

Targeted Mutation of the DNA Methyltransferase Gene Results in Embryonic Lethality

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Summary

Gene targeting in embryonic stem (ES) cells has been used to mutate the murine DNA methyltransferase gene. ES cell lines homozygous for the mutation were generated by consecutive targeting of both wild-type alleles; the mutant cells were viable and showed no obvious abnormalities with respect to growth rate or morphology, and had only trace levels of DNA methyltransferase activity. A quantitative end-labeling assay showed that the level of m⁵C in the DNA of homozygous mutant cells was about one-third that of wild-type cells, and Southern blot analysis after cleavage of the DNA with a methylation-sensitive restriction endonuclease revealed substantial demethylation of endogenous retroviral DNA. The mutation was introduced into the germline of mice and found to cause a recessive lethal phenotype. Homozygous embryos were stunted, delayed in development, and did not survive past midgestation. The DNA of homozygous embryos showed a reduction of the level of m⁵C similar to that of homozygous ES cells. These results indicate that while a 3-fold reduction in levels of genomic m⁵C has no detectable effect on the viability or proliferation of ES cells in culture, a similar reduction of DNA methylation in embryos causes abnormal development and embryonic lethality.

Introduction

The DNA of vertebrates contains tissue-specific patterns of methylated cytosine residues. 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 early embryogenesis and gametogenesis (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. Analysis of cloned murine DNA MTase cDNA (Bestor et al., 1988) has shown that the enzyme contains a C-terminal domain of 500 aa

that is closely related to those bacterial type II restriction methyltransferases that form 5-methylcytosine (m⁵C) (Lauster et al., 1989; Posfai et al., 1989); this catalytic domain is linked to an N-terminal domain of 1000 aa that has been predicted to have regulatory functions (Bestor et al., 1988; Bestor, 1990).

Heritable, tissue-specific methylation patterns have been proposed to form the basis of a stable regulatory system (Riggs, 1975). Several lines of evidence support this suggestion. The distribution of methylated cytosine residues in DNA undergoes characteristic changes during differentiation within a cell lineage, and 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* (reviewed by Cedar, 1988). Methylation of promoter regions inhibits transcription of cloned genes both in transfected cells and in transcription assays *in vitro* (Ben-Hattar and Jiricny, 1988; Watt and Molloy, 1988; Iguchi-Ariga and Schaffner, 1989). Gene reactivation occurs in certain cell types upon treatment with 5-azacytidine, an inhibitor of cytosine methylation (Jones et al., 1983). Alleles on the inactive X chromosome are often methylated in promoter regions, while alleles on the active X chromosome are normally unmethylated (reviewed by Grant and Chapman, 1988). Allele-specific methylation has been proposed to be involved in the phenomenon of genomic imprinting, and several transgenes have been observed to be inherited in the unmethylated state from parents of one sex and in the methylated state from parents of the other sex (Sapienza et al., 1987; Swain et al., 1987; Reik et al., 1987; Hadchouel et al., 1987; Sasaki et al., 1991).

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. The correlation between methylation and gene inactivation does not prove causation, and the effects of *in vitro* methylation on expression of transfected genes may not reflect true developmental regulation. 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 5-azacytidine have been found to be unmethylated in non-expressing tissues (Jones et al., 1990). The drug 5-azacytidine has methylation-independent side effects (Jones, 1985) and can also perturb protein-DNA interactions directly (G. Verdine, personal communication), so that its effects cannot be ascribed to demethylation of DNA alone. While most experimental evidence is consistent with cytosine modification playing an important role in developmental gene expression, it is worth pointing out that small-genome metazoan 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 mam-

malian gene control and development are needed to address the issue.

The importance of cytosine modification in vertebrate development can be assessed by mutating the gene for DNA MTase so as to reduce its amount or activity to limiting levels; developmental abnormalities in embryos containing lower than normal levels of genomic m⁵C would confirm that unperturbed methylation patterns are essential for normal development. Recent advances in genetic manipulation of pluripotent mouse embryonic stem (ES) cells have made it possible to construct strains of mouse that bear precise mutations in any gene for which cloned probes are available (Capecchi, 1989). We report here the mutation of the DNA MTase gene by homologous recombination in ES cells and establishment of the mutation in the germline of mice. The level of m⁵C in the DNA of ES cells and of embryos homozygous for the mutation was reduced to about one-third of that found in heterozygous or wild-type cells or embryos. While no discernible phenotype was apparent in homozygous ES cells in culture, homozygous mutant embryos displayed severe stunting, developmental delay, and death at mid-gestation at a time when organogenesis and rapid growth proceed in normal embryos.

Results

Disruption of the DNA MTase Gene by Homologous Recombination

DNA corresponding to the DNA MTase locus was isolated from a genomic DNA library prepared from mouse strain 129/sv and used for construction of the targeting vectors. The structure of the DNA MTase protein and the locations of the ATG start codon and an Nael restriction site in the cDNA are diagrammed in Figure 1A. A replacement type targeting vector termed pMT(N)neo (Figure 1B) was used to disrupt the DNA MTase gene by homologous recombination (Thomas and Capecchi, 1987). The construct contained a 900 bp deletion extending from the Nael site just upstream of the translation start site to a second Nael site in an intron (Figures 1A and 1B). The deleted sequences were replaced with a neomycin-resistance gene under the control of the phosphoglycerate kinase-1 (PGK1) promoter (Adra et al., 1987). The neo gene was in the same orientation as the DNA MTase gene (Figure 1B). Upon homologous recombination, this construct would remove 20 bp of 5' untranslated sequence, the first 27 codons, and the 5' splice site at the end of the first coding exon. A herpes simplex virus thymidine kinase gene was added to the targeting vector to allow selection against cells that had undergone nonhomologous integration (Mansour et al., 1988).

pMT(N)neo was transfected into ES cells of the J1 line (E. L. and R. J., unpublished data) and cells were plated in medium containing both G418 and the pyrimidine derivative FIAU (McMahon and Bradley, 1990). Resistant colonies were picked after 8–10 days of drug selection and DNA was prepared from pools of three independent clones (see Experimental Procedures). DNA was digested with

KpnI and hybridized with a genomic DNA probe, pBB, which is external to the 5' end of the targeting vector (Figure 1B). Homologous recombination was observed in 5 out of 36 pools of doubly-resistant ES cells (Figure 1C). As FIAU selection gave a 2.5-fold enrichment, and assuming each pool had only one positive clone, the frequency of homologous recombination was calculated to be 1/54 G418^r clones. The targeting frequency in the second experiment, when cells were selected with G418 alone, was 1/30 (4 of 40 pools). Four independent positive clones (clones 4.1, 8.1, 27.1, and 30.2) were identified by another round of Southern blot analysis of the 12 clones from the four positive pools from the first transfection. DNA from each targeted clone was further characterized by digestion with XbaI and hybridization of Southern blots with probe pBB, which detected fragments of 5.3 kb from the wild-type allele and 6.1 kb from the targeted allele (Figure 1B). Fragments of the predicted size for a disrupted MTase gene were detected with hybridization probes derived from the neo gene (data not shown).

