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# De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells

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## SUMMARY

**It has been a controversial issue as to how many DNA cytosine methyltransferase mammalian cells have and whether de novo methylation and maintenance methylation activities are encoded by a single gene or two different genes. To address these questions, we have generated a null mutation of the only known mammalian DNA methyltransferase gene through homologous recombination in mouse embryonic stem cells and found that the development of the homozygous embryos is arrested prior to the 8-somite stage. Surprisingly, the null mutant embryonic stem cells are viable and contain low but stable levels of methyl cytosine and methyltransferase activity, suggesting the existence of a second DNA methyltransferase in mammalian cells. Further studies indicate that de novo methylation activity is not impaired by the mutation as integrated provirus DNA in MoMuLV-infected homozygous embryonic stem cells become methylated at a similar rate as in wild-type cells. Differentiation of mutant cells results in further reduction of methyl cytosine levels, consistent with the de novo methylation activity being down regulated in differentiated cells. These results provide the first evidence that an independently encoded DNA methyltransferase is present in mammalian cells which is capable of de novo methylating cellular and viral DNA in vivo.**

Key words: DNA cytosine methyltransferase, de novo methylation, genomic imprinting, X-inactivation, gene targeting, embryonic stem cells

## INTRODUCTION

Methylation of DNA at the C5 position of cytosine residues plays an important role in the regulation of gene expression in vertebrates (Eden and Cedar, 1994) and is essential for mammalian development (Li et al., 1992). Tissue-specific methylation patterns are established during embryonic development through a highly regulated process that involves de novo methylation and demethylation and are transmitted in a clonal fashion from cell to cell through the action of the maintenance DNA methyltransferase. During the preimplantation stage of mouse embryonic development, a process of genome-wide demethylation occurs, resulting in hypomethylation of the genome at the blastula stage (Monk et al., 1987; Kafri et al., 1992, 1993). This process erases the methylation patterns inherited from gametes of both parents and may have a role in reformatting the genome prior to the initiation of normal program of embryonic development (Razin and Cedar, 1993). Shortly after implantation, a wave of de novo methylation takes place that adds methyl groups to unmodified cytosine residues in DNA, resulting in a rapid increase in genomic methylation levels during postimplantation development (Jähner et al., 1982; Monk et al., 1987; Kafri et al., 1992). De novo methylation of the genome also occurs during differentiation and maturation of gametes of both sexes and may play a critical role in the establishment of genomic imprinting in the gametes (Chaillet et al., 1991; Stöger et al., 1993; Brandeis et al., 1993; Tremblay et al., 1995; Zuccotti and Monk 1995; Ariel et al., 1995; Tucker et al., 1996). Undifferentiated embryonic carcinoma (EC) cells and embryonic stem (ES) cells have been shown to have relatively high de novo methylation activity (Stewart et al., 1982; and this study) while the de novo methylation activity is low or undetectable in differentiated EC cells, postgastrulation embryos and adult somatic tissues (Jähner et al., 1982; Stewart et al., 1982; reviewed by Jähner and Jaenisch, 1984).

The evidence summarized above is consistent with two different DNA methyltransferase activities being expressed in mammalian cells. A maintenance methyltransferase activity must be present in all proliferating cells to ensure the maintenance of methylation patterns during cell division. A de novo methyltransferase activity, specifically expressed in early embryonic cells, would serve to re-establish the DNA methylation patterns following implantation. For an understanding of how DNA methylation patterns are established during embryogenesis and how these patterns are maintained throughout the life of the animal, it is important to define the DNA methyltransferase activities expressed at different stages

of development and to identify the gene or genes that encode these activities. Biochemical studies in the past have shown that mammalian DNA methyltransferases purified from somatic cells prefer hemimethylated DNA as substrate as would be expected for a maintenance methyltransferase (Gruenbaum et al., 1982; Bestor and Ingram, 1983; Pfeifer et al., 1983; reviewed by Adams et al., 1993). This property of the mammalian DNA methyltransferases is in striking contrast to the bacterial DNA cytosine methyltransferases which do not discriminate between unmethylated and hemimethylated target sequences. The cloning of the first mammalian DNA methyltransferase (MTase) has revealed that it has, in addition to the C-terminal catalytic domain, a large N-terminal domain that is not present in bacterial enzymes (Bestor et al., 1988). Proteolysis of partially purified mammalian DNA methyltransferases results in increased rate of de novo methylation (Adams et al. 1983; Bestor, 1992), proposed to be the consequence of separation of the catalytic domain from the inhibitory N-terminal domain (Bestor, 1992). Although this hypothesis remains to be tested, it raises an interesting possibility that the known MTase may carry out both maintenance and de novo methylation through posttranslational modification of the enzyme. It is not known, however, whether posttranslational modification of the MTase occurs in vivo and if so, whether it has functional relevance. The focus of this study is to investigate the alternative possibility, i.e. that an independently encoded and developmentally regulated DNA methyltransferase is responsible for de novo methylation during early embryogenesis.

Previous experiments showed that a partial loss-of-function mutation of the DNA MTase gene (*Dnmt*) resulted in embryonic lethality but had no effect on the viability of ES cells homozygous for the mutation (Li et al., 1992). Here, we describe the generation of a true null allele of the Dnmt gene. ES cells homozygous for the null mutation proliferate normally and display very low levels of genomic DNA methylation. Infection of mutant ES cells with the MoMuLV results in de novo methylation of the integrated provirus DNA with a similar rate to that seen in wild-type ES cells, suggesting that the de novo methylation process is not impaired by the mutation. These results provide strong evidence that an independently encoded DNA methyltransferase, likely a de novo cytosine methyltransferase, is expressed in early embryonic cells.

## MATERIALS AND METHODS

### Construction of gene targeting vectors

A targeting vector, pMT(S)neo, was constructed in which a PGK-neo-polyA cassette isolated from plasmid pKJ-1 was inserted into the unique *Sal*I site of an 8.5 kb *Xho*I genomic DNA fragment isolated from a 129/Sv genomic DNA library (Fig. 1B). The *Sal*I site is in an exon in the 5′ region of the cDNA(Fig. 1A). A pMC1-tk-poly(A) cassette was added for negative selection. To disrupt the catalytic domain, a targeting vector, pMT(C)neo, was constructed in which a 0.8 kb genomic DNA fragment (from *Bst*EII to *Bam*HI) in a 8.0 kb *Xho*I-*Xba*I genomic fragment was deleted and replaced with the same neomycin cassette (Fig. 1C). The deleted DNA sequences contain two exons coding for 98 amino acid residues (TNSLGQ.....TFGVLQ) which include the most conserved PC motif (motif 4) and the ENV motif (motif 6) (Lauster et al., 1989; Posfai et al., 1989; Fig. 1A). For targeted disruption of the remaining wild-type allele, pMT(C)hyg was constructed in the same way as pMT(C)neo except that the neomycin gene was replaced with a hygromycin-resistance gene (Li et al., 1992). The pMC1-tk-poly(A) cassette was also included in the construct for negative selection. The neomycin/hygromycin genes were in the same transcriptional orientation as the *Dnmt* gene in all targeting constructs.

### Generation of mutant mice and ES cell lines

ES cell culture, transfection and selection were carried out as described previously (Li et al., 1992). The targeting vectors, pMT(S)neo or pMT(C)neo, were linearized and transfected into J1 ES cells via electroporation and transfected cells were selected with G418 and FIAU. Drug-resistant clones were screened by Southern blot hybridization using probes external to the targeting constructs (Fig. 1B,C). The targeting frequency was 1/52 and 1/112 doubly resistant clones for pMT(S)neo and pMT(C)neo, respectively. Chimeric mice and $F_1$ heterozygous mice were produced as described previously (Li et al., 1992).

