dnmt* mutant (-/-), and rescued ES cells (-/- + cDNA) are shown. (C) *Igf2r* RNase protection assay performed on subcloned lines of immortalized fibroblasts prepared from a P7 chimera made between rescued EG cells and a wild-type BALB/c host. (Lanes 1,2) Two wild-type BALB/c lines; (lanes 3-6) four puromycin-resistant lines derived from the injected rescued EG cells. Antisense RNA probes for *Igf2r* and γ actin (as a loading control) were used.

type embryos has been shown to be caused by biallelic expression of the gene (Li et al. 1993). The ES cell lines used for these studies were not marked by any polymorphism to distinguish the alleles of *H19*, but the overexpression seen in rescued ES cells is consistent with a biallelic transcriptional profile.

To determine whether expression of these imprinted genes returned to wild-type levels in terminally differentiated cells in vivo, RNA was prepared from wild-type and rescued mutant fibroblasts derived from a P7 (P0 = day of birth) mouse. *Igf2r* expression, which was found at high levels in wild-type fibroblasts (Fig. 4C, lanes 1,2), was found to be completely extinguished in rescued mutant fibroblasts (lanes 3–6). No significant expression of *H19* and *Igf2* was seen in either wild-type or rescued mutant fibroblast clones (data not shown). The lack of expression of *Igf2* and *H19* in wild-type cells was probably caused by a developmental down-regulation of these genes, for the following reasons: (1) Expression of *Igf2* was observed in wild-type fibroblast cultures derived from a 16.5-day-old embryo (data not shown), and (2) *H19* is known to be switched off in most tissues soon after birth (Poirier et al. 1991).

Xist This gene, in contrast to the imprinted genes described above, is imprinted only in extraembryonic tissues. The maternal allele is methylated and the paternal allele remains unmethylated throughout cleavage and in the extraembryonic lineage (Ariel et al. 1995; Zuccotti and Monk 1995). Following implantation, however, the maternal imprint is erased and one of two alleles becomes randomly methylated and silent at the time of X chromosome inactivation (Norris et al. 1994). *Xist* becomes differentially methylated in a 1.5-kb region surrounding its promoter (Norris et al. 1994), with two clusters of CpG sites showing gamete-specific methylation—one cluster just upstream of the transcriptional start site (Zuccotti and Monk 1995) and the other 600-bp downstream (Ariel et al. 1995). In male ES cells, with a single, maternally derived X-chromosome, this region was almost completely methylated (Fig. 3D, lane 2). In *Dnmt* mutant ES cells, this methylation was completely erased (lane 4), including the gamete-specific methylation sites. The same degree of partial methylation seen in wild-type ES cells was observed in the rescued ES cells (lanes 6,8). As shown before (Beard et al. 1995), in vitro differentiation of wild-type J1 cells resulted in an increase in methylation (lane 3), and this was also seen for the rescued ES cells (lanes 7,9). Also as shown previously (Beard et al. 1995), this demethylation resulted in the expression of *Xist* upon differentiation of *Dnmt* mutant ES cells (Fig. 4E, lane 4) but not J1 (lane 2) or rescued ES cells (lane 6).

Our results show that methylation and expression of the imprinted genes *H19* and *Igf2r* were not restored to normal levels following expression of MTase in homozygous *Dnmt* mutant ES cells or their differentiated derivatives, in contrast to repetitive DNA sequences and *Xist*. These observations suggest that once the genomic DNA has been demethylated by deletion of the maintenance MTase, the de novo methylation activity in ES cells can distinguish between two classes of genes: (1) the majority of genes, as evidenced by the repetitive probes in Figure 2 or *Xist*, which can be remethylated to normal levels so as to assure normal expression patterns during subsequent differentiation; and (2) imprinted genes such

as *H19* and *Igf2r*, which cannot be remethylated and functionally restored once demethylated.