Generation of ES Cell Lines Homozygous for the Mutation

The remaining wild-type allele of the DNA MTase gene in ES cells heterozygous for the mutation was disrupted by a second round of gene targeting. The targeting vector pMT(N)hyg (Figure 1B) was constructed by replacement of the PGK-neo-pA segment of pMT(N)neo with a PGK-hyg-pA cassette (te Riele et al., 1990), which confers resistance to hygromycin B. ES cells (clone 27.1) heterozygous for the pMT(N)neo mutation were subjected to a second round of gene targeting with pMT(N)hyg and hygromycin B selection. As the PGK-hyg-pA cassette is 200 bp larger than the PGK-neo-pA cassette, the diagnostic bands derived from a targeted allele would be 10.2 kb and 6.3 kb for KpnI or XbaI digestion, respectively (Figure 1B). After screening 96 hyg^r clones by Southern blot hybridization with probes pBB, neo, and hyg (data not shown), 5 clones were found to have undergone homologous recombination. Digestion of DNA with KpnI yielded only the 10 kb band expected for a second targeting event in the wild-type allele for two clones (clones 10 and 52, Figure 2), while the vector had integrated into the mutant allele in the other three clones (one of these, clone 12, is shown in Figure 2). Digestion with XbaI produced the expected bands of 6.1 and 6.3 kb in DNA from clone 52, but a fragment 1 kb longer than expected was seen in DNA from clone 10. This suggested an unexpected DNA rearrangement at the integration site of the pMT(N)hyg vector in this particular clone (Figure 2).

DNA MTase Activity in the 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. Whole-cell lysates were analyzed by immunoblotting to compare levels of DNA MTase protein in wild-type, heterozygous, and homozygous mutant cells. The antibody used had

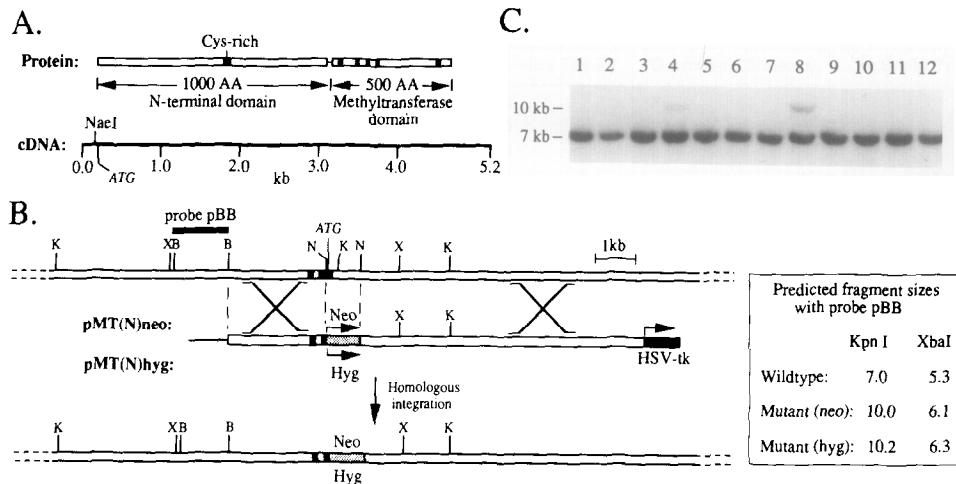


Figure 1. Disruption of the 5' End of the DNA MTase Locus

(A) 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 C-terminal domain is closely related to bacterial DNA (cytosine-5) restriction methyltransferases; closed squares indicate positions of conserved motifs (Bestor et al., 1988; Posfai et al., 1989; Lauster et al., 1989). A Cys-rich region within the N-terminal domain is indicated. The Nael site 20 bp upstream of the ATG initiation codon was chosen for gene targeting.

(B) Homologous recombination of the targeting vectors pMT(N)neo and pMT(N)hyg at the DNA MTase locus. The top line represents the structure of the 5' end of the DNA MTase gene. Exons identified by DNA sequencing are shown in black. The middle line represents the targeting vectors pMT(N)neo and pMT(N)hyg, in which a 900 bp Nael fragment containing part of the first coding exon is replaced with either a 1.8 kb PGK-neo-poly(A) cassette (top) or a 2.0 kb PGK-hyg-poly(A) cassette (bottom). The 5' Nael site and ATG initiation codon of the deleted sequences correspond to those shown in the cDNA. The targeting vector was linearized at a XbaI site in the plasmid polylinker 3' to the pMC1-tk-poly(A) cassette shown as a hatched box. Arrows indicate transcriptional orientation of the marker genes. At bottom is the predicted structure of the locus following targeted integration of pMT(N)neo or pMT(N)hyg. Homologous recombination was detected by probing Southern blots with probe pBB, and the expected sizes of diagnostic restriction fragments are given at lower right. K, KpnI; X, XbaI; B, BamHI; N, Nael.

