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DNA Methylation, Genomic Imprinting, and Mammalian Development

E. Li,* C. Beard,† A.C. Forster,‡ T.H. Bestor,‡ and R. Jaenisch†
*Massachusetts General Hospital - East, Cardiovascular Research Center, Charlestown, Massachusetts 02129; †Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142; ‡Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115

About 60% of all CpG dinucleotides in the DNA of vertebrates are methylated at the C5 position, but the frequency with which the modified base is found at particular sites varies between cell types. These methylation patterns are transmitted by clonal inheritance (Wigler et al. 1981) through the strong preference of mammalian DNA (cytosine-5)-methyltransferase (DNA MTase) for hemimethylated DNA (Gruenbaum et al. 1982; Bestor and Ingram 1983). Methylation patterns are established during gametogenesis and early embryogenesis (Jähner et al. 1982; Chaillet et al. 1991), although little is known of the molecular mechanisms that control sequence-specific de novo methylation and demethylation. The cDNA coding for the murine MTase has been cloned, and sequence analysis (Bestor et al. 1988) has shown that the enzyme contains a carboxy-terminal catalytic domain of 500 amino acids (Lauster et al. 1989; Posfai et al. 1989) linked to an amino-terminal regulatory domain of 1000 amino acids (Bestor et al. 1988; Bestor 1990). The enzyme is associated with the replication foci of S-phase cells (Leonhardt et al. 1992), and recent results have demonstrated that high levels of MTase are present in oocytes and preimplantation embryos (Carlson et al. 1992).

Numerous studies have shown that the transcriptional control regions of genes are undermethylated in tissues where the gene is expressed relative to the same sequences in tissues where the gene is not expressed. Several lines of evidence support the notion that tissue-specific methylation patterns may be involved in the control of developmental gene regulation. (1) Changes in methylation status (usually the loss of methylated cytosines from promoter regions) are correlated with the activation of many tissue-specific genes during differentiation in vivo (for review, see Cedar 1988). (2) Gene reactivation occurs in certain cell types upon treatment with 5-azacytidine (azaC), an inhibitor of cytosine methylation (Jones et al. 1983). (3) Alleles on the inactive X chromosome are often methylated in promoter regions, whereas alleles on the active X chromosome are normally unmethylated (for review, see Grant and Chapman 1988). (4) Allele-specific methylation has also been observed for many imprinted transgenes which are inherited in the unmethylated state from parents of one sex and in the methylated state from parents of the other sex (Hadchouel et al. 1987; Reik et al. 1987; Sapienza et al. 1987; Swain et al. 1987; Sasaki et al. 1991). The functional role of DNA methylation in genomic imprinting was strengthened by the recent finding that three imprinted genes, *H19*, *Igf2*, and *Igf2r*, are differentially methylated depending on the parental origin of inheritance (Sasaki et al. 1992; Ferguson-Smith et al. 1993; Stöger et al. 1993).

Despite a large body of evidence, the importance of cytosine modification in vertebrate gene control remains a controversial issue because of the necessarily indirect or correlative nature of many of the studies in the field. For example, tissue-specific genes in established lines of cultured cells often undergo de novo methylation at sites that are not methylated in animal tissues (Antequera et al. 1990), and genes that are demethylated and reactivated in cultured cells treated with azaC have been found to be unmethylated in nonexpressing tissues (Jones et al. 1990). Although most experimental evidence is consistent with cytosine modification playing an important role in developmental gene expression, it is worth pointing out that small-genome metazoa such as *Drosophila* and *Caenorhabditis elegans* develop in the apparent absence of cytosine modification (Urieli-Shoval et al. 1982; Simpson et al. 1986). Direct tests of the importance of DNA modification in mammalian gene control and development are needed to address the issue.

In this paper, we summarize our efforts to assess the importance of DNA methylation in vertebrate development and genomic imprinting. Using gene targeting in embryonic stem (ES) cells, we have generated mice that are unable to maintain normal levels of CpG methylation due to a defect in the DNA MTase gene. ES cells carrying two mutant MTase alleles are viable, whereas mutant embryos die at midgestation with their genomic DNA substantially demethylated. To assess the role of DNA methylation in genomic imprinting, we have examined the expression of the three imprinted genes, *H19*, insulin-like growth factor 2 (*Igf2*), and *Igf2* receptor (*Igf2r*), in mutant mice. Expression of all three imprinted genes was affected in mutant embryos, demonstrating that maintenance of DNA methylation is required for differential expression of

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imprinted genes during embryogenesis. The results summarized in this paper have been published in Li et al. 1992, 1993).

RESULTS

Generation of ES Cell Lines Carrying a Targeted Mutation in Both MTase Alleles

The DNA MTase gene was disrupted by homologous recombination as described previously (Li et al. 1992). The targeting vector contained a 900-bp deletion extending from the *Nae*I site just upstream of the translation start site (Fig. 1) to a second *Nae*I site in an intron (Li et al. 1992). Upon homologous recombination, 20 bp of 5′ untranslated sequence, the first 27 codons, and the 5′ splice site at the end of the first coding exon were deleted from the targeted allele. To generate cells homozygous for the mutation, the remaining wild-type allele was disrupted by a second round of gene targeting using a targeting vector carrying the hygromycin resistance gene as a selectable marker. Two clones, clones 10 and 52, were identified that had undergone homologous recombination in both alleles of the DNA MTase gene. Southern analysis with flanking probes confirmed clean homologous recombination events in both alleles of clone 52, but revealed that an unexpected DNA rearrangement had occurred in clone 10 at the integration site of the pMT(N)hyg vector (see Li et al. 1992).

DNA MTase Activity and Genomic DNA Methylation in Homozygous Mutant ES Cells

The homozygous mutant cells showed normal morphology and growth rates in tissue culture with no discernible phenotype after more than 30 rounds of cell division. Western analysis revealed that no full-length MTase polypeptide was synthesized in the homozygous mutant cells, but small amounts of a form of the enzyme having slightly higher electrophoretic mobility were detected in both homozygous mutant lines (Li et al. 1992). Enzyme assays were performed to test for DNA MTase activity in lysates of homozygous mutant cells (Li et al. 1992). As shown in Figure 2, DNA methylase activity was reduced to about 50% in the heterozygous cells and severely reduced in the homozygous cells. In homozygous mutant ES clone 10, which has an unexpected DNA rearrangement at the site of integration in one allele, enzyme activity was lower than in ES clone 52, which has the predicted sequence arrangement at both alleles. These data suggest that the mutant allele targeted by pMT(N)hyg in clone 10 may contain a more severe mutation than the counterpart in clone 52.