Normal methylation and expression patterns of imprinted genes are restored after passage through the germ line

To test whether germ-line transmission would restore normal methylation and expression patterns to imprinted genes, five male chimeras, generated from rescued ES cells, were mated to BALB/c females. Three of these chimeras, derived from two independently transfected ES cell lines, produced agouti offspring, indicating contribution of the rescued genome to the germ line. The *Dnmt* allele that had undergone homologous recombination was transmitted at the expected frequency, and pups carrying the *Dnmt* cDNA appeared identical to their littermates. Paternal transmission of the imprinted genes contributed by the rescued ES cells makes clear predictions regarding the methylation and expression status of imprinted genes in the offspring. For example, after germ-line transmission the paternal *H19* allele should be methylated and the paternal *Igf2* allele should be expressed. Consistent with this prediction, the same methylation pattern was seen in pups derived from rescued ES cells (Fig. 5D, lanes 2–4) as in pups derived from the host–blastocyst (lane 1) when *H19* methylation in tail preparations was examined as described above. As the examined promoter–proximal region of *H19* is known to be unmethylated on the maternally derived allele (Tremblay et al. 1995), we conclude that methylation of the paternally derived allele had occurred during germ-line transmission. Methylation of *Igf2r* and *Igf2* was examined in the same regions described above. A wild-type methylation pattern was seen for *Igf2r* (Fig. 5F), which is consistent with the paternally derived allele of this gene remaining hypomethylated after germ-line passage. Finally, a wild-type methylation pattern was observed for *Igf2* (Fig. 5E), indicating full methylation of the paternally derived allele.

RNAse protection analysis was performed on total RNA prepared from gestational day 19.0 embryos. Identical levels of *Igf2* RNA were observed in pups derived from the rescued ES cells (Fig. 5B, lane 1) as in pups derived from the host–blastocyst (lane 2). Because *Igf2* is not expressed from the maternally derived allele (DeChiara et al. 1991), we conclude that this expression was derived from the paternally transmitted allele. Similar results were observed for expression levels of *H19* (Fig. 5A) and *Igf2r* (Fig. 5C).

These results indicate that normal methylation patterns were restored to both alleles of *H19* in the male gonad, resulting in the appropriate expression of both *H19* and *Igf2* from the paternally derived alleles in the germ-line pups (see Discussion). The presence of an unmethylated *Igf2r* allele in these pups, and wild-type levels of *Igf2r* expression in germ-line-transmitted embryos, indicates that this gene was not remethylated in the male gonad. By extension, methylation of both alleles of *Igf2r* would presumably occur upon germ-line transmis-

Figure 5. Normal methylation and expression patterns of imprinted genes are restored after passage through the germ line. Southern blot and RNase protection analysis of germ-line embryos and pups produced by a chimera made from rescued ES cells. (A–C) RNase protection analysis performed on whole RNA from embryos, using probes indicated in Fig. 4. A male chimera was mated to BALB/c females, and embryos analyzed at embryonic day 19.0. Black-eyed embryos (R), derived by germ-line transmission of the rescued ES cells, were compared to albino wild-type littermates (+/+). (D–F) Southern blot analysis of genomic DNA from postnatal mice. DNA prepared from tails of 3-week old mice was digested with methylation-sensitive enzymes and probed with *H19*, *Igf2*, or *Igf2r* sequences, as described in Fig. 3, A, C, and B, respectively. A wild-type F_1 (BALB/c $\times$ 129/Sv) mouse (lane 1) was compared to three germ-line pups derived from a cross between a chimera generated with rescued ES cells and a BALB/c female (lanes 2–4). *HindIII*-digested λ DNA size standards are indicated to the left of each blot.

sion through a female chimera, but none of the four female chimeras produced agouti offspring. In conclusion, our results suggest that de novo methylation of the im-

printed genes *H19* and *Igf2r*, in contrast to “bulk” DNA and the *Xist* and *Igf2* genes, can only occur after passage through the germ line.