To generate ES cell lines homozygous for the $Dnmt^s$ mutation, delayed blastocysts were isolated from heterozygous females mated with heterozygous male mice and ES cell lines were derived as described previously (Robertson 1987; Li et al., 1992). $Dnmt^{c/c}$ ES cell lines were generated by targeted disruption of the second wild-type allele with the pMT(C)hyg construct in one of the $Dnmt^{c/+}$ ES cell lines. Cells transfected with pMT(C)hyg were selected with hygromycin B (110 μg/ml) and FIAU (0.2 μM) on a feeder layer of hygromycin-resistant STO cells.

### Growth competition by co-culture of wild-type and mutant ES cells

Wild-type and mutant ES cells were cultured individually for two passages and then mixed together at about 1:1 ratio and cultured for 2 days. Cells were then trypsinized and divided into three fractions. One fraction was used for DNA isolation (i.e. d0 in Fig. 8, which represents the inital ratio of mutant and wild-type ES cells), the second fraction of cells was cultured as undifferentiated ES cells on a feeder layer of embryonic fibroblast cells in medium containing LIF (500 u/ml) and the third fraction of cells was cultured as embryoid bodies under the differentiation condition as described previously (Robertson, 1987). Undifferentiated ES cells and embryoid bodies were harvested at days 2, 4, 8, 10, 12 and 15 for genomic DNA preparation. The ratio of wild-type versus mutant cells was measured by Southern blot analysis of the DNA using probes that could differentiate wild-type fragments from mutant fragments (Li et al., 1992 for $Dnmt^n$; and Fig. 4A,B for the $Dnmt^s$ and $Dnmt^c$ alleles).

### DNA purification and measurement of methyl cytosine in genomic DNA

Purification of genomic DNA and Southern blot analysis were carried out as described previously (Li et al., 1992). For measurement of methyl cytosine by thin layer chromatography (TLC), DNA was treated with RNaseA (200 μg/ml) at 37°C for 2 hours, extracted once with phenol/chloroform, precipitated and resuspended in TE buffer.

To examine the methylation status of genomic DNA, DNA was digested overnight with restriction enzymes, *Hpa*II or *Msp*I (Boehringer) using 100 units of enzyme per 10 μg DNA. An additional 20 units of enzyme was added the next morning to each sample to ensure complete digestion. Southern blot hybridization was conducted as described previously (Li et al., 1992). Complete digestion of genomic DNA with *Hpa*II was achieved under these conditions as judged by probing digested DNA with a mitochondiral DNA probe (see Fig. 6C for an example).

To detect methyl cytosine directly, RNA-free genomic DNA was analyzed by end-labeling/TLC assay as described previously (Cedar et al., 1979) except that Polygram Cel 300 plates (Macherey-Nagel) was used for TLC assay.

### Infection of ES cells with a replication-competent Molony murine leukemia virus

A replication-competent retrovirus, Mo-MuLV$^{sup}$-1 (Reik et al., 1987), was used to infect ES cells. Virus-producing cells (NIH3T3 cells) were cultured in ES cell medium. Freshly collected medium (24-hour incubation) was filtered through a 0.45 μm filter and used for infection of ES cells. Rapidly growing ES cells were seeded at $1.0\times10^6$ cells per 25 $cm^2$ flask and infection was initiated 16 hours later using the following protocol. 2 ml fresh virus-containing medium in the presence of polybrene (8 μg/ml) was added to each flask of ES cells and cells were incubated at 37°C for 1 hour. The virus-containing medium was then replaced with ES cell medium and the cells were incubated at 37°C for 1 hour to allow recovery. This alternating infection and recovery procedure was repeated three more times. After 4 rounds of infection, cells were cultured in ES cell medium and split every other day. Cells were harvested at 1 day, 2 days, 4 days and 8 days after infection and DNA was isolated for Southern blot analysis.

## RESULTS

### A null mutation of the *Dnmt* gene results in hypomethylation of genomic DNA and postimplantation lethality

Two mutant alleles of the DNA MTase gene (*Dnmt*) have been reported previously (Li et al., 1992, 1993). The *Dnmt*$^n$ allele (previously termed MTase$^n$, n stands for N-terminal disruption) is a partial loss-of-function mutation, which results in reduction of methyl cytosine to 30% of the normal level in homozygous embryos and midgestation lethality (Li et al., 1992). The *Dnmt*$^s$ allele (previously termed MTase$^s$, s stands for *Sal*I site), which has not been described in detail before (Li et al., 1993), disrupts a region known to be involved in targeting DNA MTase to DNA replication foci (Leonhardt et al., 1992) (Fig. 1B) and results in embryonic lethality at an earlier stage. However, it remains unresolved whether the *Dnmt*$^s$ allele is a null mutation because the C-terminal catalytic domain was uninterrupted in the mutant gene and two aberrant transcripts were detected in the homozygous ES cells (this study). To assess the effect of complete inactivation of DNA MTase on embryonic development and ES cell viability, a null allele, termed *Dnmt*$^c$ (c stands for disruption of the catalytic or C-terminal domain), was generated by deletion of two highly conserved motifs, the PC motif (motif 4) and the ENV motif (motif 6), within the catalytic domain (Fig. 1A,C), in which the amino acid residues P-C and E-N-V are conserved among all cytosine methyltransferases from bacteria to mammals (Lauster et al., 1989; Posfai et al., 1989, Kumar et al., 1994). The cysteine residue in the PC motif has been identified as the active site nucleophile of bacterial cytosine methyltransferases and mutations of this cysteine residue completely abolishes the enzyme activity (Wu and Santi, 1987; Chen et al., 1991; Friedman and Ansari, 1992; Wyszynski et al., 1992; Kumar et al., 1994).

While mice heterozygous for either the *Dnmt*$^s$ or the *Dnmt*$^c$ allele showed no discernible abnormalities and were fertile, embryos homozygous for either mutation were readily distinguished from wild-type embryos as early as 8.5 days post-coitum (d.p.c.) (data not shown). At 9.5 d.p.c, both *Dnmt*$^{s/s}$ and *Dnmt*$^{c/c}$ embryos frequently displayed a distorted neural tube and lacked visible somites and a few most advanced mutant embryos developed eight pairs of somites (Fig. 2). Analysis of genomic methylation levels using a methylation-sensitive enzyme such as *Hpa*II showed that DNA of endogenous C-type retroviruses and pericentromeric minor satellite repeats was highly demethylated in both *Dnmt*$^{s/s}$ and *Dnmt*$^{c/c}$ embryos at 9.5 d.p.c. with the methylation level significantly lower than in the less severe *Dnmt*$^{n/n}$ mutant embryos (Fig. 3). Similar results were obtained when DNA methylation of the L1 repeats, the major satellite repeats, and several unique genes were analyzed (data not shown). The *Dnmt*$^{s/c}$ compound heterozygous embryos showed very similar defects and methylation levels as compared to embryos homozygous for either allele (data not shown), while the *Dnmt*$^{n/s}$ and *Dnmt*$^{n/c}$ embryos had intermediate levels of methylation and their development was arrested at about 15-somite stage (Fig. 3 and data not shown). These results confirm our previous finding that the maintenance of a normal DNA methylation pattern is essential for mammalian development.