To assess whether the mutation affected the overall level of 5-methylcytosine (m5C) in genomic DNA, we analyzed endogenous retroviruses which are present in multiple copies and are known to be highly methylated (Stuhlmann et al. 1981). Figure 3 shows that digestion of DNA from wild-type (lane 2) and heterozygous cells (lanes 4 and 5) with *Hpa*II resulted only in bands of high molecular weight, whereas digestion with *Msp*I yielded multiple small fragments (lane 1). In contrast to wild-type or heterozygous ES cells, DNA from the homozygous ES cells yielded small fragments when digested with *Hpa*II (lanes 3 and 6). These results indicate that the MTase mutation resulted in substantial demethylation of genomic DNA. We note that the level of retroviral DNA methylation was slightly lower

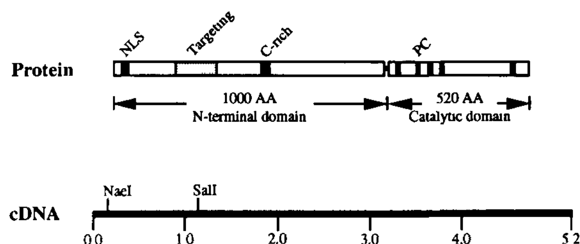

Figure 1. The structure of the DNA MTase protein and cDNA. DNA MTase consists of two domains linked by a run of alternating lysyl and glycyl residues. The carboxy-terminal domain is closely related to bacterial DNA (cytosine-5) restriction methyltransferases; filled squares in the diagram indicate 5 conserved motifs (Bestor et al. 1988; Lauster et al. 1989; Posfai et al. 1989). The amino-terminal domain contains a nuclear localization signal (NLS), a domain required for targeting the MTase protein to the sites of DNA replication (Targeting), and a cysteine-rich region (C-rich). The *Nae*I site 20 bp upstream of the putative translation initiation codon and a *Sal*I site at codon 330 were disrupted by the integration of the neomycin gene through homologous recombination.

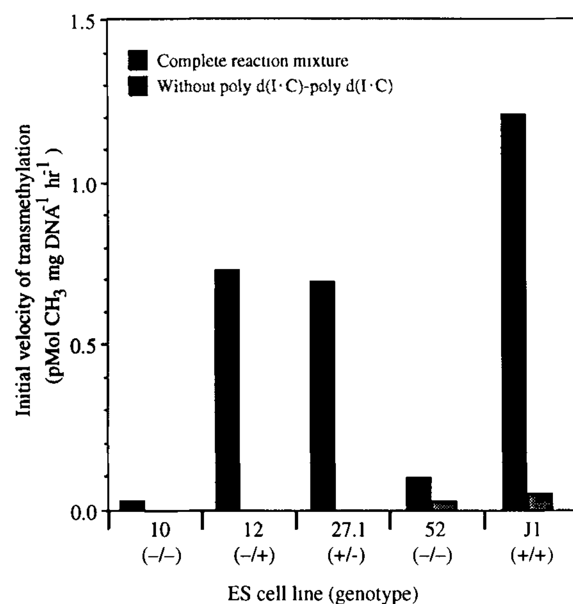

Figure 2. DNA MTase enzyme activity in wild-type and mutant ES cell lysates. Cell lysates were prepared and enzyme assays were carried out as described previously (Li et al. 1992). Heterozygous mutants can be seen to have lower, and the homozygous mutants severely reduced, levels of enzyme activity; in agreement with the immunoblot studies, clone 52 had more activity than clone 10.

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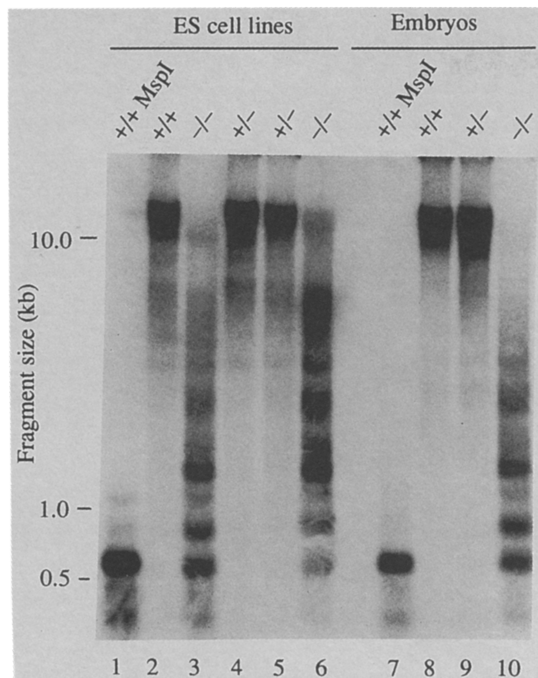

Figure 3. Southern blot analysis of DNA methylation of endogenous retroviral genome in wild-type and mutant ES cell lines and embryos. DNA from ES cell lines or day-10.5 embryos was digested with the methylation-sensitive restriction endonuclease *Hpa*II and hybridized with Mo-MLV cDNA. As a control, DNA from wild-type ES cells (J1 in lane *1*) or a wild-type embryo (lane *7*) was digested with a methylation-insensitive isoschizomer, *Msp*I. Lanes *2–6* contain *Hpa*II-digested DNA from clones J1, 10, 12, 27.1 (12 and 27.1 are heterozygous ES cell lines), and 52, respectively. Comparison of intensity of bands between 0.5 kb and 1 kb in lanes *3* and *6* shows that DNA from cell line 10 appears to be more sensitive to *Hpa*II than DNA from line 52, in agreement with the lower levels of DNA MTase observed in line 10. Lanes *8–10* contain *Hpa*II-digested DNA from wild-type, heterozygous, and homozygous embryos, respectively.

in clone 10 ES cells than in clone 52 cells (compare lanes 3 and 6 of Fig. 3), which would be predicted if the mutation in clone 10 was more severe than that in clone 52.