Discussion

The experiments in this study were designed to examine the developmental specificity of DNA methylation activity in the mouse. Because gross genomic hypomethylation is tolerated by *Dnmt* mutant ES cells, we could start at a specific point in the murine life cycle with a near-complete deficiency of genomic methylation and determine the sites and times of methylation activity. Our results allow the two following conclusions regarding the function of the DNA methyltransferase. (1) Genomic methylation levels in ES cells, which were decreased substantially as a result of the targeted inactivation of the *Dnmt* gene, could be restored to normal by expression of a functional *Dnmt* gene. That this remethylation process has restored normal methylation patterns to the genome is inferred by the ability of these remethylated cells to undergo normal differentiation in vitro, to form teratomas, and contribute extensively to chimeras in vivo. In contrast, the parental mutant cells fail to differentiate normally in each of these assays. (2) Upon *Dnmt* cDNA expression in ES cells, the imprinted genes *H19* and *Igf2r* were not remethylated at either allele and, as a consequence, were not appropriately expressed. Despite this deficiency, the cells were able to contribute substantially to chimeras, including the gonad. Importantly, normal methylation and expression patterns of the two imprinted genes *Igf2* and *H19* were restored after passage through the male germ line.

Expression of functional MTase in the fully hypomethylated *Dnmt* mutant ES cells complemented the mutant phenotype, leading to a substantial restoration of normal genomic DNA methylation levels, as assayed by various repetitive sequence probes. In vitro, the enzyme prefers hemimethylated over unmethylated substrates (Gruenbaum et al. 1982), consistent with the notion that the *Dnmt* gene encodes the maintenance methyltransferase that is expressed in every cell. How might re-expression of a maintenance methyltransferase activity result in de novo methylation of the unmethylated genomic DNA in mutant ES cells? We have postulated previously that early embryos as well as undifferentiated ES cells express a de novo DNA methyltransferase that is not active in postgastrulation embryos (Jähner et al. 1982) or in differentiated ES cells (Stewart et al. 1982). The low but stable level of genomic DNA methylation in *Dnmt* mutant ES cells is likely caused by the activity of a separately encoded enzyme (H. Lei, S.P. Oh, R. Jüsterman, R. Jaenisch, K.A. Goss, and E. Li, in prep.), which is insufficient to maintain normal methylation levels in the absence of the maintenance DNA methyltransferase. Re-expression of the maintenance methyltransferase would, however, be expected to lead to an increase in methylation levels as it would maintain the methylation levels set by the embryonic de novo methyltransferase activity.

Dnmt mutant ES cells, while proliferating normally when undifferentiated, grow poorly and die upon induction of differentiation, with the level of genomic DNA methylation decreasing (H. Lei, S.P. Oh, R. Jüttermann, R. Jaenisch, K.A. Goss, and E. Li, in prep.). This is consistent with the embryonic de novo DNA methyltransferase becoming inactive upon differentiation, leading to further hypomethylation. An important issue was whether re-expression of MTase not only would restore genome-wide methylation levels but also lead to rescue of the mutant phenotype, which is a reduction in differentiation capacity in vitro and in vivo (Li et al. 1992; H. Lei, S.P. Oh, R. Jüttermann, R. Jaenisch, K.A. Goss, and E. Li, in prep.). Remethylated cells have clearly regained the ability to differentiate, as evidenced by three different assays: in vitro differentiation as embryoid bodies, teratoma formation, and contribution to the tissues of chimeras, which represents the most stringent functional test. The extensive coat color contribution to these normal chimeras suggests that single-copy genes had become appropriately remethylated and were correctly expressed, resulting in normal embryonic development. The restoration of wild-type methylation levels and consequent transcriptional inactivation was observed for the single-copy gene *Xist*. *Xist* is not imprinted in male ES cells and embryonic derivatives because the single copy of this gene is normally methylated and unexpressed (Norris et al. 1994). The partially methylated pattern seen in normal male ES cells (Beard et al. 1995) was restored to the same degree in rescued ES cells. Importantly, the CpG sites showing gamete-specific methylation (Ariel et al. 1995; Zuccotti and Monk 1995) were remethylated, supporting the notion that methylation of these sites leads to transcriptional inactivity of *Xist*. The remethylation of *Xist*, coupled with the functional complementation assays, suggests that the methylation of all or most other nonimprinted genes is fully restored. It is possible, however, that some genes, whose misexpression would not compromise normal development, are not becoming appropriately remethylated. A detailed study of remethylation at individual loci will address this issue and yield information regarding the functional importance of methylation at specific sites as compared to overall methylation patterns.