### ES cells homozygous for either the *Dnmt*$^s$ or the *Dnmt*$^c$ mutation are viable

ES cell lines homozygous for the *Dnmt*$^s$ mutation were derived from delayed blastocysts by mating heterozygous mice. Of the 28 ES cell lines generated, 8 lines were homozygous for this mutation. A Southern blot representative of the *Dnmt*$^s$ mutant ES cell lines is shown in Fig. 4A. *Dnmt*$^c$ homozygous ES lines were generated by targeting the wild-type allele in the heterozygous ES cells with a vector containing the hygromycin-resistance gene (Fig. 1C). Of the 96 selected ES clones, 8 were homozygous for the mutation as shown by Southern blot analysis (Fig. 4B). We also performed Southern analysis using the deleted *Bst*EII-*Bam*HI genomic fragment as a probe (i.e. pBBs) and showed that no hybridizing bands were detected in the *Dnmt*$^{c/c}$ DNA (Fig. 4C), confirming that the PC and ENV motifs were deleted. In addition, no aberrant DNA rearrangement or deletion was detected in the mutant *Dnmt* locus as proven by Southern analysis using a cDNA probe that hybridized to about 30 kb genomic DNA flanking the deleted region (data not shown). At least two independent homozygous ES cell lines for each mutation were analyzed in all experiments described below and identical results were obtained. Northern blot analysis using a full-length cDNA probe showed that two aberrant transcripts of approximately 5.5 kb and 9 kb were synthesized from the *Dnmt*$^s$ allele, a weak and slightly smaller transcript was detected in *Dnmt*$^{n/n}$ cells, and no MTase-specific transcript was detected in *Dnmt*$^{c/c}$ cells (Fig. 4D). Western blot analysis using antibodies specific to the N-terminal domain of the DNA MTase (Bestor, 1992; Li et al., 1992) detected a slightly smaller protein in *Dnmt*$^n$ mutant cells as shown previously (Fig. 4E, lane 4; Li et al., 1992) but no MTase protein was detected in either the *Dnmt*$^{s/s}$ or the *Dnmt*$^{c/c}$ mutant cells (Fig. 4E, lanes 5 and 6). As antibodies specific to the C-terminal domain were not available, we could not determine whether a truncated MTase was produced by the *Dnmt*$^s$ allele.

### The null mutant ES cells contain low but stable levels of methyl cytosine and methyltransferase activity

To test whether methyl cytosine was present in the *Dnmt*$^{s/s}$ and the *Dnmt*$^{c/c}$ mutant ES cells, we examined the methylation

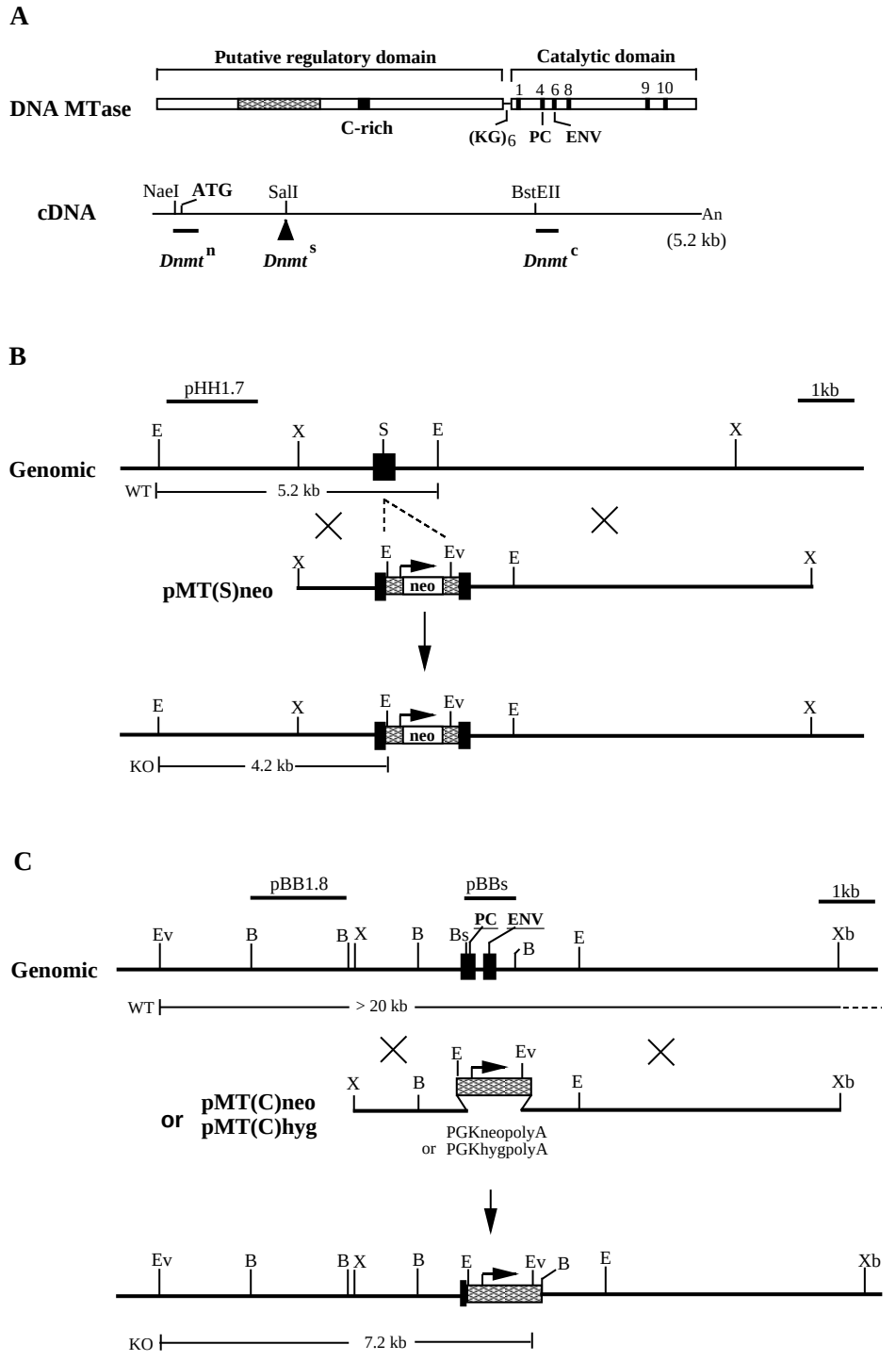



**Fig. 1.** The structure of DNA MTase protein and the targeting vectors. (A) The DNA MTase protein (at top) consists of two domains linked by a run of 13 alternating lysyl and glycyl residues, $(KG)_6$. The C-terminal catalytic domain contains ten conserved sequence motifs among which six (motifs 1, 4, 6, 8, 9, 10, shown as black bars) are highly homologous to those of bacterial cytosine methyltransferases. The invariant prolylcysteinyl residues (PC) in motif 4 function as the active site nucleophile which is required for the function of cytosine methyltransferases. The N-terminal domain contains a Zn-binding cysteine rich region (C-rich) and a region required for DNA MTase to associate with replication foci (shaded box). The cDNA (at bottom) is lined up with the protein and the ATG indicated was previously thought to be the translation initiation site (Bestor et al., 1988). The three restriction enzyme sites, *Nae*I, *Sal*I and *Bst*EII, where the Dnmt gene was disrupted, are indicated. The short bars at the *Nae*I and *Bst*EII sites represent the deleted regions in $Dnmt^n$ and $Dnmt^c$ mutants while the arrowhead at the *Sal*I site represents the $Dnmt^s$ mutation resulting from insertion of a neomycin gene in the *Sal*I site. (B) Generation of the $Dnmt^s$ allele. The top line is the genomic DNA surrounding the *Sal*I (S) site in an exon (solid box). The *Xho*I (X) fragment was used for the construction of the targeting vector, pMT(S)neo (middle line), in which a PGK-neo-poly(A) cassette containing a *Eco*RI (E) site at its 5′ end was inserted into the *Sal*I site. The mutant allele resulting from homologous recombination is shown at the bottom. For screening recombinant clones by Southern analysis, DNA was digested with *Eco*RI and hybridized to a pHH probe that detects a 5.2 kb wild-type (WT) allele and a 4.2 kb mutant allele (KO). (C) Generation of the $Dnmt^c$ allele. The top line represents the wild-type genomic DNA containing the exons (solid boxes) that encode the highly conserved motifs 4 (PC) and 6 (ENV). The targeting vector pMT(C)neo or pMT(C)hyg (middle line) contains a 9 kb genomic fragment from *Xho*I (X) to *Xba*I (Xb), in which a 0.8 kb fragment from *Bst*EII site (Bs) to the 3′ *Bam*HI site (B) was deleted and replaced with a PGK-neo-poly(A) cassette or a PGK-hyg-poly(A) cassette. The mutant locus resulting from homologous recombination is shown at the bottom. For screening recombinant clones, DNA was digested with *Eco*RV (Ev) and hybridize to a 1.8 kb *Bam*HI fragment, pBB1.8, which detects the wild-type allele (WT) of larger than 20 kb in size and a 7.2 kb mutant allele (KO). The *Bst*EII-*Bam*HI fragment, pBBs, was used as a probe for Southern analysis of cell lines homozygous for the $Dnmt^c$ allele (Fig. 4C).