Altered Splicing of the Pre-mRNA Transcribed from the Disrupted DNA MTase Gene

As described above, the disrupted DNA MTase gene underwent a deletion of about 1 kb of genomic sequence lying between *Nae*I sites near the 5′ end of the transcription unit; the deleted sequences were replaced with a *Pgk1*-neomycin resistance cassette (Li et al. 1992). The deletion affects one exon and removes peptide sequences that have no known function (Leonhardt et al. 1992). Northern hybridization and S1 nuclease analysis showed the presence of an altered form of mRNA derived from the mutant allele (C. Beard, unpubl.). Reverse transcription-polymerase chain reaction (RT-PCR) was used to amplify and clone the alternative forms of DNA MTase mRNA that might encode the slightly faster migrating band seen on immunoblots (Fig. 4) and might be responsible for the residual DNA MTase activity found in homozygous mutant ES cells (Fig. 2).

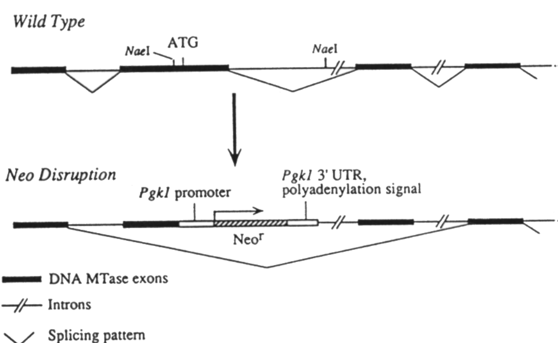

Figure 4. An altered splicing pattern in the DNA MTase pre-mRNA transcribed from the disrupted DNA MTase gene. Purified RNA was subjected to RT-PCR, and the products were cloned into pDK101 as described previously (Kovalik et al. 1991) and sequenced by the dideoxy chain termination method. In the pre-mRNA derived from the disrupted allele, splicing occurs between the first and fourth exons shown in the figure; the reading frame is preserved by the splice. The exons shown in the diagram comprise 500 nt. There are no in-frame ATG codons in the region shown, so translation initiation may occur at a non-ATG codon. The lengths of the second and third introns are not known.

Analysis of RT-PCR products showed that an altered splicing pattern deleted the exon that was disrupted by the neo cassette, and the next 3′ exon (Fig. 4; A.C. Forster and T.H. Bestor, unpubl.). A total of 363 nucleotides were deleted from the mRNA, which is consistent with the reduced apparent mass of the immunoreactive protein seen on immunoblots (Fig. 5). The deletion also removes the first in-frame ATG codon, which was regarded as the most likely translational start site (Bestor et al. 1988). This finding sug-

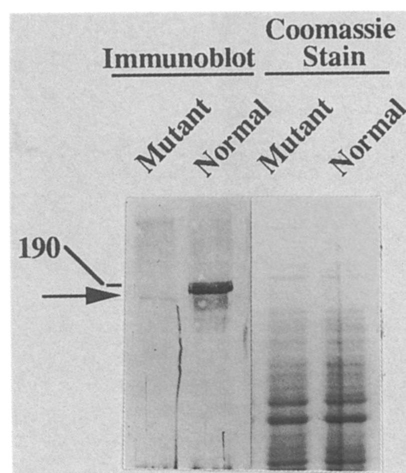

Figure 5. Immunoblot analysis of wild-type and mutant embryos. The immunoblot to the left shows that the normal M_r 190,000 species of DNA MTase is absent from the mutant embryos and replaced by small amounts of a slightly faster-migrating protein. On the right is a Coomassie blue R250-stained gel of the extracts used in preparation of the immunoblot.

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gests that there is another start codon in the DNA MTase mRNA and that the normal start site may be a non-ATG codon.

The results of the RT-PCR studies support the notion that the MTase mutation is a partial loss-of-function mutation, as originally proposed by Li et al. (1992). A form of DNA MTase mRNA which lacks only 363 nucleotides is produced through the use of an altered splicing pattern; the peptide sequences encoded by the deleted sequences have no known function, and the affected sequences are not strongly conserved between mouse and human DNA MTase. We conclude that this mutation allows the production of an essentially normal DNA MTase at about 5% of the level of the wild-type protein.

DNA Methylation Is Essential for Embryonic Development

Mice carrying the mutation in the germ line were generated by injection of targeted ES cell clones into blastocysts. Whereas heterozygous animals were phenotypically normal, embryos that were homozygous for the mutation died by day 11 of gestation. When isolated at day 10.5, mutant embryos were stunted and of a developmental stage characteristic of normal embryos at day 9.5 or younger. The best-developed homozygous mutant embryos had about 20 somites, distinct forelimb buds, and a closed anterior neuropore. Histological analysis revealed that major organ rudiments were present but smaller than in wild-type littermates. Close inspection revealed significantly increased numbers of dead or dying cells and considerably fewer mitotic figures in homozygous as compared to wild-type embryos (Li et al. 1992).

The m5C content of DNA from homozygous and normal embryos was analyzed by examining the methylation of *Hpa*II sites at endogenous retroviral loci as described above. Figure 3 shows a similar extent of demethylation in homozygous embryos as in the homozygous ES cell lines (compare lane 10 with lanes 3 and 6). No differences in methylation patterns were detected between heterozygous and wild-type embryos. As shown in Figure 5, an anti-MTase antibody detected a strong band of M_r 190,000 on immunoblots of lysates of normal embryos, whereas homozygous mutant embryos displayed small amounts of a slightly smaller protein. A similar result was observed in control and mutant ES cells (data not shown).

The data described so far indicate that mice carrying targeted mutations of the DNA MTase gene derived from two independently established ES clones showed similar developmental abnormalities and a recessive lethal phenotype. Both DNA and protein analyses are consistent with the hypothesis that the recessive lethal phenotype was caused by the mutation of the DNA MTase gene so as to preclude the normal establishment or maintenance of methylation patterns.