The imprinted genes *H19* and *Igf2r* were not remethylated after introduction of the *Dnmt* cDNA into mutant ES cells. The regions examined for methylation show a gamete-specific methylation difference and are believed to constitute the primary imprinting mark (Barlow 1995). The methylation of these regions and expression status of these genes in rescued ES cells were identical to that seen previously in *Dnmt* mutant ES cells and embryos (Li et al. 1993). Furthermore, hypomethylation persisted in ES cell-derived fibroblasts isolated from chimeras, and methylation was restored to normal only after germ-line transmission. In contrast to *H19* and *Igf2r*, *Igf2* was remethylated extensively in an area that has been shown to be methylated differentially in the adult (Sasaki et al. 1992; Brandeis et al. 1993; Feil et al. 1994) but not in the gametes (Brandeis et al. 1993). Why

methylation levels of *Igf2* decreased in fibroblasts derived from rescued ES cells is not clear at this time. It has been suggested that *Igf2* expression depends on the availability of two enhancers shared with the closely linked *H19* gene, *Igf2* being expressed only when the promoter of *H19* is methylated and inaccessible to its downstream enhancers (Bartolomei et al. 1993). In mice lacking the *H19* gene, a maternally transmitted deletion results in both the methylation and the transcription of *Igf2* (Leighton et al. 1995a), whereas in mice lacking the downstream enhancers, a paternally transmitted deletion results in an abrogation of *Igf2* transcription from that allele (Leighton et al. 1995b). In this study methylation was restored to *Igf2*, but little expression of the gene was seen in differentiated ES cells, similar to the *Dnmt* mutant ES cells and embryos. Our results support the idea that the methylation and expression status of *H19* is the primary determinant of *Igf2* expression, and that methylation of *Igf2* in the region examined does not affect its expression.

Our observations are of general significance in defining the stage in development for, and the mechanisms involved in, the establishment of genomic imprinting. Figure 6 contrasts the developmental changes in methylation of bulk DNA and *Xist* with that of the imprinted genes *H19* and *Igf2r*. Although it is generally assumed that imprinted genes acquire their methylation mark during gametogenesis, no direct evidence of an enzyme activity involved in gamete-specific methylation has been demonstrated. Our results show conclusively that passage through the male germ line is necessary to obtain the allele-specific methylation mark on at least one imprinted gene, *H19*. This suggests the existence of a male-specific de novo methyltransferase activity that may specifically recognize all paternally modified imprinted genes. Presumably a female-specific de novo methyltransferase activity exists, which would be active during oogenesis and would be expected to recognize maternally imprinted genes such as *Igf2r*. Although other results strongly suggest that the de novo methyltransferase expressed in ES cells is an independently encoded enzyme (H. Lei, S.P. Oh, R. Jüttermann, R. Jaenisch, K.A. Goss, and E. Li, in prep.), we have no evidence whether the gamete-specific activities are encoded by separate genes, are attributable to modification of the maintenance MTase or the separately encoded de novo methyltransferase activity (H. Lei, S.P. Oh, R. Jüttermann, R. Jaenisch, K.A. Goss, and E. Li, in prep.), or are reflective of a change in chromatin structure affecting substrate accessibility.