status of genomic DNA by Southern blot hybridization using a full-length MoMuLV cDNA probe. To eliminate possible contamination of wild-type feeder cells, Dnmt mutant cells were passaged at least five times in LIF-containing medium in the absence of feeder cells prior to DNA preparation. As shown in Fig. 5, DNA from wild-type and all heterozygous ES cell lines was highly methylated, while DNA from the $Dnmt^{s/s}$ and $Dnmt^{c/c}$ ES cells was extensively demethylated with methylation levels even lower than that of the $Dnmt^{n/n}$ mutant cells (Fig. 5A, compare lanes 6 and 7 with lanes 1-5).

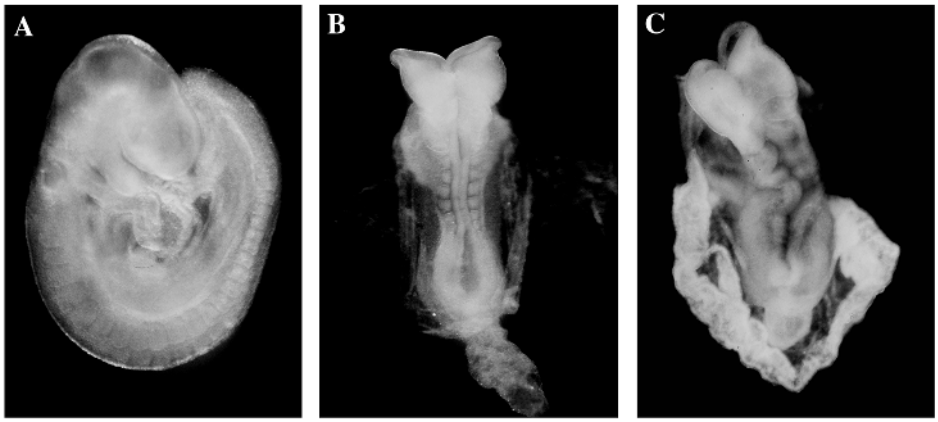
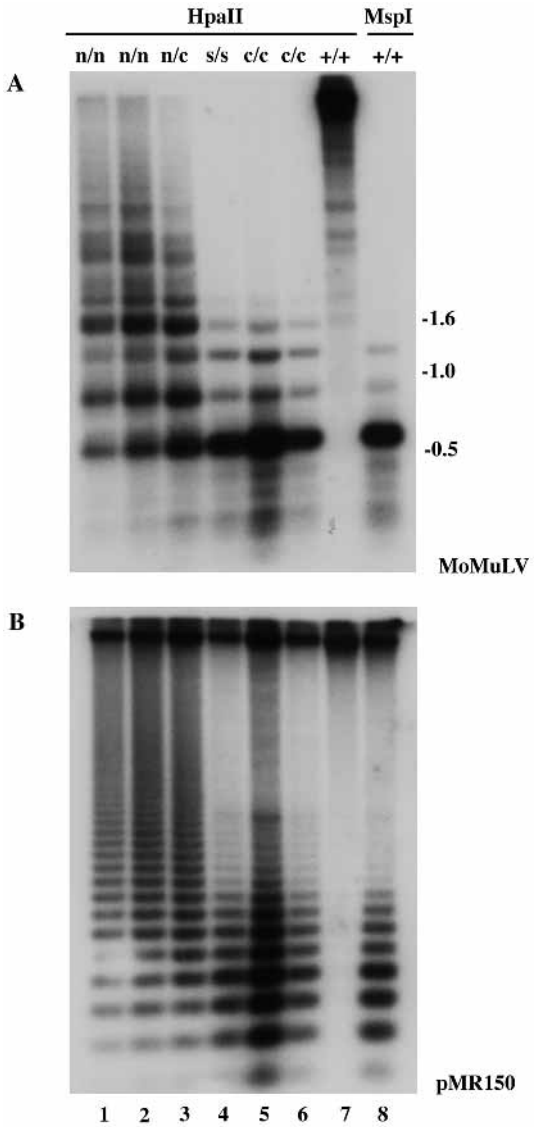



**Fig. 2.** Gross morphology of Dnmt mutant embryos. Wild-type and mutant embryos of 9.5 d.p.c. were dissected out of decidua and examined using a stereo dissecting microscope. The embryos are: (A) wild-type, (B) a most advanced *Dnmt*$^{s/s}$ or *Dnmt*$^{c/c}$ embryo with up to 8 pairs of somites and (C) a most frequently observed type of *Dnmt*$^{s/s}$ or *Dnmt*$^{c/c}$ mutant embryo, which has a distorted neural tube and lacks somites.

The *Dnmt*$^{s/s}$ and *Dnmt*$^{c/c}$ cells, however, were not completely demethylated. Comparison of the digestion patterns of the methylation-sensitive enzyme *Hpa*II (lanes 6, 7) with its methylation insensitive isoschizomer *Msp*I (lane 8) indicates that methylated CpG sites are still present in both *Dnmt*$^{s/s}$ and *Dnmt*$^{c/c}$ ES cells.




To confirm the presence of methyl cytosine in the *Dnmt*$^{s/s}$ and the *Dnmt*$^{c/c}$ ES cells by an independent method, genomic methyl cytosine content was directly measured by radioactive end-labeling of *Msp*I-digested genomic DNA followed by thin layer chromatography (TLC) of labeled mononucleotides as described previously (Cedar et al., 1979). DNA was also purified from feeder-free ES cells. As shown in Fig. 5B, a residual amount of methyl cytosine was indeed detected in both *Dnmt*$^{s/s}$ and *Dnmt*$^{c/c}$ cells (Fig. 5B, lanes 3 and 4). Similar results were obtained when DNA was digested with *Taq*I restriction enzyme (data not shown). Since non-specific DNA degradation during sample preparation would significantly elevate the intensity of the dCMP spot but not that of the methyl-dCMP spot (as methyl cytosine accounts for only 1-2% of total cytosine in wild-type DNA), quantification of the methyl cytosine in *Dnmt*$^{s/s}$ and *Dnmt*$^{c/c}$ cells by this method would be very difficult. Nevertheless, the result demonstrated the presence of methyl cytosine in the null mutant ES cells. To confirm that the low level of methylation detected was not due to contamination of wild-type ES cells and could be stably maintained, 48 subclones of a *Dnmt*$^{c/c}$ line were derived from single cells by limiting dilution of *Dnmt*$^{c/c}$ cells and methylation levels were analyzed by either Southern analysis or direct measurement of methyl cytosine as described in Fig. 5. We found that all subclones had very similar levels of methyl cytosine as the parental line (data not shown), thus eliminating the possibility of contamination by wild-type ES cells. This result also indicates that the low level of methyl cytosine can be stably maintained in null mutant cells for more than 20 cell generations. We have also measured methyltransferase activity in nuclear extracts prepared from wild-type and

**Fig. 3.** Southern blot analysis of methylation levels of genomic DNA of the Dnmt mutant embryos. DNA was isolated from embryos of 9.5 d.p.c. and digested with *Hpa*II (lanes 1-7) and *Msp*I (lane 8), and then fractionated on 1% agarose gel, blotted and hybridized to a MoMuLV cDNA probe (Jähner et al., 1982) (A), or to a centromeric minor satellite repeat, pMR150 (Chapman et al., 1984) (B). The genotype of each embryonic DNA sample is shown as n/n (lanes 1, 2), n/c (lane 3), s/s (lane 4), c/c (lanes 5, 6) and +/+ (lanes 7, 8), in which symbols n, s, c and + represent *Dnmt*$^{n}$, *Dnmt*$^{s}$, *Dnmt*$^{c}$ and wild-type alleles, respectively. The same symbols are used in the following figures to represent wild-type and the three *Dnmt* mutant alleles.