Normal DNA Methylation Is Required for Genomic Imprinting

The observations summarized in the introduction correlate differential expression of imprinted genes with the inheritance of parental-specific methylation patterns, but no direct evidence has been obtained so far linking the expression of imprinted genes with differential DNA methylation. We therefore examined the effect of reduced genomic methylcytosine levels on the expression of the *H19*, *Igf2*, and *Igf2r* genes. Analysis of RNA from mutant embryos indicated that the expression of all three genes was altered.

H19 *gene*. Previous work has shown that the paternal allele of the *H19* gene is silent and the maternal allele is expressed in normal embryos (Bartolomei et al. 1991). To assess the effect of a reduced level of DNA methylation on *H19* expression, RNA was isolated from normal and DNA MTase mutant embryos at embryonic day 10.5 and analyzed by Northern blot hybridization. Figure 6A shows that expression of the *H19* gene was significantly elevated in all four homozygous mutant embryos (Fig. 6A, lanes 2, 3, 4, and 7) when compared to wild-type and heterozygous embryos (lanes 1, 5, and 6). To determine whether this increased expression in mutant embryos was due to overexpression of the maternal allele or to the activation of the silent paternal allele, embryos with distinguishable paternal and maternal alleles were obtained by crossing 129/Sv mice heterozygous for the DNA MTase mutation with *Mus castaneus* (*M. cas.*) which carry a polymorphic marker on the H19 mRNA (Bartolomei et al. 1991). F_1 (129 × *M. cas.*) males heterozygous for the DNA MTase mutation were then crossed with heterozygous 129 females, and embryos were isolated at day 10.5. It was expected that 50% of the embryos would carry a paternally derived *M. cas.* *H19* allele, and a quarter of those would be homozygous for the DNA MTase mutation. The embryos were genotyped for the presence of the *M. cas. H19* allele and for the DNA MTase mutation by Southern analysis (data not shown), and only those embryos which inherited the *H19 M. cas.* allele were used in the RNA analysis. RNase protection assays were performed using an RNA probe which could distinguish the 129/*M. cas.* polymorphism in the H19 mRNA (Bartolomei et al. 1991). If the reduced genomic methylcytosine levels led to activation of the paternal allele, the *M. cas.* transcript, in addition to the 129 transcript, would be present in homozygous mutant embryos but not in wild-type or heterozygous embryos. Figure 6B shows an example of such an RNase protection assay, in which the paternal *M. cas.* allele was found to be expressed at the same level as the 129 maternal allele in homozygous embryos (Fig. 6B, lane 3), whereas only the 129 maternal allele was expressed in the heterozygous embryo (lane 2). These results indicate that unperturbed DNA methylation patterns

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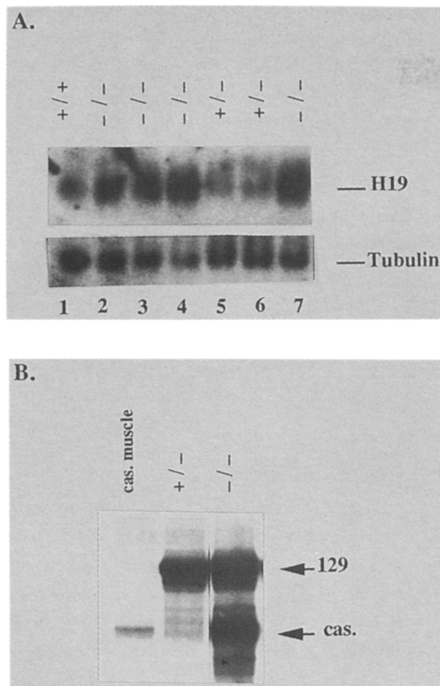

Figure 6. Expression of *H19* gene in DNA MTase mutant embryos.(*A*) Northern blot analysis of total RNA (10 μg per lane) from day-10.5 embryos hybridized with a 3-kb genomic *Eco*RI/*Sal*I fragment which spans the mouse *H19* gene and a tubulin cDNA probe as an RNA loading control. Embryos homozygous for the MTase^n mutation (lanes *2*, *3*, *4*, and *7*); heterozygous embryos (lanes *5* and *6*); and wild-type embryos (lane *1*). (*B*) RNase protection assay of total RNA (2 μg per lane) prepared from day-10.5 embryos either homozygous (−/−) or heterozygous (+/−) for the MTase mutation using a riboprobe generated from a clone containing the 754-bp *Bam*HI-*Stu*I genomic DNA fragment of the *H19* gene as described previously (Bartolomei et al. 1991). Only the allele-specific protected fragment (129 or cas., with arrows) from exon 5 is shown. Embryos are obtained from mating F_1(*M. cas.*/129) male mice heterozygous for the MTase^n mutation to 129 female MTase^n heterozygous mice, and genotyped for the presence of *M. cas.* allele of the *H19* gene and the MTase^n mutation. Embryos that carry a paternally inherited *M. cas.* allele and a maternally inherited 129 allele of the *H19* gene were used for the RNase protection assay. Muscle RNA from an adult *M. cas.* mouse was included as a control.

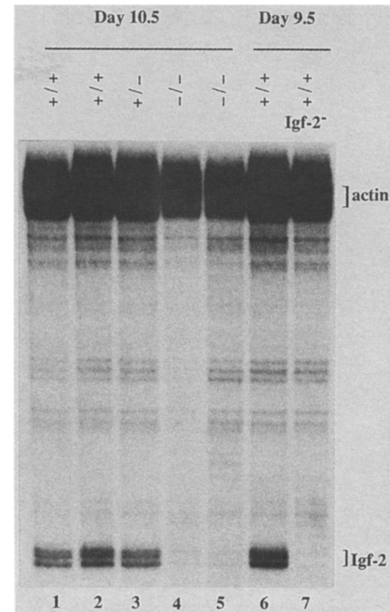

Figure 7. RNase protection assay of expression of the *Igf2* gene. Total cell RNA was prepared from embryos. Samples of RNA (10 μg per lane) from day-10.5 embryos homozygous for the DNA MTase mutation (lanes *4* and *5*), wild-type (lanes *1* and *2*), and heterozygous (lane *3*) littermates, and day-9.5 wild-type control embryos (lanes *6* and *7*) were analyzed by RNase protection using the E2 probe containing exon 2 of the *Igf2* gene and the mouse cytoplasmic actin control probe. The assay was performed according to the method of DeChiara et al. (1991). RNA from a day-9.5 embryo carrying a paternally inherited disrupted allele of the *Igf2* gene (Igf-2$^-$, lane *7*) represents a negative control for *Igf2* expression, as exon 2 is deleted in the paternal allele, and the maternal allele is transcriptionally silent.

are essential for maintaining the transcriptionally inactive state of the paternal *H19* allele.