(C) Southern blot analysis of ES cell clones transfected with pMT(N)neo. DNA from pools of three independent neo' clones was digested with KpnI, blotted, and hybridized with pBB. The 7 kb fragment was derived from the wild-type allele, and the 10 kb fragment seen in lanes 4 and 8 is diagnostic of homologous recombination.

been raised against a bacterial fusion protein (pATH52), which contained aa 137–635 of DNA MTase fused to the C-terminus of *E. coli* TrpE. On immunoblots of somatic cell lysates, the antibody detected a protein of apparent M_r 190,000, the known M_r of DNA MTase in human and murine cells (Figure 3 and Pfeifer and Drahovsky, 1986). Figure 3 shows that a protein of the expected size was present

at lower levels in whole cell extracts from the heterozygous cell lines 12 and 27.1 as compared with wild-type cells. In extracts from the homozygous mutant lines 10 and 52, a barely detectable band of slightly lower molecular weight was noticed (Figure 3; more easily seen in lysates of homozygous embryos in Figure 9). The bands just below the main band in the lanes containing wild-type and heterozygous lysates are the result of cleavage of extremely protease-sensitive sequences near the N-terminus; cleavage of these sequences occurs both in vitro during preparation of extracts and under some conditions in vivo (Bestor and Ingram, 1983, 1985).

Enzyme assays were performed to test for DNA MTase activity in lysates of homozygous mutant cells. Cell extracts were incubated with poly d(IC)-poly d(IC), a synthetic DNA substrate which has high methyl-accepting activity (Pedrali-Noy and Weissbach, 1986), and the rate of transfer of tritium from [*methyl-³H*]S-adenosyl methionine into DNA was measured. As shown in Figure 4, DNA methyltransferase activity was reduced about 50% in the heterozygous cells and severely reduced in the homozygous cells. The residual enzyme activity in lysates of ES cell clone 10 was reproducibly lower than that of clone 52. In homozygous mutant ES clone 10, which has an unexpected DNA rearrangement at the site of integration in one allele, both enzyme activity (Figure 4) and immunoreactive protein (Figure 3) were lower than in ES clone 52, which has

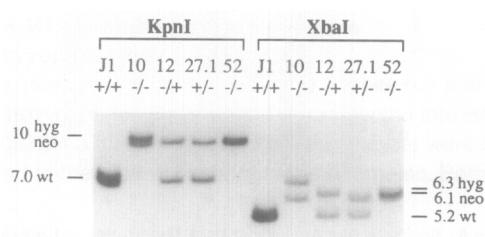


Figure 2. Southern Blot Analysis of ES Cell Clones Targeted with pMT(N)hyg

J1 is the wild-type parental cell line, 27.1 is the heterozygous neo' clone used for the second round targeting with pMT(N)hyg, and 10 and 52 are homozygous clones in which the wild-type alleles were targeted. Clone 12 is a heterozygous neo-sensitive clone in which the pMT(N)neo allele was targeted by pMT(N)hyg. The predicted size of fragments derived from the three different alleles are indicated (also see Figure 1B). Notice that a rearrangement has occurred in the pMT(N)hyg allele in clone 10, as detected by an increase in size of 1 kb in a diagnostic XbaI DNA fragment.

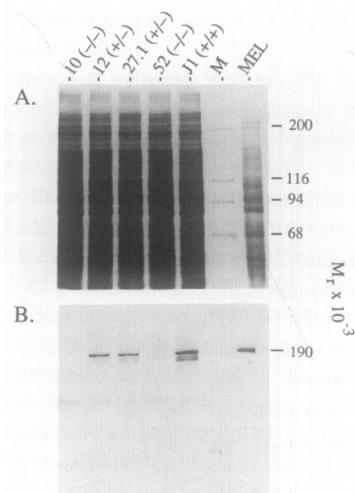


Figure 3. Immunoblot Analysis of DNA MTase in Wild-Type and Mutant ES Cell Lines

(A) A photograph of ES cell lysates fractionated on SDS-8% polyacrylamide gels and stained with Coomassie blue R250. Clone numbers and genotypes are given above. Lane M contains size markers, and MEL denotes a whole-cell lysate of Friend murine erythroleukemia cells, which express high levels of DNA MTase (Bestor et al., 1988). (B) An immunoblot of an identical gel probed with anti-pATH52, an antibody to DNA MTase. Levels of DNA MTase can be seen to be decreased in heterozygous mutant cell lysates. The band just detectable in the homozygous clone 52, and nearly undetectable in homozygous clone 10, migrates faster than the main M, 190,000 band, which is not detected in both clones. The bands migrating slightly faster than the main M, 190,000 band in the heterozygous and wild-type lanes are the result of cleavage of very protease-sensitive sequences near the N-terminus of DNA MTase.

the predicted sequence arrangement at both alleles. These data suggest that the mutant allele targeted by pMT(N)hyg in line 10 may contain a more severe mutation than the counterpart in clone 52.

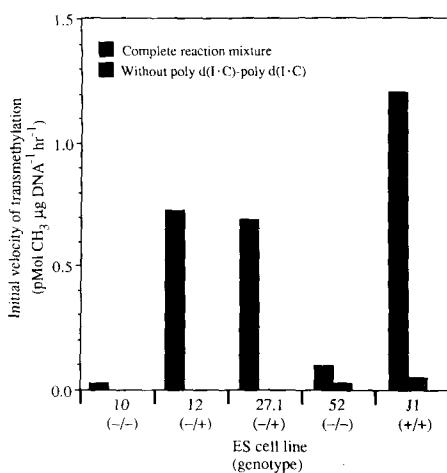


Figure 4. DNA MTase Enzyme Activity in Wild-Type and Mutant ES Cell Lysates

Cell lysates were prepared and enzyme assays were carried out as described in Experimental Procedures. Heterozygous mutants can be seen to have lower levels and the homozygous mutants severely reduced levels of enzyme activity; in agreement with the immunoblot studies, clone 52 had more activity than clone 10.

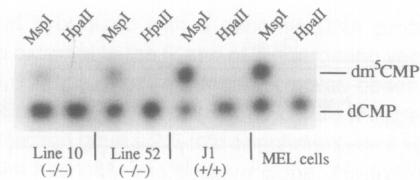


Figure 5. Quantitation of Genomic m⁵C Content in Wild-Type and Mutant Cells

The end-labeling assay described in Experimental Procedures revealed that 60% of CCGG sequences were methylated in wild-type ES DNA and 20% in homozygous mutants 10 and 52. Heterozygous mutants did not differ from wild type in terms of m⁵C content (data not shown).