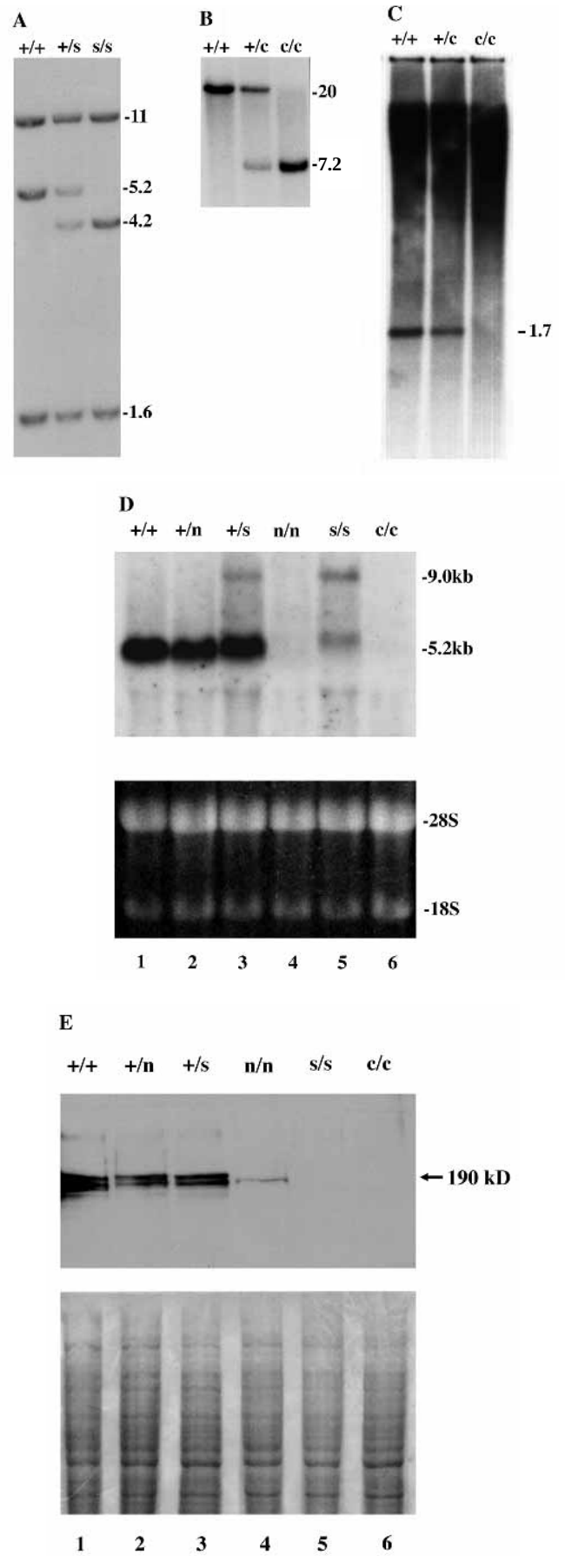
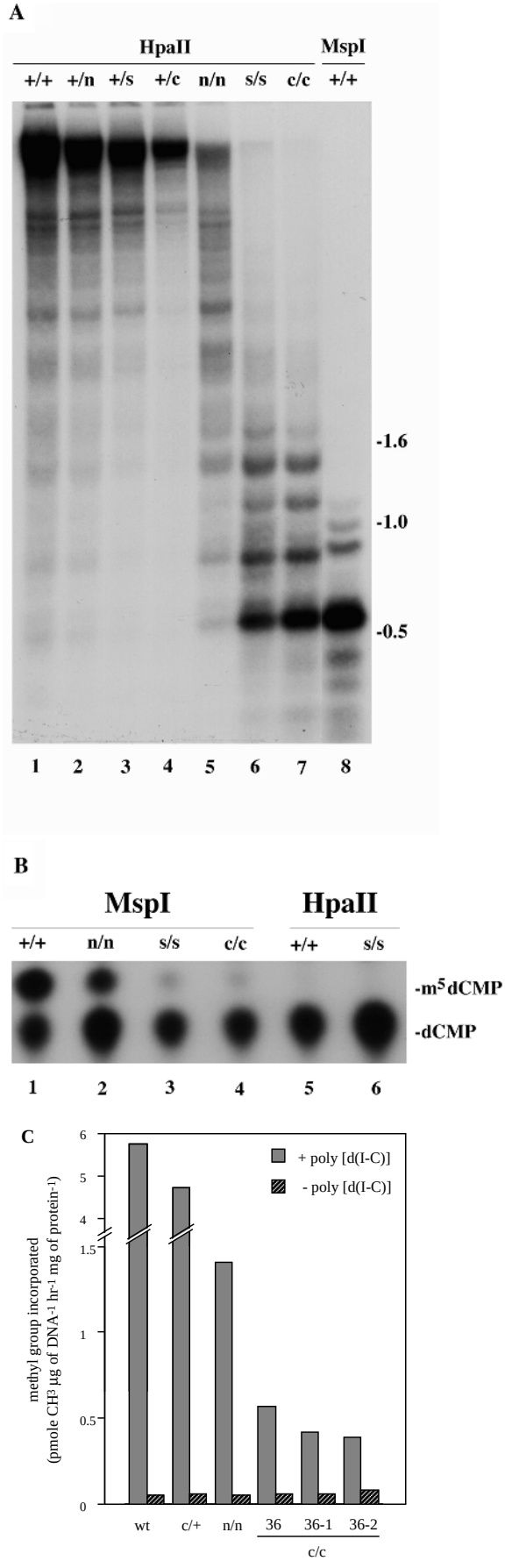



**Fig. 4.** Molecular analysis of the *Dnmt* mutant ES cell lines. (A) Southern blot analysis of the *Dnmt*$^{s}$ mutant cells: DNA digested with *Eco*RI was blotted and hybridized to a cDNA fragment from 5′ end of the cDNA to the *Sal*I site (see Fig. 1A). The pHH1.7 genomic probe (see Fig. 1B) used in the initial screening of recombinant clones gave high background (data not shown). The recombinant clones were confirmed with the 5′ cDNA probe, which detects the same wild-type and mutant bands (5.2 kb and 4.2 kb, respectively) as pHH1.7. The 5′ cDNA probe also contains exons that hybridize to 1.6 kb and 11 kb bands in the 5′ region of the genomic locus (the genomic locus not shown). (B) Southern blot analysis of the *Dnmt*$^{c}$ mutant cells: DNA digested with *Eco*RV was blotted and hybridized to the pBB1.8 probe (Fig. 1C). The probe detects a larger than 20 kb band from the wild-type allele and a 7.2 kb band from the knockout allele. (C) DNA from wild-type, heterozygous and *Dnmt*$^{c}$ homozygous ES cells was digested with *Bam*HI, blotted and hybridized to a genomic fragment, pBBs (see Fig. 1C). Since the pBBs fragment, which contains the exons encoding the PC and ENV motifs, was deleted in the *Dnmt*$^{c}$ mutation, it detects a 1.7 kb band in wild-type and heterozygous DNA as predicted but no discrete bands in DNA of homozygous cells. The smear at the top of the blot indicates the presence of repetitive sequences in the pBBs fragment. (D) Northern blot analysis of DNA MTase transcripts in wild-type (lane 1), heterozygous (lanes 2 and 3) and homozygous (lanes 4-6) ES cell lines. Approximately 20 μg total RNA was loaded in each lane, blotted and hybridized to a full-length cDNA probe. 18S and 28S rRNA bands are shown at the bottom as RNA loading controls. A very weak band, which is slightly smaller than the 5.2 kb wild-type transcript, is visible in n/n RNA (lane 4) on the original film. No bands were seen in c/c RNA (lane 6). (E) Immunoblot analysis of DNA MTase in wild-type and mutant ES cells. The bottom panel shows ES cell lysates fractionated on SDS-8% polyacrylamide gels and stained with Coomassie blue R250. The top panel shows an immunoblot of an identical gel probed with an anti-pATH52 antibody, which is specific to the N-terminal region from aa 137-635 of the MTase enzyme (Li et al., 1992) and stained using an ECL immunodetection kit. A $190\times10^{3}$ $M_{r}$ protein is detected in wild-type and heterozygous cells. A slightly smaller protein is detected in the *Dnmt*$^{n/n}$ cells while no MTase protein is detected in *Dnmt*$^{s/s}$ or *Dnmt*$^{c/c}$ mutant cells.