It is of interest to compare the rescued mutant ES cells with cells derived from androgenetic (AG) or parthenogenetic (PG) embryos (Fundele and Surani 1994). Chimeras between wild-type embryos and either PG- or AG-derived embryonic cells are generally inviable and show highly skewed and complementary distribution of the uniparental cells to different tissues of the chimera (Thomson and Solter 1988; Fundele et al. 1989, 1990; Nagy et al. 1989; Mann et al. 1990). The phenotype of the chimeras has been proposed to be caused by unbalanced

Figure 6. (A) Developmental changes in methylation of bulk DNA and imprinted genes. Bulk DNA and nonimprinted genes undergo consecutive waves of demethylation and de novo methylation in early development before the adult somatic patterns are established (Razin and Shemer 1995). *Xist* is depicted as nonimprinted as its expression is only imprinted in extraembryonic tissues. The monoallelic methylation patterns of the imprinted genes *H19* and *Igf2r* are established in the male and female gametes, respectively, and are not altered throughout development. (B) Methylation is almost completely erased for all DNA sequences examined in *Dnmt* mutant ES cells and embryos. Expression of the *Dnmt* cDNA in mutant ES cells restores the methylation of bulk DNA and single-copy genes such as *Xist* but does not restore methylation to imprinted genes such as *H19* and *Igf2r*. Methylation of these genes is restored only after transmission through the germ line of male and female chimeras, respectively, implying the presence of gamete-specific DNA methylation processes for imprinted genes. The degree of shading in the boxes indicates the extent of DNA methylation. Maternally and paternally derived alleles are both displayed for *H19* and *Igf2r*.

expression of the imprinted genes in the uniparental component (Allen et al. 1994; Szabo and Mann 1994). In contrast to PG- or AG-derived embryonic cells, the rescued ES cells described here have methylation erased at both paternally and maternally methylated loci, and the subsequent biallelic expression patterns of these genes resulted in a qualitatively different transcriptional profile. Chimeras produced from the rescued ES cells had extensive coat color contribution and were normal. Analysis of organ-specific contribution in chimeras and the possibility of producing embryos derived exclusively

from the rescued ES cells (Nagy et al. 1990) will allow us to fully assess the developmental potential of a mouse lacking an imprinted genome.

Materials and methods

Tissue culture

The wild-type J1 (Li et al. 1992), *Dnmt*^{n/n} clone 10, (Li et al. 1992), *Dnmt*^{s/s}, and *Dnmt*^{c/c} (Li et al. 1993; H. Lei, S.P. Oh, R. Jüttmann, R. Jaenisch, K.A. Goss, and E. Li, in prep.) ES cell lines were used. ES cell lines were cultured as described by Li et al. (1992). ES cells were induced to differentiate into embryoid bodies by suspension culture in bacteriological petri dishes, as described (Robertson 1987), and were collected at day 15 for analysis. A *Dnmt*^{s/s} EG cell line was derived from a cross between *Dnmt*^{s/s} mice. Primordial germ cells were cultured from gestational day 8.5 embryos on SF⁺-m220 feeder cells with 1000 U/ml of LIF and 20 ng/ml of bFGF, as described (Matsui et al. 1992). The three homozygous mutant ES cell lines and the EG cell line all showed equivalent molecular characteristics and functional capacity before and after transfection of the *Dnmt* cDNA, and they are collectively referred to as rescued ES cells in the text.

Fibroblasts were prepared by maceration and trypsinization of eviscerated, decapitated embryonic and postnatal carcasses and grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 0.1 mM nonessential amino acids (GIBCO), 0.1 mM β -mercaptoethanol, and antibiotics. SV40 virus infection was as described (Jat et al. 1986). To enrich for fibroblasts derived from the rescued ES cells, puromycin (Sigma) selection (2 μ g/ml) was applied after infection. Puromycin-selected populations were plated at 0.5–1.0 cell/well density to produce subcloned lines. Subclones were chosen from those plates with less than one colony per three wells. RNase protection experiments were performed on subcloned fibroblasts prepared from a P7 (P0 = day of birth) chimera made between a rescued *Dnmt*^{s/s} EG cell line (described above) and a BALB/c host. Methylation data were obtained from these cells, and fibroblasts prepared from two 3-month-old chimeras made with a rescued *Dnmt*^{s/s} ES cell line.