Decreased DNA Methylation in Homozygous Mutant ES Cells

The level of m⁵C in the DNA of mutant cells was measured by a quantitative end-labeling method described previously (Bestor et al., 1984). Genomic DNA from wild-type and mutant cells was digested with Mspl, a restriction endonuclease that cleaves the sequence CCGG on the 5' side of the central CpG dinucleotide. Mspl is insensitive to methylation at the CpG site. DNA fragments were ³²P-labeled at their 5' ends and digested to 5' mononucleotides with nuclease P1, and the labeled mononucleotides were separated by thin-layer chromatography (see Experimental Procedures). The level of methylated cytosines in CCGG sites was quantitated by measuring the radioac-

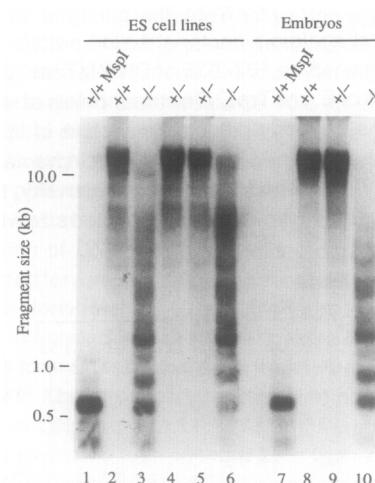


Figure 6. Southern Blot Analysis of DNA Methylation of Endogenous Retroviral Genome in Wild-Type and Mutant ES Cell Lines and Embryos

DNA from ES cell lines or day 10.5 embryos was digested with the methylation-sensitive restriction endonuclease HpaII and hybridized with MoMuLV 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, Mspl. Lanes 2–6 contain HpaII-digested DNA from clones J1, 10, 12, 27.1, and 52, respectively. Comparison of intensity of bands between 0.5 and 1 kb in lanes 3 and 6 shows that DNA from cell line 10 appears to be more sensitive to HpaII than DNA from line 52, in agreement with the lower levels of DNA MTase observed in line 10. Lanes 8–10 contain HpaII-digested DNA from wild-type, heterozygous, and homozygous embryos, respectively.

Table 1. Generation of Chimeric Mice and Germline Transmission of Targeted DNA MTase Alleles

Clone	Embryos Injected	Chimeras Born	M/F	Males Tested	Germline Chimeras	Complete Transmitters	Sterile Males
8.1	30 (a)	17	10/7	4	3	1	1
27.1	20 (a)	12	10/2	5	3	2	2
	17 (b)	13	12/1	3	3	3	0

Fifteen to twenty ES cells were injected into either BALB/c (a) or C57Bl/6 (b) blastocysts. M/F stands for male/female chimeric mice. Germline transmission was scored by the presence of agouti offspring from matings to BALB/c or C57Bl/6 females. Complete transmitters are those male chimeras that produce only agouti offspring.

tivity within the two major spots, 5' [³²P]dCMP and 5' [³²P]dm⁵CMP. DNA from the homozygous mutant cells was found to have an m⁵C content of about one-third of that of DNA from wild-type ES cells, in which about 60% of all CCGG sites were methylated (Figure 5). The content of m⁵C in the DNA of the heterozygous ES cells was not detectably different from that of wild-type cells (data not shown).

The DNA of endogenous retroviruses is known to be highly methylated (Stuhlmann et al., 1981). DNA of mutant and wild-type cells was purified and digested with Hpall, a methylation-sensitive isoschizomer of Mspl. DNA fragments were subjected to Southern blotting and hybridized to an Mo-MuLV cDNA probe, which allows detection of multiple endogenous retroviruses in the mouse genome (Stuhlmann et al., 1981; Jähner and Jaenisch, 1984). Digestion of DNA from wild-type (Figure 6, lane 2) and heterozygous cells (Figure 6, lanes 4 and 5) with Hpall resulted only in bands of high molecular weight, whereas digestion with Mspl yielded multiple small fragments (Figure 6, lane 1). In contrast to wild-type or heterozygous ES cells, DNA from the homozygous ES cells yielded small fragments when digested with Hpall (Figure 6, lanes 3 and 6). These results indicate that the DNA MTase mutation resulted in substantial demethylation of genomic DNA. We note that the level of retroviral DNA methylation was slightly lower in clone 10 ES cells than in clone 52 cells (compare lanes 3 and 6 of Figure 6), in agreement with the data of Figures 3 and 4.

DNA MTase Mutation Is a Recessive Embryonic Lethal

Two independent lines of ES cells (clones 8.1 and 27.1) heterozygous for the mutation were injected into BALB/c or C57Bl/6J blastocysts, and the blastocysts were transferred to the uteri of pseudopregnant females. Both lines

generated offspring with extensive coat color chimerism, and all of the fertile male chimeras tested transmitted the mutant allele to their offspring (Table 1).

Mice heterozygous for the mutation were indistinguishable from wild-type littermates. Heterozygotes were intercrossed and several litters of newborn mice were genotyped by Southern blot hybridization with probe pBB. None of 18 liveborn offspring were found to be homozygous (Table 2), a result consistent with prenatal lethality of the homozygous mutation. Of a total of 44 conceptuses analyzed at 12.5–15.5 days of gestation, 19 were observed as necrotic implantation sites and no homozygotes were detected among the remaining normal embryos (Table 2). To determine the time of death, embryos were isolated at days 9.5 and 10.5 of gestation. Of a total of 39 embryos dissected at this stage, 13 were retarded in development. Subsequent genotype analysis revealed that all 13 abnormal embryos, but none of the normal embryos, were homozygous for the mutation (Table 2).

Examination in the dissecting microscope revealed that homozygous embryos isolated at day 10.5 were stunted and of a developmental stage characteristic of normal embryos at day 9.5 or younger. Protein analysis of whole embryo lysates showed that homozygous mutant embryos had a mass of about one-eighth that of heterozygous or wild-type littermates. The best-developed homozygous mutant embryos had about 20 somites, distinct forelimb buds, and a closed anterior neuropore (Figure 7A, middle embryo). The majority of the homozygous embryos were comparable to normal day 8.5–9 embryos with 10–20 somites. Most had turned within the amnion, whereas a few were still in the process of turning, and they often had an open anterior neural pore and lacked visible forelimb buds (Figure 7A, right embryo). The yolk sacs in the homozygous embryos lacked visible blood or blood vasculature and were smaller than in normal embryos (Figure 7B).