mutant ES cell lines using poly d(I·C)-poly d(I·C) as DNA substrate. The results showed that a significant level of methyltransferase activity was detected in a *Dnmt*$^{c/c}$ line and two of the subclones (Fig. 5C). Taken together, these results provide compelling evidence for the existence of a second DNA cytosine methyltransferase in ES cells.

## De novo methylation of provirus DNA in null mutant ES cells

If a de novo methyltransferase were encoded by a gene other than the known MTase gene, then its activity should be detectable in the *Dnmt*$^{c/c}$ ES cells. To test this possibility, wild-type and MTase mutant ES cells were infected multiple times with a replication-competent retrovirus, MoMuLV$^{sup}$-1, which contains a supressor F gene in its long terminal repeats (LTR) (Fig. 6A; Reik et al., 1987). De novo methylation of integrated viral DNA was analyzed by Southern blot hybridization using the supressor F gene probe, which distinguishes the infected virus from the endogenous retroviral sequences. Fig. 6B shows that the viral DNA was already partially methylated in wild-type, *Dnmt*$^{n/n}$, and *Dnmt*$^{c/c}$ ES cells 24 hours after infection. The methylation levels increased in the following days in both wild-type and mutant ES cells and reached a stable level 8 days




after infection. The *Hpa*II sites in the LTR region were almost completely methylated in wild-type ES cells but partially methylated in mutant cells due to inactivation of the known MTase in mutant cells, which prevented maintenance methylation of the provirus DNA. To ensure that *Hpa*II digestion was complete, the same blot was rehybridized with a mitochondrial DNA probe. Since the mitochondrial genome is not methylated, the result that the *Hpa*II digestion pattern was identical to the *Msp*I digestion pattern (Fig. 6C) confirmed that *Hpa*II digestion was complete. Although the methylation level of the provirus genome was lower in mutant ES cells than in wild-type cells, the methylation level reached the highest point within 4-8 days for all cell lines tested (Fig. 6B). This suggests that de novo methylation was not affected by the complete inactivation of the known enzyme and that an independently encoded methyltransferase activity is involved in de novo methylation of provirus DNA.

### Growth deficiency of the null mutant cells upon induction of differentiation

While ES cells homozygous for either the $Dnmt^{s}$ or the $Dnmt^{c}$ mutation grew normally, the growth of the mutant embryos was arrest prior to the 8-somite stage and the delay in development of the mutant embryos correlated with the severity of the mutation. These observations suggest that DNA methylation is not essential for the growth of undifferentiated ES cells but required for the proliferation of differentiated somatic cells. To test this hypothesis, we induced the MTase mutant ES cells to differentiate in culture by formation of embryoid bodies. Although the $Dnmt^{n/n}$ ES cells were able to differentiate as extensively as wild-type cells to give rise to various cell types, the $Dnmt^{s/s}$ and $Dnmt^{c/c}$ cells underwent only limited differentiation and grew more slowly (data not shown). To directly compare cell growth rate between mutant and wild-type cells before and after differentiation, the mutant

**Fig. 5.** DNA methylation levels in wild-type and Dnmt mutant ES cells. (A) Methylation status of endogenous retrovirus DNA. DNA was digested with *Hpa*II (lane 1-7) or *Msp*I (lane 8), fractionated on a 1.0% agarose gel, blotted and hybridized to a MoMuLV full-length cDNA probe. DNA of wild-type and all heterozygous cell lines is highly methylated (lane 1-4), DNA of $Dnmt^{n/n}$ cells is partially demethylated (lane 5) and that of $Dnmt^{s/s}$ and $Dnmt^{c/c}$ cells is highly demethylated (lanes 6-7). A control *Msp*I digestion, indicative of complete demethylation, is shown in lane 8. Size markers (kb) of part of the 1 kb ladder (BRL) are shown on the right. (B) Direct detection of methyl cytosine. RNA-free genomic DNA was digested with *Msp*I (lanes 1-4) or *Hpa*II (lanes 5, 6) and end-labeling/TLC analysis was carried out as described (Cedar et al., 1979). The genotype of each DNA sample is indicated as described in Fig. 3. Only methyl dCMP ($m^5$dCMP) and dCMP spots are shown. The control *Hpa*II digestion of the wild-type (lane 6) and $Dnmt^{s/s}$ cells (lane 7) did not produce methyl CMP spots with counts above background indicating that the assay was highly specific. (C) DNA methyltransferase activity in wild-type and mutant ES cells. Nuclear extracts from wild-type (wt), heterozygous (c/+), $Dnmt^{n/n}$ (n/n) and $Dnmt^{c/c}$ (c/c) lines were prepared and DNA methyltransferase activity was assayed as described (Li et al., 1992). The subclones 36-1 and 36-2 were derived from one of the original $Dnmt^{c/c}$ clones, clone 36, by limiting dilution. The reaction was carried out with (shaded bars) or without (hatched bars) poly d(I-C). The activities of the wild-type and heterozygous ES cells are presented on a higher scale.