Igf2 ***gene.*** The *Igf2* gene is closely linked to the *H19* gene but reciprocally imprinted, i.e., expressed only from the paternal allele (DeChiara et al. 1991). To examine *Igf2* expression in DNA MTase-deficient embryos, RNA was isolated from day-10.5 mutant and control embryos and analyzed by RNase protection as described previously (DeChiara et al. 1991). Figure 7 shows that although the *Igf2* gene was expressed in wild-type and heterozygous embryos (lanes 1–3), its expression was almost undetectable in the homozygous embryos (lanes 4 and 5). Because the development of DNA MTase mutant embryos at day 10.5 is delayed by approximately one day when compared to normal littermates (Li et al. 1992), we also determined *Igf2* expression in younger embryos. Figure 7, lane 6 shows that *Igf2* was expressed in wild-type day-9.5 embryos at the same level as in day-10.5 embryos. Our results, therefore, suggest that a normal level of DNA methylation is required for the expression of the active paternal allele.

Igf2r ***gene.*** It has been shown that the *Igf2r* gene, which is located on chromosome 17, is expressed exclusively from the maternal allele (Barlow et al. 1991). To examine whether *Igf2r* expression was also affected by a reduction in DNA methylation, RNA was isolated from day-9.5 embryos and analyzed by RNase protection. Figure 8 shows that *Igf2r* expression was detected at almost the same level in the DNA MTase mutant embryos (lane 2) as in the wild-type embryos (Fig. 8, lane 1). Similar results were obtained with day-10.5 embryos (data not shown). This indicated that *Igf2r* transcription, in contrast to transcription of the *H19* and the *Igf2* genes, was unaffected by the DNA MTase mutation, suggesting that the maintenance of the *Igf2r* gene imprint might not involve DNA methylation.

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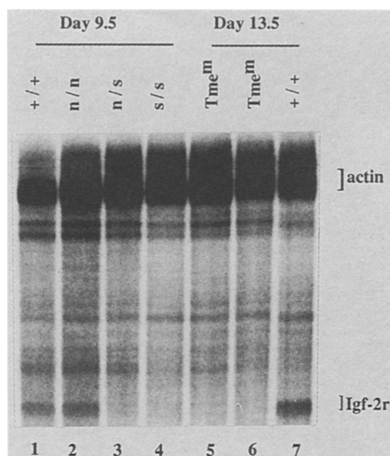

Figure 8. RNase protection analysis of *Igf2r* expression. Embryos were genotyped by Southern blot analysis of DNA from half of the yolk sac membranes using pBB (for MTase^n) and pHH (for MTase^s) probes. Embryos plus the other half of the yolk sac membranes of identical genotype were pooled for RNA preparation. Samples of RNA (10 μg per lane) from day-9.5 wild-type (lane *1*), MTase^n homozygotes (lane *2*, n/n), compound heterozygotes MTase^n/MTase^s (lane *3*, n/s), and MTase^s homozygotes (lane *4*, s/s), and day-13.5 Tme^m (lane *5* and *6*) and wild-type (lane *7*) embryos (RNA kindly provided by Denise Barlow) were analyzed by RNase protection. Tme^m stands for maternally inherited Tme mutation, which contains a large chromosomal deletion spanning the *Igf2r* gene. RNA from Tme^m embryos serves as negative control because the maternally inherited *Igf2r* allele is deleted, and the paternal allele is silent. The probe was prepared from a plasmid SN that contains a 347-bp *Sma*I-*Not*I fragment comprising the transcription start sites and part of the first exon of the *Igf2r* gene (Stoger et al. 1993). A 190-base antisense riboprobe was synthesized in vitro from a *Hin*fI linearized DNA template using T3 RNA polymerase and [^{32}P]CTP. The size of the protected fragment was 155 bases.

Alternatively, it seemed possible that the maintenance of the *Igf2r* gene imprint, although dependent on DNA methylation, was quantitatively less affected by demethylation of genomic DNA than that of the other two imprinted genes. In this context, it is significant to emphasize that the DNA MTase mutation, generated by insertion of a targeting vector into the *Nae*I site at the 5′ end of the DNA MTase gene (termed MTase^n allele), is a partial loss-of-function mutation which resulted in a reduced but stable level of genomic methylcytosine in homozygous MTase^n/MTase^n ES cells (see above). To address whether the level of DNA methylation was critical for the maintenance of the *Igf2r* imprint, we used a second mutant allele that was generated by targeting the DNA MTase gene further downstream at a *Sal*I site (termed MTase^s) corresponding to amino acid residue 330 (see Fig. 1). In contrast to MTase^n/MTase^n embryos, which survive up to the 20-somite stage, embryos homozygous for the DNA MTase^s allele develop to the 5–10-somite stage at day 9.5 and die around day 10. Their genomic DNA is much less methylated than DNA of homozygous MTase^n/MTase^n embryos (E. Li et al., unpubl.). The MTase^s allele is, therefore, a more severe mutation than the MTase^n allele and possibly represents a null mutation. RNA was isolated from day-9.5 MTase^n/MTase^s and MTase^s/MTase^s mutant embryos and tested for *Igf2r* expression by RNase protection. As shown in Figure 8, the *Igf2r* gene was repressed in both MTase^n/MTase^s and the MTase^s/MTase^s mutant embryos (lanes 3 and 4), in contrast to the MTase^n/MTase^n embryos. Because development of the MTase^s/MTase^s mutant embryos is delayed by approximately one day as compared to wild-type embryos, RNA isolated from wild-type day-8.5 embryos was analyzed as a control and found to contain a similar level of expression as in day-9.5 embryos. These results indicate that DNA methylation is required for expression of the maternal *Igf2r* allele. *Igf2r* gene expression appears, however, to be quantitatively less sensitive to DNA demethylation than the *Igf2* gene expression.