Table 2. Genotype of Offspring Derived from Heterozygous Parents

Stage	Litter	Total	Resorption	Genotype of Live Embryos		
				+/+	+/-	-/-
p1–7	5	18	0	9	9	0
E12.5–15.5	5	44	19	11	14	0
E9.5–10.5	5	39	0	6	20	13

p1–7 represents mice of postnatal day 1–7, while E12.5–15.5 and E9.5–10.5 represent embryos of gestation day 12.5–15.5 and day 9.5–10.5, respectively. Resorbed embryos were excluded from genotyping. +/+ , +/- , and -/- refer to mice that were wild-type, heterozygous, and homozygous for the mutant allele, respectively. All 13 homozygous mutant embryos of E9.5–10.5 were distinguishable from their normal littermates in that they were severely stunted.



Figure 7. Gross Morphology of Wild-Type and Mutant Embryos at 10.5 Days of Gestation

(A) Whole view of a wild-type embryo (left) and two homozygous mutant littermates.

(B) Whole view of the yolk sac of a wild-type embryo (left) and that of a homozygous mutant littermate.

(C) Side view of a homozygous embryo showing an abnormal structure (arrow) near the tail bud.

About two-thirds of the mutant embryos displayed a structure protruding from the lower abdomen (Figure 7C, arrow), which is similar to an abnormal structure observed in XO parthenogenetic embryos that may be derived from the allantois (Mann and Lovell-Badge, 1988). Histological analysis of homozygous embryos revealed that major organ rudiments were present but smaller than in wild-type littermates (Figures 8C and 8D). Close inspection revealed significantly increased cell deaths (Figure 8B, arrows) and considerably fewer mitotic figures in homozygous as compared with wild-type embryos (Figures 8A and 8B).

The m^5C content of DNA from homozygous and normal embryos was analyzed by the quantitative end-labeling method as applied to the ES cells. Figure 9B shows that

total cytosine methylation was reduced to a similar level (~30% of wild type, or 20% of all CCGG sequences) in homozygous mutant embryos as in the homozygous ES cell lines (Figure 5). In addition, when methylation of HpaII sites at endogenous retroviral loci were examined by Southern blot analysis, a similar extent of demethylation was observed in homozygous embryos as in the homozygous ES cell lines (Figure 6, 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 9A, anti-pATH52 detected a strong band of M_r 190,000 on immunoblots of lysates of normal embryos, while homozygous mutant embryos displayed small amounts of a slightly smaller protein, as previously ob-

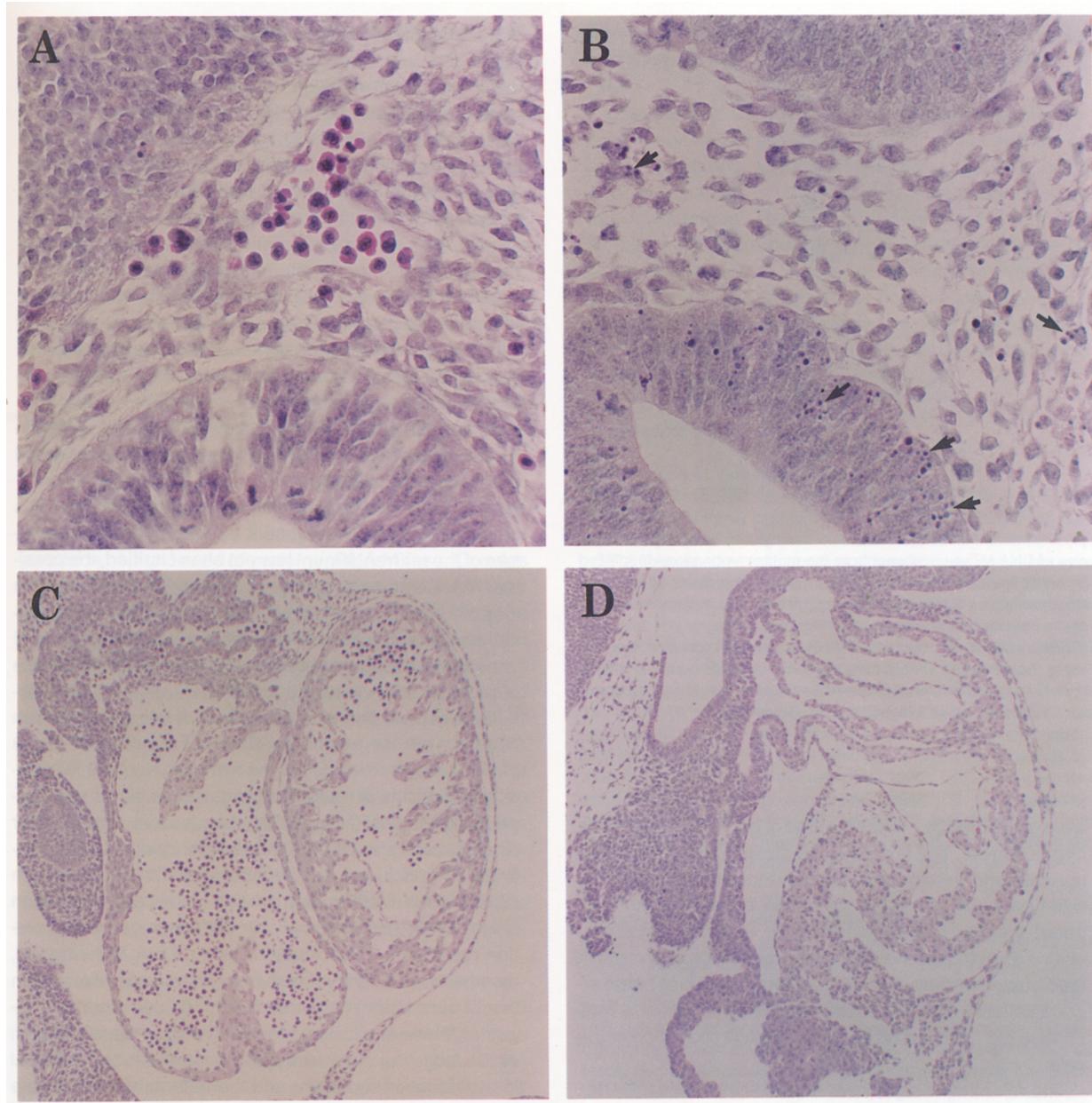


Figure 8. Histological Analysis of Wild-Type and Homozygous Embryos

(A and B) Brain tissues in normal (A) and mutant (B) embryos. Arrows indicate regions with cell deaths in mutant embryos.
(C and D) Developing hearts in normal (C) and mutant (D) embryos.

served in mutant ES cells. Embryo proteins were separated by electrophoresis on SDS-6% polyacrylamide gels; these higher-porosity gels allowed resolution of the normal and mutant proteins.