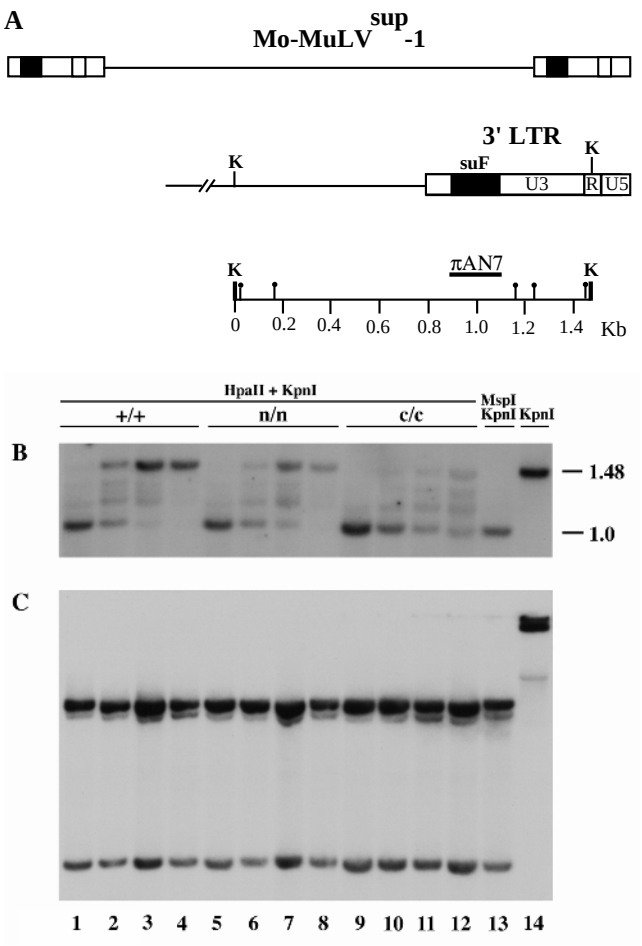



**Fig. 6.** De novo methylation of integrated provirus DNA in MoMuLV-infected wild-type and Dnmt mutant ES cells. (A) The MoMuLV$^{sup}$-1 provirus genome is shown at top and an enlarged 3′ LTR is shown in the middle. The supressor F gene (filled box) is located in the U3 region of the LTR. The 1.48 kb *Kpn*I (K) fragment that contains five *Hpa*II/*Msp*I sites (vertical bars with circles at the ends) is shown at bottom. The size marker (kb) and the suppressor F gene probe, πAN7, are indicated. (B) Genomic DNA isolated from infected wild-type (lane 1-4), *Dnmt*$^{n/n}$ (lanes 5-8) and *Dnmt*$^{c/c}$ (lanes 9-12) ES cells was digested with *Kpn*I and *Hpa*II (lane 1-12), blotted and hybridized to the πAN7 probe. Lanes 13 and 14 contain infected wild-type DNA digested with *Kpn*I and *Msp*I or *Kpn*I alone, respectively. DNA was isolated from infected cells 24 hours (lanes 1, 5 and 9), 2 days (lanes 2, 6 and 10), 4 days (lanes 3, 7 and 11) and 8 days (lanes 4, 8 and 12) after infection. (C) The same blot was re-hybridized with a mitochondrial DNA probe.

and wild-type ES cells were mixed and co-cultured for a period of 15 days. DNA was isolated at different days and analyzed by Southern blot hybridization using DNA probes that could distinguish the wild-type alleles and the mutant alleles. The ratio of mutant versus wild-type cells was measured by comparing the intensity of the mutant band to that of the wild-type band on a Southern blot as shown in Fig. 7. It was found that, while undifferentiated *Dnmt*$^{s/s}$ or *Dnmt*$^{c/c}$ cells proliferated at the same rate as wild-type cells, they were quickly outgrown by the wild-type cells and almost completely eliminated after growing in differentiation medium for 15 days (Fig. 7B,C). In contrast, differentiated *Dnmt*$^{n/n}$ ES cells proliferated at a slightly lower rate as compared to the wild-type cells (Fig. 7A). These results suggest that DNA methylation is probably required for efficient differentiation and/or proliferation of differentiated cells. Further studies are being conducted in chimeric embryos derived from different mutant ES cell lines to determine the extent of differentiation, the rate of proliferation and the cell death process of the MTase mutant cells.

### In vitro differentiation of mutant ES cells results in further demethylation of genomic DNA

It has been shown that de novo methylation activity is high in undifferentiated EC cells, but low or undetectable in differentiated EC cells and somatic cells (Stewart et al., 1982; Jähner and Jaenisch, 1984). This result suggests that the de novo methyltransferase is down regulated upon differentiation of EC cells. To test whether the de novo methyltransferase activity detected in the homozygous ES cells was also down-regulated after differentiation, we compared the methylation status of endogenous C-type retrovirus DNA before and after differentiation. Wild-type and mutant ES cells were induced to differentiate by formation of embryoid bodies in suspension culture. While the methylation level remained unchanged in differentiated wild-type cells (Fig. 8, lanes 1 and 2), it was significantly reduced in mutant ES cells 15 days after induction of differentiation (Fig. 8, lanes 3-8). This observation is consistent with the de novo DNA methyltransferase activity being downregulated in differentiated cells. Note that the methylation level in *Dnmt*$^{s/s}$ and *Dnmt*$^{c/c}$ cells was substantially reduced but not completely eliminated upon differentiation. This result was expected because a large number of mutant ES cells remained undifferentiated in embryoid bodies. The result described in this section is consistent with the prediction of regulated expression of the de novo methyltransferase activity in ES cells.

## DISCUSSION

We report here the generation and characterization of a new allele, *Dnmt*$^{c}$, of the only known mammalian DNA MTase. The *Dnmt*$^{c}$ mutation deleted two highly conserved motifs containing amino acid residues P-C (in motif 4) and E-N-V (in motif 6) which are invariant in all cytosine methyltransferases from bacteria to mammals (Lauster et al., 1989; Posfai et al., 1989, Kumar et al., 1994). The PC motif forms the active site, which is absolutely required for the enzyme activity (Wu and Santi 1978; Chen et al., 1991; Friedman and Ansari, 1992; Wyszynski et al., 1992). Furthermore, the MTase catalytic domain was also interrupted by the neomycin gene and no protein was detected by the antibody specific to the N-terminal domain of the MTase. Based on these lines of evidence, we conclude that the *Dnmt*$^{c}$ represents a null allele. Although transcripts of 9 kb and 5.4 kb were detected in the *Dnmt*$^{s/s}$ cells that could potentially encode the catalytic domain of the MTase, the DNA methylation level in these mutant cells is identical to that of the *Dnmt*$^{c/c}$ cells. Moreover, we have also derived ES cell lines that are homozygous for the *Dnmt*$^{s}$ mutation and heterozygous for the *Dnmt*$^{c}$ mutation (i.e. *Dnmt*$^{s/s,c/+}$) by targeted disruption of the C-terminal domain in a *Dnmt*$^{s/s}$ cell line through homologous recombination and

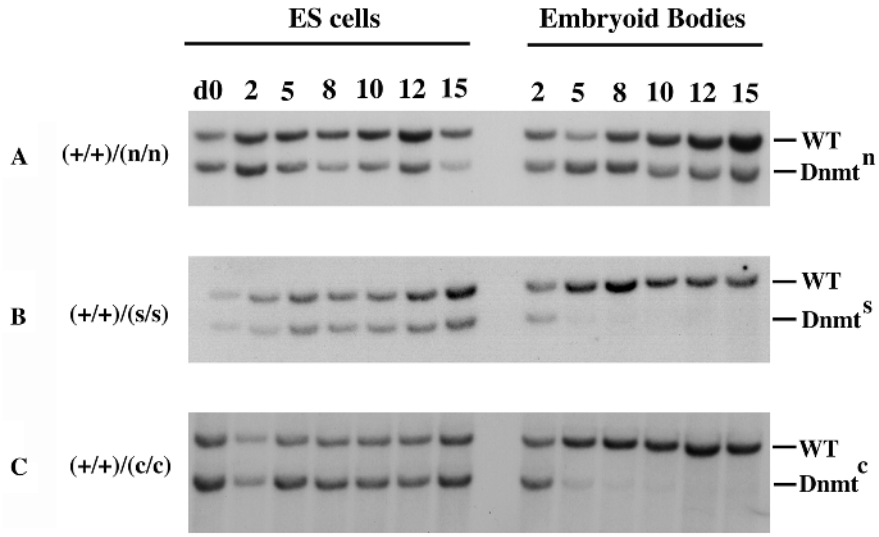



**Fig. 7.** Growth competition of wild-type and Dnmt mutant ES cells before and after differentiation. A mixture of wild-type ES cells (+/+) and mutant ES cells, *Dnmt*$^{n/n}$ (A), *Dnmt*$^{s/s}$ (B), or *Dnmt*$^{c/c}$ (C), was cultured on a feeder layer of embryonic fibroblast as undifferentiated ES cells (on the left) or grown in suspension to differentiate as embryoid bodies (on the right). Cells or embryoid bodies were harvested for DNA isolation at days 2, 5, 8, 10, 12 and 15 as labeled. DNA of cells at day 0 (d0) was isolated from the cell mixture at the beginning of differentiation, representing the initial ratio of mutant and wild-type cells. DNA was then digested with *Kpn*I (A), *Eco*RI (B), or *Eco*RI + *Eco*Rv (C), blotted and hybridized to probes that could distinguish between wild-type alleles and mutant alleles (Li et al., 1992, and Fig. 4A,B). (C) DNA was digested with *Eco*RI and *Eco*Rv, blotted and probed with pBB1.8 that detects an 7.5 kb wild-type band and a 5.8 kb mutant band (see Fig. 1C). The wild-type and mutant bands are labeled on the right.

found that the methylation level of *Dnmt*$^{s/s,c/+}$ cells is identical to that of *Dnmt*$^{s/s}$ and *Dnmt*$^{c/c}$ cells (data not shown). These results strongly suggest that both *Dnmt*$^{c}$ and *Dnmt*$^{s}$ are null alleles of the DNA MTase.