CHIP

All plasmids were prepared by alkali lysis from bacterial cultures and purified by centrifugation in continuous CsCl–ethidium bromide gradients (Sambrook et al. 1989). pMWT–PURO was made as follows. The *Dnmt* cDNA was derived from pMG (gift of T. Bestor, Columbia University, New York, NY), which comprises a 4934-bp *Dnmt* cDNA, starting at an *Eco*RI site 203 bp upstream of the first ATG and terminating at a *Bgl*II site 226 bp downstream of the stop codon TAG. This cDNA was inserted into pPGK–CAS, to utilize the *Pgk*-1 polyadenylation tract, which comprises the 500-bp *Pvu*II–*Hind*III fragment at the 3' end of the *Pgk*-1 cDNA (Adra et al. 1987). pPGK–CAS is described as the *Pgk*-1-driven expression vector in van der Lugt et al. (1991). A 4.8-kb *Dnmt* *Nae*I genomic fragment (Li et al. 1992) was cloned into a unique *Nae*I site in the open reading frame (ORF) of the *Dnmt* cDNA, such that the *Dnmt* cDNA sequence removed upstream of the *Nae*I site was restored in its genomic context. Finally, a *puro*^R cassette from pPgk–Puro was inserted downstream of the *Dnmt* cDNA. pPgk–Puro consists of the 660-bp *Hind*III–*Cl* fragment of pBabePuro (Morgenstern and Land 1990) cloned into pPGK–CAS through several intermediate cloning steps.

This construct was designed to act as an insertion vector (Thomas and Capecchi 1987) using a unique *Hind*III site (Fig.

1A), which lies 700 bp upstream of the *NaeI* site marking the genomic-cDNA junction and 4.1 kb downstream of the 5' end of the genomic *Dnmt* fragment. The construct was linearized at this site and then electroporated into ES cells. Upon homologous insertion at the *Dnmt* locus, the cDNA-genomic hybrid configuration of pMWT-PURO fused the *Dnmt* promoter and any missing upstream exons to the *Dnmt* cDNA. *Dnmt* mRNA transcripts were produced of the same size and amount as the endogenous gene, and they were spliced appropriately to allow production of the full-length protein. For electroporation, $\sim 10^7$ ES cells were trypsinized and washed and resuspended in 600 μ l of electroporation buffer, made as described (Thomas and Capecchi 1987), except with 137 mM NaCl and 10 μ g of linearized pMWT-PURO. The cell-DNA mixture was electroporated in a Bio-Rad Gene Pulser at 400 V and 25 μ F. The cells were plated on 10-cm dishes preplated with γ -irradiated, puromycin-resistant embryonic fibroblasts prepared from pPgk-Puro transgenic mice (kind gift of E. Simpson, Jackson Laboratory, Bar Harbor, ME). Media containing 2 μ g/ml of puromycin (Sigma) were changed every day, and isolated colonies picked 7–9 days after electroporation. DNA and RNA were analyzed by standard procedures (see below).