The results described in this section indicate that mice carrying targeted mutations of the DNA MTase gene derived from two independently established ES clones showed similar severe 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.

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; the deleted sequences were replaced by a neo expression cassette in the same transcriptional orientation as the DNA

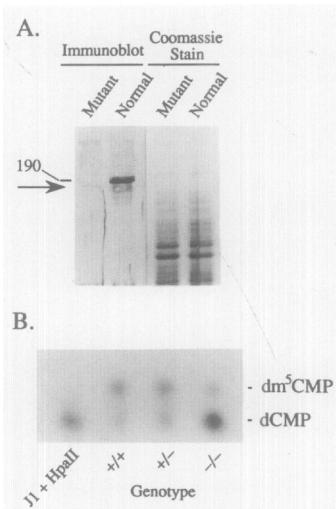


Figure 9. Immunoblot Analysis and m^5C Quantitation of Wild-Type and Mutant Embryos

(A) The immunoblot to the left shows that the normal M, 190,000 species of DNA MTase is absent from the mutant embryos and replaced by small amounts of a slightly faster migrating protein. At right is a Coomassie blue R250-stained gel of the extracts used in preparation of the immunoblot.

(B) Reduction of m^5C contents in DNA from homozygous mutant embryos. As previously seen in homozygous mutant ES cells, the DNA of mutant embryos contained 30% of the m^5C seen in wild-type embryos, which showed 60% methylation of CCGG sites.

MTase gene. ES cell lines homozygous for the mutation were generated by a second round of gene targeting with a hyg^r construct. The homozygous mutant cells proliferated normally and were morphologically indistinguishable from wild-type cells, despite a reduction of genomic m^5C 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 m^5C content did not detectably change with propagation of the cells.

The retention of significant amounts of m^5C in the DNA of the homozygous mutant ES cells could be due to the activity of a previously uncharacterized DNA methyltransferase or an altered, but enzymatically active, form of the known DNA MTase derived from the mutated gene. While there is no firm evidence for the presence of more than one DNA methyltransferase in mammals, the possible existence of additional enzymes cannot be excluded. *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 embryo-specific forms of DNA MTase. Such enzymes might be able to maintain reduced but stable levels of DNA methylation in homozygous mutant ES cells, but be unable to sustain methylation patterns in differentiating somatic tissues of mutant embryos. The following considerations raise, however, the possibility that the targeted mutation of the MTase gene did not completely inactivate the gene but resulted in the production of small amounts of truncated

but enzymatically active polypeptide. First, the nature of the targeting construct used in this study does not assure a null mutation; the deletion introduced into the DNA MTase gene removed only 20 bp of 5' untranslated region, one 5' splice site, and the first 27 codons. The first ~300 aa of DNA MTase can be removed by proteolysis without detectable effect on in vitro enzymatic activity (Bestor and Ingram, 1985), and it is possible that internal reinitiation of translation, or initiation from a site within the resistance marker in a chimeric or alternatively spliced mRNA, could have resulted in synthesis of an active enzyme. Anti-DNA MTase antibodies did detect small amounts of a protein of slightly higher mobility on immunoblots of mutant cell and embryo lysates. Second, two independent homozygous mutant ES lines, 10 and 52, differed in that immunoreactive material, m^5C content in DNA, and enzyme activity in lysates were reproducibly lower in cell line 10. It was found that one disrupted allele in line 10 contained an unexpected DNA rearrangement that changed the size of a diagnostic restriction fragment by 1 kb. Although the nature of the rearrangement has not been clarified, it is possible that line 10 contains one partial and one more severe loss of function mutation that result in lower enzyme activity. We cannot, however, exclude the possibility of clonal variation between independent ES cell clones as a cause of reduced enzyme levels in line 10. Finally, we have derived a second line of mice bearing a targeted insertion mutation in a region of the DNA MTase gene 3' of the pMT(N)neo insertion. Embryos homozygous for this mutation have a similar phenotype to that of embryos homozygous for the pMT(N)neo disruption but die a day earlier (E. L., unpublished data). This independent mutation confirms our conclusion that impairment of DNA MTase gene activity leads to embryonic lethality at the postgastrulation stage. While we do not know yet whether this second insertion resulted in a null mutation of the DNA MTase gene, the phenotype of the mutant embryos is more severe than that of mice homozygous for the pMT(N)neo mutation. Together, these results suggest that homologous integration of the targeting construct pMT(N)neo produced a partial loss of function mutation, and that a form of DNA MTase with alterations in the N-terminal region was produced at low levels from the mutant gene. However, we do not know whether the residual enzyme activity produced by the pMT(N)neo mutant allele is sufficient to stably maintain the reduced genomic DNA methylation level in homozygous ES cells or whether another DNA methyltransferase not affected by the mutation is responsible.

Consequences of Reduced Levels of DNA MTase in Mouse Embryos

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 m^5C level in DNA from day 10 homozygous embryos was found to be 30% of that of wild-type embryo DNA; given the frequency of the CpG dinucleotide in mammalian DNA (Schwartz et al., 1962), this means that homozygous mutants had $\sim 1 \times 10^7$ methylated CpG sites per haploid genome versus 3×10^7 for wild type. The finding that a 3-fold reduction in m^5C completely prevented development beyond mid-gestation suggests an essential role of DNA modification in normal mammalian development.