DISCUSSION

Mutant ES Cells with Reduced Levels of DNA Methylation

The murine DNA MTase gene was mutated by introduction of a deletion that removed part of the first coding exon. ES cell lines homozygous for the mutation proliferated normally and were morphologically indistinguishable from wild-type cells, despite a reduction of genomic m5C to about one-third of the wild-type level. Substantial demethylation of restriction sites within the multiple, highly methylated copies of retrovirus DNA in the genome of homozygous ES cells was also observed. The reduction in m5C content did not detectably change with propagation of the cells.

The retention of significant amounts of m5C in the DNA of the homozygous mutant ES cells indicates that methyltransferase activity is expressed in mutant cells. We consider two possibilities to account for the reduced but stable methylation levels in the genomic DNA of homozygous mutant ES cells. (1) The mutation at the *Nae*I site did not result in a null mutation and the residual enzyme activity produced by the mutant allele was sufficient to maintain the lower level of methylation. Indeed, RNA analyses showed that altered forms of mRNA were produced in mutant cells, which could give rise to an active enzyme by in-frame alternative splicing around the disrupted exon. Small amounts of a protein are detectable by anti-DNA MTase antibodies on immunoblots of mutant cell and embryo lysates. Independent genetic evidence supports the conclusion that the MTase^n allele was a partial loss-of-function mutation. The targeted insertion mutation (MTase^s) at a downstream *Sal*I site that lies in a region of the DNA MTase gene which is predicted to encode an essential domain resulted in a more severe phenotype: Embryos homozygous for this anticipated severe loss-of-function mutation die at earlier stages than do those homozygous for the MTase^n allele (E. Li, unpubl.). The DNA of mutant embryos is almost completely de-

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methylated, consistent with the MTase^s allele possibly representing a null mutation of the DNA MTase gene. (2) Alternatively, the observed m5C levels in the homozygous mutant cells could be due to another, previously uncharacterized DNA methyltransferase. For example, de novo methylation in early development (Jähner et al. 1982; Shemer et al. 1991), in gametogenesis (Chaillet et al. 1991), and in cultured embryonal carcinoma cells (Stewart et al. 1982) could be the result of an embryo-specific form(s) of DNA MTase which is not active in somatic cells. Such an enzyme might be able to maintain the reduced but stable level of DNA methylation in homozygous mutant ES cells, but be unable to sustain methylation patterns in differentiating somatic tissues of mutant embryos. Support for this hypothesis is provided by the recent derivation of ES cells which are homozygous for the MTase^s allele. These cells, similar to MTase^n homozygous cells, proliferate normally but exhibit a level of genomic methylation which is stable but slightly lower than in the homozygous N allele clone 10 cells (E. Li, unpubl.). If the MTase^s allele represents a null mutation of the known DNA MTase gene, these results strongly support the notion that embryonic cells express a methyltransferase which is different from the mutated DNA MTase and which is able to stably maintain the reduced m5C level in the homozygous cells. However, it has not yet been proven that the DNA MTase mutation represents a null allele.

DNA Methylation and Genomic Imprinting

Although numerous experiments have correlated DNA methylation with gene repression and demethylation with gene activation (Bird 1986; Cedar 1988), experimental proof that DNA methylation controls the activity of cellular genes in vivo has been lacking. The activation of the normally silent paternal *H19* allele in DNA MTase-deficient embryos provides the first in vivo evidence for a causal link between DNA methylation and gene activity. It has been shown recently that the CpG island in the promoter region of the paternal *H19* allele becomes de novo methylated after fertilization (Ferguson-Smith et al. 1993). The simplest interpretation of our results is that interference with the maintenance of this CpG island methylation leads to activation of the gene and loss of the imprint.

In contrast to the activation of the *H19* gene, it is less clear how genomic DNA demethylation could lead to the inactivation of the expressed *Igf2* and *Igf2r* alleles. Unlike *H19*, the closely linked *Igf2* gene shows no obvious parental-origin-specific methylation of the promoter CpG island (Sasaki et al. 1992). It has been proposed that expression of the two reciprocally imprinted genes *H19* and *Igf2* is functionally and/or mechanistically related and that the imprinting of a single chromosomal site might control the activity of both genes (Zemel et al. 1992). Our results are consistent with the possibility that *H19* is the "primary" imprinted gene whose activity is directly controlled by the parental-specific methylation. *H19* transcription may, in turn, suppress in *cis* the expression of the closely linked *Igf2* gene either by competing for a common set of regulatory elements shared by the *Igf2* gene or by directly inhibiting the transcription of the *Igf2* gene. Mutually exclusive expression is also seen in X chromosome inactivation, where the *Xist* gene, located at the X inactivation center, is expressed exclusively from the inactive X chromosome (Brockdorff et al. 1992). It has been suggested that the Xist mRNA, which lacks a conserved open reading frame (as does the H19 mRNA), may act in *cis* to cause X chromosome inactivation (Kay et al. 1993).

It is interesting to consider the repression of the *Igf2r* gene in MTase mutant embryos in relation to a recently proposed model that identified a downstream CpG island as the "imprinting box" (Stöger et al. 1993). It was shown that this island becomes specifically methylated in the expressed maternal allele during oogenesis. Stöger et al. hypothesized that the maternal-specific methylation of this box may represent the primary imprinting signal for the maternal *Igf2r* allele and that the CpG island may act as a "gene silencer," for example, by binding a repressor protein when not methylated and thus inhibiting expression of the paternal allele. Methylation of CpG islands may play a crucial role in establishing and maintaining genomic imprinting patterns (Bird 1993). Methylated CpG islands have also been detected on the inactive X chromosome and have been suggested to stabilize repression (Riggs and Pfeifer 1992). Methylation of CpG islands may be a general mechanism to control differential expression of identical alleles of genes such as imprinted and X-linked genes.