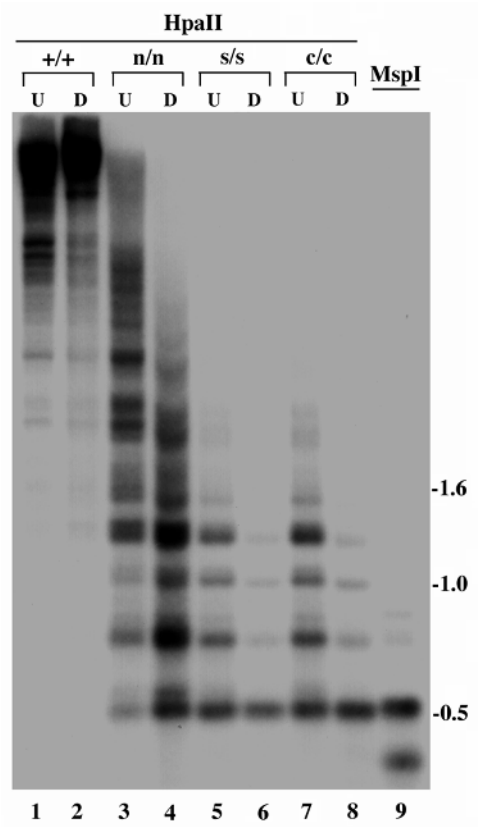



**Fig. 8.** Further reduction of methyl cytosine levels in differentiated Dnmt mutant cells. DNA was digested with *Hpa*II (lane 1-8) or *Msp*I (lane 9), blotted and hybridized with the MoMuLV probe. The genotype of the cell lines is labeled as described in Fig. 3. Lanes 1, 3, 5 and 7 contain DNA from undifferentiated (U) wild-type and mutant ES cell lines while lane 2, 4, 6 and 8 contain DNA isolated from day 15 embryoid bodies (D). The size markers are indicated on the right.

The survival of null mutant ES cells made it possible to examine whether an independently encoded DNA methyltransferase is expressed in ES cells. We demonstrated by two independent methods that low levels of methyl cytosine were present in the *Dnmt*$^{c/c}$ cells (Fig. 5A,B) and, furthermore, this methylation was stably maintained after more than 20 cell generations. We also showed that a significant amount of methyltransferase activity was detected in the null mutant cells by standard in vitro *trans*-methylation assays (Fig. 5C). These results strongly suggest that a second DNA cytosine methyltransferase is present in ES cells, which is responsible for 'maintaining' a low level of methyl cytosine in the *Dnmt*$^{c/c}$ cells. Since methylation levels are stable in *Dnmt*$^{c/c}$ cells, it is predicted that de novo methylation should occur in order to compensate the loss of methyl cytosine as a result of DNA replication. We suspect that de novo methylation in ES cells is not affected by our DNA MTase mutations. Unfortunately, de novo methylation of natural DNA substrate (e.g. λ phage DNA) in vitro using nuclear extract of both wild-type and *Dnmt*$^{c/c}$ cells was very inefficient (data not shown). It seems necessary to purify the second DNA methyltransferase from *Dnmt*$^{c/c}$ cells before its de novo methylation activity can be fully characterized.

De novo methylation activity can also be measured by introduction of unmodified foreign DNA into the cells and subsequent analysis of its methylation status. We infected wild-type and mutant ES cell lines with the murine retrovirus MoMuLV and found that newly integrated provirus DNA was almost completely methylated in wild-type cells 8 days after infection. Although the provirus DNA remained partially methylated in mutant ES cells due to the lack of the maintenance DNA MTase, DNA methylation also reached highest levels in 8 days. The similar progress of de novo methylation of the viral DNA in mutant and wild-type cells suggests that disruption of the Dnmt gene has little or no effect on de novo methylation. In other words, the remaining DNA methyltransferase activity

in *Dnmt*$^{c/c}$ cells is capable of de novo methylating cellular and foreign DNA. The finding that active de novo methylation of the provirus DNA in infected mutant ES cells and differentiation of mutant ES cells induces further demethylation is reminiscent of a previous observation that provirus DNA is fully methylated after infection of preimplantation mouse embryos but remains unmethylated when postgastrulation embryos are infected (Jähner et al., 1982). This second DNA methyltransferase is likely an early embryo specific de novo methyltransferase that is responsible for active de novo methylation of the genome shortly after implantation. Although our results do not exclude the possibility that the known DNA MTase has de novo methylation activity in vivo, we favor the hypothesis that de novo methylation and maintenance methylation are catalyzed by two separate enzymes such that these two activities can be regulated independently. Alternatively, it is possible that the newly identified activity may function synergistically with the known DNA MTase to carry out rapid de novo methylation during early postimplantation development.

The expression of an independently encoded de novo methyltransferase in early embryos provides an explanation as to why *Dnmt*$^{c/c}$ embryos are able to develop and survive until 8.5 d.p.c.. Previous studies have indicated that de novo methylation occurs in early embryos at a time between 3.5 d.p.c. and 6.5 d.p.c. (Razin and Cedar, 1993). Active de novo methylation in postimplantation embryos would slow down the net loss of methylation in mutant embryos and thus delaying the time when methylation of genomic DNA has decreased below a level critical to cell growth. An alternative explanation is that early embryonic cells are less dependent on DNA methylation for their growth. Consistent with this view, we found that ES cells homozygous for *Dnmt*$^{c}$ and *Dnmt*$^{s}$ alleles were viable and grew normally despite very low levels of DNA methylation while differentiation of these cells results in growth arrest (Fig. 7).

The cause of embryonic lethality of the mutant embryos is still unknown. One of the possibilities is that demethylation induces inappropriate expression of developmentally regulated genes such as the imprinted genes (Li et al., 1993; Beard et al., 1995). It seems unlikely that the known imprinted genes such as *H19*, *Igf-2* and *Igf-2* receptor are responsible for the embryonic lethality because inactivation of these genes does not affect early embryonic development (DeChiara et al., 1991; Lau et al., 1995; Leighton et al., 1995; Wang et al., 1995). However, it is possible that other as yet unknown imprinted genes that are required for early development could be involved. We recently showed that the *Xist* gene is abnormally expressed in male embryos that are homozygous for the *Dnmt*$^{s}$ mutation (Beard et al., 1995). If activation of the *Xist* gene results in X chromosome inactivation, then other X-linked genes on the single X chromosome in male embryos or both X chromosomes in female embryos would be silenced and this could result in embryonic lethality of both male and female mutant embryos. A second possibility is that DNA methylation may be an essential component of high order chromatin structure and deficiency in DNA methylation may lead to abnormal chromatin structure that perturbs replication and compaction of the chromatin DNA and causes cell cycle arrest. DNA methylation has been implicated in chromatin organization. For example, cytosine methylation is required for a methyl CpG-binding protein, MeCP-2, to bind to the centromeric region of the chromosomes (Nan et al., 1996). Hypomethylation is associated with the lack of H1 histone and acetylation of other nucleosome histones in the chromatin (Tazi and Bird 1990; Jeppesen and Turner, 1993). Our finding that the severity of developmental delay of mutant embryos correlates with the severity of the mutation and the degree of demethylation of genomic DNA suggests that DNA methylation deficiency has a negative effect on cell growth. How DNA methylation is involved in chromatin function and cell growth regulation is currently under investigation.

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