Southern blot analysis

Genomic DNA was prepared from cell cultures and pup tails as described (Laird et al. 1991). Ten micrograms of DNA was digested with 40 units of restriction endonuclease, except for *HhaI* and *HpaII*, which were used at double this amount. Digested DNA was electrophoresed in agarose gels, and blotted onto Zetabind nylon membranes (Cuno Laboratory Products). Usual Southern blot procedures were used (Sambrook et al. 1989), with hybridization buffer (Church and Gilbert 1984) and [α - 32 P]dCTP-labeled probe (Feinberg and Vogelstein 1983) prepared as described. Blots were washed at 65°C with $0.1 \times$ SSC/0.1% SDS, and exposed to AR-X film (Kodak). The following probes were used: HV is a 600-bp *HindIII*-*EcoRV* fragment of genomic DNA that includes the first exon of *Dnmt*; pMO contains a 5.5-kb MoMuLV cDNA fragment (Jähner et al. 1982); pMR150 contains a minor satellite centromeric repeat (Chapman et al. 1984; gift of V. Chapman, Roswell Park Memorial Institute, Buffalo, NY); pBSIIISac contains a 3.8-kb genomic *SacI* fragment from a region of the *H19* gene starting 750 bp upstream of the transcriptional start site, identical to the probe used in Tremblay et al. (1995) (generously donated by M. Bartolomei, University of Pennsylvania, Philadelphia); p1269 contains a 720-bp *EcoRI*-*XbaI* genomic *Igf2* fragment whose 5' end is ~ 4.5 kb from the first exon, identical to probe 2 in Feil et al. (1994) (gracious gift of S. Tilghman, Princeton University, NJ); pPP4 contains a 1.1-kb *BstBI*-*MluI* genomic *Igf2r* fragment located within the intronic region 2, identical to the probe used in Stöger et al. (1993) (gift of D. Barlow, IMP, Vienna, Austria); and pXistMc1 contains a 1.5-kb *Xist* cDNA fragment that includes the transcriptional start site, 1.5 kb of exon 1, and the gamete-specific methylation sites as reported in Ariel et al. (1995) and Zuccotti and Monk (1995) (provided by H. Willard, Case Western Reserve University, Cleveland, OH). A mitochondrial probe was used to assay for completeness of *HhaI* and *HpaII* digestions, because the mitochondrial genome does not become methylated. This was a gift of P. Laipis (University of Florida, Gainesville) and comprises bp 4013 to 7720 of the published sequence from mouse L cells (Bibb et al. 1981).

Northern blot analysis

RNA was prepared as described (Chomczynski and Sacchi 1987), from confluent ES cell cultures passaged without a fibro-

blast feeder layer. Denatured RNA was electrophoresed in formaldehyde-containing agarose gels and blotted onto Zetabind nylon membranes (Cuno) as described (Sambrook et al. 1989). Hybridization, washing, and exposure occurred as described above for DNA analysis. The *Dnmt* cDNA from pMG was used for a probe, and an α -tubulin cDNA probe (pIL α T1) (Lemischka et al. 1981) was used to control for loading amounts.

RNase protection assays

Assays were performed as described for *H19*, *Igf2*, and *Igf2r* (Li et al. 1993), and for *Xist* (Beard et al. 1995).

Teratoma formation and generation of germ-line chimeras

Teratomas were produced by preplating 3×10^6 ES cells for 30 min to remove embryonic fibroblasts, resuspending them in 300 μ l of PBS, and injecting them subcutaneously into the flanks of adult male 129/Sv mice. Wild-type J1, *Dnmt*^{+/+}, and rescued *Dnmt*^{+/+} EG and *Dnmt*^{-/-} ES cells were used. Tumors were excised at 3 weeks. Chimeras were made as described (Li et al. 1992), except that two to three ES or EG cells were injected into blastocysts. Male chimeras derived from the two rescued *Dnmt*^{+/+} ES cell lines were mated to BALB/c females, and progeny were analyzed with Southern blot and RNase protection assays.

Acknowledgments

We thank Klaus Willecke and Edwin Cuppen for pMWT-Puro, Elizabeth Simpson for providing pPgk-Puro transgenic mice, Tim Bestor for pMG, Shirley Tilghman for the *H19* and *Igf2* probes, Denise Barlow for the *Igf2r* probes, Marisa Bartolomei for the *H19* probe, Huntington Willard for the 5' *Xist* probe, Argiris Efstratiadis for the *Igf2* probes, Verne Chapman for the centromeric repeat probe, Nathalie van der Lugt for pPGK-CAS, Michael Rudnicki for pIL α T1, and Phil Laipis for the mitochondrial DNA probe. L.J.-G. was supported by a Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation Fellowship (DRG-1223). P.W.L. was the recipient of a National Service Research Award (F32 CA 09097) from the National Cancer Institute. E.L. was supported by a grant from Bristol-Myers and Squibb. R.J. was supported by National Institutes of Health grant R35-CA44339.

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Germ-line passage is required for establishment of methylation and expression patterns of imprinted but not of nonimprinted genes.

K L Tucker, C Beard, J Dausmann, et al.

Genes Dev. 1996, **10**:

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