Our results clearly establish that DNA methylation plays a critical role in genomic imprinting. More specifically, DNA methylation is required for maintaining monoallelic expression of three imprinted genes, *H19*, *Igf2*, and *Igf2r*, during embryonic development. It will be particularly interesting to study whether methylation changes are involved in the formation of Wilms' tumors, which in some cases show biallelic expression of the *H19* and *Igf2* genes (Ogawa et al. 1993; Rainier et al. 1993).

DNA Methylation and Embryonic Development

The phenotype caused by integration of pMT(N)neo at the DNA MTase gene was recessive lethality; homozygous embryos failed to develop beyond the stage characteristic of normal day-9.5 embryos and died prior to day 11. Histological analysis showed that major organ rudiments, such as heart, brain, and in some cases forelimb buds, were present but less well developed than gestational age would indicate. Homozygous embryos also showed an increased number of dead cells and few mitotic figures among live cells. The m5C level in DNA from day-10 homozygous embryos was found to be 30% of that of wild-type embryo DNA. The finding that a threefold reduction in m5C com-

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pletely prevented development beyond midgestation suggests an essential role for DNA modification in normal mammalian development.

The obvious phenotype at the histological level in homozygous embryos was widespread cell death and reduced cell proliferation. The underlying cause of cell lethality is not known, although one possibility is inappropriate gene expression. Overexpression of *H19* in transgenic mice has been shown to result in embryonic lethality at day 14, but it appears unlikely that deregulation of imprinted genes would lead to a cell-lethal phenotype (Brunkow and Tilghman 1991). Alternatively, it is possible that normal chromatin configuration and/or cellular DNA replication are disturbed when the genomic m5C content drops below a critical level. Consistent with this possibility is the observation that demethylation induced by treatment of cells with azaC has been shown to lead to decondensation of heterochromatin and to affect the replication timing of the inactive X chromosome (Jablonka et al. 1987; Haaf and Schmid 1989).

Embryonic lethality contrasts with the lack of a discernible mutant phenotype in homozygous ES cells, suggesting that a reduction in m5C levels might be cell-lethal in differentiated tissues but not in pluripotent ES cells. Homozygous embryos do demonstrate, however, significant morphogenesis and tissue differentiation prior to death, indicating that initial cellular differentiation is not affected by lack of methylation. The following hypothesis may explain the apparently normal development of homozygous embryos up to the stage of organogenesis. Sufficient levels of genomic methylation might be maintained early in development by two sources for MTase activity: (1) A de novo DNA methyltransferase, as discussed above, is active in ES cells and in pregastrulation embryos but not in differentiated cells. In this context, it is significant to note that the acquisition of adult levels of genomic m5C by de novo methylation is achieved only at the time of gastrulation (Monk et al. 1987). (2) It has been shown that large maternal stores of methyltransferase are present in oocytes and blastocysts (Carlson et al. 1992). Once the embryo has developed postgastrulation, the embryonic de novo MTase would be inactivated and the level of maternally derived MTase would become more limiting at each consecutive cell division. This would successively reduce genomic methylation to a level which is incompatible with cell survival. It is interesting to note that demethylation induced by long-term azaC treatment of cells never reaches levels below 20% of the normal level (Michalowsky and Jones 1989). The m5C content in homozygous ES cells is well above this possibly critical level but reaches lower levels in somatic cells of the dying mutant embryo.

DNA methylation has been hypothesized to be involved in numerous processes, which include X inactivation, genomic imprinting, virus latency, carcinogenesis, aging, and the regulation of tissue-specific gene expression during development. The mutant ES cells and animals described here make possible rigorous tests of these hypotheses. The mutant ES cells may also aid in isolating other methyltransferases whose function might be to establish the methylation patterns in early development and to set up genomic imprints in the gametes.

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REFERENCES

Antequera, F., J. Boyes, and A. Bird. 1990. High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. *Cell* **62:** 503.

Barlow, D.P., R. Stöger, B.G. Herrmann, K. Saito, and N. Schweifer. 1991. The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the *Tme* locus. *Nature* **349:** 84.

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

Bestor, T. 1990. DNA methylation: Evolution of a bacterial immune function into a regulator of gene expression and genome structure in higher eukaryotes. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **326:** 179.

Bestor, T.H. and V.M. Ingram. 1983. Two DNA methyltransferases from murine erythroleukemia cells: Purification, sequence specificity, and mode of interaction with DNA. *Proc. Natl. Acad. Sci.* **80:** 559.

Bestor, T.H., A.P. Laudano, R. Mattaliano, and V.M. Ingram. 1988 Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. *J. Mol. Biol.* **203:** 971.

Bird, A. 1986. CpG rich islands and the function of DNA methylation. *Nature* **321:** 209.

———. 1993. Imprints on islands. *Curr. Biol.* **3:** 275.

Brockdorff, N., A. Ashworth, G.F. Kay, V.M. McCabe, D.P. Norris, P.J. Cooper, S Swift, and S. Rastan. 1992. The product of the mouse *Xist* gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. *Cell* **71:** 515.

Brunkow, M.E. and S.M. Tilghman. 1991. Ectopic expression of the *H19* gene in mice causes prenatal lethality. *Genes Dev.* **5:** 1092.

Carlson, L.L., A.W. Page, and T.H. Bestor. 1992. Properties and localization of DNA methyltransferase in preimplantation mouse embryos: Implications for genomic imprinting. *Genes Dev.* **6:** 2536.

Cedar, H. 1988. DNA methylation and gene activity. *Cell* **53:** 3.

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.

DeChiara, T., E. Robertson, and A. Efstratiadis. 1991.

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Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* **64:** 849.

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

Grant, S.G. and V.M. Chapman. 1988. Mechanisms of X-chromosome regulation. *Annu. Rev. Genet.* **22:** 199.

Gruenbaum, Y., H. Cedar, and A. Razin. 1982. Substrate and sequence specificity of a eukaryotic DNA methylase. *Nature* **295:** 620.