Significant morphogenesis and tissue differentiation were observed in homozygous mutant embryos. We consider two possibilities to account for the apparently normal development of homozygous embryos up to the stage of organogenesis. Normal methylation patterns might be maintained early in development by additional DNA methyltransferases, although as previously mentioned, there is currently no direct evidence for more than one species of DNA MTase in mammals. Alternatively, early cell lineage determination and tissue differentiation may occur in the presence of high levels of maternal DNA MTase. Oocytes and early embryos contain very large stores of DNA MTase protein (Monk et al., 1991; Howlett and Reik, 1991); the amount per nucleus is $\sim 5 \times 10^3$ greater in oocytes than in proliferating mouse fibroblasts, and the total amount declines by only a small factor between the oocyte and blastocyst stages (L. L. Carlson and T. H. B., unpublished data). Together with the large maternal store of DNA MTase, residual enzyme produced by the partial loss of function mutation resulting from integration of pMT(N)neo may delay the developmental stage at which DNA MTase becomes limiting for development. There is also evidence for the acquisition of adult levels of genomic m^5C only at the time of gastrulation (Monk et al., 1987), and the effects of reduced levels of DNA MTase may be especially severe at that time.

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 a likely possibility is inappropriate gene expression. Genes that are normally repressed by methylation would be expected to be activated in the presence of limiting levels of DNA MTase, and expression of many genes at high levels or in inappropriate tissues might result in cell death or a reduced ability of affected cells to participate in tissue formation. Embryonic lethality contrasts with the lack of a discernible mutant phenotype in homozygous ES cells. This raises the possibility that a reduction in m^5C levels is cell-lethal in differentiated tissues but not in pluripotent ES cells. To address this question, tissue distributions of homozygous ES cells in live chimeric mice derived from normal blastocysts injected with homozygous ES cells will be examined. We are also attempting to derive differentiated cell lines from explanted mutant embryos by infection of dispersed cells with oncogenic viruses. It is also conceivable that gene expression in embryonic cells is less dependent on DNA methylation than in somatic cells, as shown by the observation that demethylation by 5-azacytidine readily results in the activation of retroviral genes in fibroblasts or other

somatic cells, but is not sufficient to activate proviruses in embryonic cells (Stewart et al., 1982; Jaenisch et al., 1985).

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 DNA MTase gene mutation created by integration of pMT(N)neo allows homozygous embryos to complete gastrulation and the early stages of organogenesis, and differentiated tissues in adequate amounts can easily be obtained for analysis.

Experimental Procedures

Construction of Targeting Vectors

Cloned genomic DNA corresponding to the DNA MTase locus was isolated from a library of strain 129 mouse DNA. A 10.5 kb fragment containing the first coding exon was subcloned into the plasmid vector pSP72 (Promega). A restriction map was established and exons were mapped by Southern blot hybridization with cDNA probes. The boundaries of exons chosen for targeting were determined by sequence analysis. The targeting vector pMT(N)neo was constructed by replacement of a 900 bp Nael fragment with a blunt-ended PGK-neo-poly(A) cassette derived from pKJ-1 (Tybulewicz et al., 1991). The 5' end of the neo cassette was ligated to an Nael site in the first coding exon; the site is 20 bp upstream of the ATG initiation codon and 102 bp upstream of an exon-intron border. The 3' Nael site was in an intron. pMT(N)hyg was identical except that the resistance marker was the PGK-hyg-poly(A) cassette (te Riele et al., 1990). The targeting vectors contained 2.5 kb of homology 5' and 7 kb 3' of the drug resistance marker (see Figure 1). The pMC1-tk-poly(A) cassette derived from PM2323 (a gift from Maarten Zijlstra) was ligated into a restriction site in the vector polylinker at the 3' end of the insert in pMT(N)neo.

Transfection and Selection of Mutant ES Cells

The J1 ES cell line (E. L. and R. J., unpublished data) was derived from a male agouti 129/terSv (Stevens, 1973) embryo essentially as described by Robertson (1987) and grown in HEPES-buffered (20 mM, pH 7.3) Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum, 0.1 mM nonessential amino acids (Gibco), 0.1 mM β -mercaptoethanol, and antibiotics. J1 cells were grown on feeder layers of γ -irradiated embryonic fibroblast (EF) cells. The G418^r EF cells were derived from day 14 embryos carrying the targeted mutation of $\beta 2$ -microglobulin (Zijlstra et al., 1989). Leukemia inhibitory factor (LIF) was not used during routine passage of J1 cells, but was added to 500 U/ml during selection and cloning. J1 cells at passage 8–10 were trypsinized and suspended at 2×10^7 ml⁻¹ in electroporation buffer as described by Thomas and Capecchi (1987), except that the concentration of NaCl was at 137 mM. Cells were electroporated in the presence of 25 μ g/ml linearized DNA at 250 V, 250 μ F in a BTX 300 electroporator. The cells were plated at a density of 4×10^6 per 10 cm plate on G418^r EF cells 10 min after electroporation. Selection with G418 (Gibco) at 350 μ g/ml (as dry powder) and FIAU (Bristol Myers) at 0.2 μ M was begun about 36 hr after plating. One of three plates from each electroporation was selected with G418 alone to allow estimation of the enrichment obtained by FIAU selection. Doubly-resistant colonies were picked 8–10 days after selection, and each colony was dissociated in a small drop of trypsin-EDTA (5 μ l of 0.25% trypsin, 1 mM EDTA in HEPES-buffered saline) and transferred to the wells of 24-well plates with continued G418 selection. After 3–4 days, half the cells in each well were frozen and the remainder pooled with cells from two other wells. After 24 hr culture, the cells were lysed with 0.5 ml of 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM EDTA, 1% SDS, and 0.5 mg/ml proteinase K. The lysate was incubated at 55°C for at least 4 hr, and the DNA was purified by phenol extraction and ethanol precipitation and redissolved in 100 μ l of 10 mM Tris (pH 7.5) and 0.1 mM

EDTA. Transfection of pMT(N)hyg was performed under the same condition, and cells were selected in hygromycin B (110 µg/ml, Calbiochem) containing medium supplemented with LIF (1000 U/ml).

Southern Blot Analysis

Purified DNA from each pool was digested with the indicated restriction endonuclease, fractionated by electrophoresis through 0.8% agarose gels, blotted with 0.4 M NaOH onto GeneScreen plus (DuPont-NEN), and hybridized with a 1.4 kb BamHI fragment 5' to the short arm of the targeting vector (probe pBB in Figure 1B). Blots were washed with 0.2 × SSC and 0.1% SDS at 65°C. Tail and embryo DNA was prepared as described by Laird et al. (1991), and genotypes were established by Southern blot analysis as described above. To examine DNA methylation at CCGG sites, DNA was purified by phenol extraction and digested with HpaII (Boehringer Mannheim, 60 U of enzyme per 5 µg of DNA per digestion) for 16 hr, blotted, and hybridized with a Mo-MuLV cDNA probe. After exposure to X-ray film, the blots were stripped and rehybridized with mitochondrial DNA probes to confirm complete digestion (data not shown).