Haaf, T. and M. Schmid. 1989. 5-Azadeoxycytidine induced undercondensation in the giant X chromosomes of *Microtus agrestis*. *Chromosoma* **98:** 93.

Hadchouel, M., H. Farza, D. Simon, P. Tiollais, and C. Pourcel. 1987. Maternal inhibition of hepatitis B surface antigen gene expression in transgenic mice correlates with de novo methylation. *Nature* **329:** 454.

Jablonka, E., R. Goitein, K. Sperling, H. Cedar, and M. Marcus. 1987. 5-aza-C-induced changes in the time of replication of the X chromosomes of *Microtus agrestis* are followed by non-random reversion to a late pattern of replication. *Chromosoma* **95:** 81.

Jähner, D., H. Stuhlmann, C.L. Stewart, K. Harbers, J. Löhler, I. Simon, and R. Jaenisch. 1982. *De novo* methylation and expression of retroviral genomes during mouse embryogenesis. *Nature* **298:** 623.

Jones, P.A., S.M. Taylor, and V.L. Wilson. 1983. Inhibition of DNA methylation by 5-azacytidine. *Recent Results Cancer Res.* **84:** 202.

Jones, P.A., M.J. Wolkowicz, W.M. Rideout, F.A. Gonzales, C.M. Marziasz, G.A. Coetzee, and S.J. Tapscott. 1990. De novo methylation of the MyoD1 CpG island during the establishment of immortal cell lines. *Proc. Natl. Acad. Sc.* **87:** 6117.

Kay, G.F., G.D. Penny, D. Patel, A. Ashworth, N. Brockdorff, and S. Rastan. 1993. Expression of *Xist* during mouse development suggests a role in the initiation of X chromosome inactivation. *Cell* **72:** 171.

Kovalik, D., J. Kwak, and B. Weisblum. 1991. General method for direct cloning of DNA fragments generated by the polymerase chain reaction. *Nucleic Acids Res.* **19:** 4560.

Lauster, R., T.A. Trautner, and M. Noyer-Widner. 1989. Cytosine-specific type II DNA methyltransferases. A conserved enzyme core with variable target-recognizing domains. *J. Mol. Biol.* **206:** 305.

Leonhardt, H., A.W. Page, H.-U. Weier, and T.H. Bestor. 1992. A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell* **71:** 865.

Li, E., C. Beard, and R. Jaenisch. 1993. Role for DNA methylation in genomic imprinting. *Nature* (in press).

Li, E., T.H. Bestor, and R. Jaenisch. 1992. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* **69:** 915.

Michalowsky, L.A. and P. Jones. 1989. Gene structure and transcription in mouse cells with extensively demethylated DNA. *Mol. Cell. Biol.* **9:** 885.

Monk, M., M. Boubelik, and S. Lehnert. 1987. Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. *Development* **99:** 371.

Ogawa, O., M.R. Eccles, J. Szeto, L.A. McNoe, K. Yun, M.A. Maw, P.J. Smith, and A.E. Reeve. 1993. Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms tumor. *Nature* **362:** 749.

Posfai, J., A.S. Bhagwat, G. Posfai, and R.J. Roberts. 1989. Predictive motifs derived from cytosine methyltransferases *Nucleic Acids Res.* **17:** 2421.

Rainier, S., L.A. Johnson, C.J. Dobry, A.J. Ping, P.E. Grundy, and A.P. Feinberg. 1993. Relaxation of imprinted genes in human cancer. *Nature* **362:** 747.

Reik, W., A. Collick, M. Norris, S. Barton, and A. Surani. 1987. Genomic imprinting determines methylation of parental alleles in transgenic mice. *Nature* **328:** 248.

Riggs, A.D. and G.P. Pfeifer. 1992. X-chromosome inactivation and cell memory. *Trends Genet.* **8:** 169.

Sapienza, C., A. Peterson, J. Rossant, and R. Balling. 1987. Degree of methylation of transgenes is dependent on gamete of origin. *Nature* **328:** 251.

Sasaki, H., T. Hamada, T. Ueda, R. Seki, T. Higashinakagawa, and Y. Sakaki. 1991. Inherited type of allelic methylation variations in a mouse chromosome region where an integrated transgene shows methylation imprinting. *Development* **111:** 573.

Sasaki, H., P. Jones, R. Chaillet, A. Ferguson-Smith, S. Barton, W. Reik, and 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.

Shemer, R., T. Kafri, A. O'Connell, S. Eisenberg, J.L. Breslow, and A. Razin. 1991. Methylation changes in the apolipoprotein AI gene during embryonic development of the mouse. *Proc. Natl. Acad. Sci.* **88:** 11300.

Simpson, V.J., T.E. Johnson, and R.F. Hammen. 1986. *Caenorhabditis elegans* DNA does not contain 5-methycytosine at any time during development or aging. *Nucleic Acids Res.* **14:** 6711.

Stewart, C., H. Stuhlmann, D. Jahner, and R. Jaenisch. 1982. *De novo* methylation, expression, and infectivity of retroviral genomes introduced into embryonal carcinoma cells. *Proc. Natl. Acad. Sci.* **79:** 4098.

Stöger, 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.

Stuhlmann, H., D. Jähner, and R. Jaenisch. 1981. Infectivity and methylation of retroviral genomes is correlated with expression in the animal. *Cell* **26:** 221.

Swain, J., T. Stewart, and P. Leder. 1987. Parental legacy determines methylation and expression of an autosomal transgene: A molecular mechanism for parental imprinting. *Cell* **50:** 719.

Urieli-Shoval, S., Y. Gruenbaum, J. Sedat, and A. Razin. 1982. The absence of detectable methylated bases in *Drosophila melanogaster* DNA. *FEBS Lett.* **146:** 148.

Wigler, M., D. Levy, and M. Perucho. 1981. The somatic replication of DNA methylation. *Cell* **24:** 33.

Zemel, S., M.S. Bartolomei, and S.M. Tilghman. 1992. Physical linkage of two mammalian imprinted genes, *H19* and insulin-like growth factor 2. *Nature Genetics* **2:** 61.

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