Generation of Germline Chimeras

Embryo manipulations were carried out as described (Bradley, 1987). The J1 ES cells were found to contribute to the germline at a high frequency after injection into BALB/c or C57Bl/6J blastocysts (E. L. and R. J., unpublished data). BALB/c blastocysts were usually cultured for 1–3 hr in ES cell culture medium before injection to allow the blastocoel cavities to fully expand. ES cells were trypsinized and washed once with injection medium, which consists of DMEM supplemented with 10% fetal calf serum and 20 mM HEPES (pH 7.3). Sodium bicarbonate was omitted and the osmolarity was adjusted to 290 mmol/kg with NaCl. ES cells (15–20) in injection medium were injected into each blastocyst, and injected blastocysts were cultured in ES cell medium for 1 hr prior to transfer of 6–10 embryos to one uterine horn of a pseudopregnant female mouse. Chimeric pups were identified by eye pigmentation (if albino BALB/c host blastocysts were used) or chimeric coat color. Chimeric males were bred to BALB/c or C57Bl/6J females, and germline transmission of the mutant allele was detected by Southern blot analysis of tail DNA from F1 offspring with agouti coat color. Male chimeras demonstrated in this way to have complete transmission of the ES cell genome were bred to 129/terSv females to obtain the mutation on an inbred background.

Immunoblot Analysis and Enzyme Activity Assays

Antibodies were prepared against pATH52, a fusion protein that contained aa 137–635 of DNA MTase fused to the C-terminus of the E. coli TrpE protein (Dieckmann and Tzagoloff, 1985). pPATH52 synthesis was induced and inclusion bodies were purified from E. coli strain JM105 as described (Klempnauer and Sippel, 1987). Fusion proteins were further purified by electroelution from SDS-polyacrylamide gels and precipitation with chloroform–methanol (Wessel and Flugge, 1984) prior to injection into rabbits.

Embryos and ES cells were lysed by sonication at 0°C in 5 vol of 20 mM Tris-HCl (pH 7.4), 0.4 M NaCl, 25% glycerol, 5 mM EDTA, 0.1% Nonidet P40, 1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride, and 100 µg/ml aprotinin. After sonication, 1 vol of a 50% (v/v) DEAE-Sephadex slurry equilibrated with 20 mM Tris-HCl (pH 7.4) was added. After 10 min, the DEAE-Sephadex was removed by centrifugation. This step removed nucleic acids from the extract, which would otherwise interfere with enzyme assays and electrophoresis. The clarified lysate was used for both immunoblot analysis and enzyme activity assays.

Proteins in a portion of the extract were denatured by heating to 100°C in the presence of 2% SDS and 100 mM DTT and separated by electrophoresis through SDS-polyacrylamide gels. Separated proteins were transferred to nitrocellulose sheets (BA85, Schleicher and Schuell) or Problott PVDF membranes (Applied Biosystems, Inc.) by electroblotting in a Hoefer semidry transfer apparatus with 0.3 M Tris-HCl (pH 10.5), 20% methanol at the anode and 0.025 M Tris, 0.04 M ε-aminocaproic acid, 20% methanol at the cathode. The transfer was carried out at 4 mA/cm² for 30 min. Blots were probed with anti-pATH52, followed by alkaline-phosphatase conjugated goat anti-rabbit IgG and chromogenic substrates as described (Harlow and Lane, 1988). The more sensitive chemiluminescent substrate AMPPD

(Gillespie and Hudspeth, 1991) was used to detect the small amounts of DNA MTase present in lysates of homozygous mutant embryos.

The remainder of the clarified lysate (~200 µg of protein in 20 µl) was added to 200 µl of a solution containing 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 25% glycerol, 5 µCi of [³H]S-adenosyl L-methionine (New England Nuclear; 12 Ci/mmol), 4 µg poly d(IC)-poly d(IC), 1 mM DTT, and 200 µg/ml bovine serum albumin. The assay mixture was incubated at 37°C for 2 hr, then extracted twice with phenol-chloroform. The aqueous phase was made 0.1 M in NaOH and incubated at 50°C for 2 hr. The solution was neutralized with HCl and the radioactivity was incorporated into DNA measured by scintillation counting after trichloroacetic acid precipitation. Control reactions lacked poly d(IC)-poly d(IC).

Quantitative Measurement of m³C in DNA

The methylation status of CCGG sites was determined as previously described (Bestor et al., 1984). Purified DNA from ES cells or embryos was cleaved with Mspl, treated with alkaline phosphatase, and labeled at 5' termini by incubation with polynucleotide kinase and [³²P]ATP. The labeled DNA was purified by extraction with phenol-chloroform, spun column chromatography on Quick Spin G-50 columns (Boehringer Mannheim), and ethanol precipitation. The DNA was dissolved in 30 mM sodium acetate (pH 5.3), 0.1 mM ZnCl₂. Nuclease P1 (U.S. Biochemicals) was added to a concentration of 100 µg/ml, and the samples were incubated at 65°C for 2 hr. The 5'-deoxymononucleotides were separated by thin-layer chromatography on cellulose plates developed in isobutyric acid–water–ammonium hydroxide (66:33:1). An autoradiogram was prepared and the radioactivity in specific spots measured by scraping and scintillation counting.

Morphological and Histological Analysis

Embryos were dissected and staged as described by Kaufman (1990). The major morphological markers used for staging were somites, anterior neuropore, limb buds, optic sulcus, otic vesicle, and degree of turning. Color micrographs were taken with a Zeiss DS dissecting microscope, a Zeiss M35 camera, and Kodak Ektachrome 64T film.

Embryos were fixed in 10% buffered formalin, dehydrated in graded alcohols and xylenes, and embedded in paraplast. Sections of 4 µm thickness were stained with Harris hematoxylin and eosin. Color micrographs were taken with an Olympus BH-2 microscope, an Olympus C-35AD-2 camera with a exposure control unit, and EKTAR 25 film.

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