Loss of methylation activates *Xist* in somatic but not in embryonic cells

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The mouse *Xist* gene, which is expressed only from the inactive X chromosome, is thought to play a role in the initiation of X inactivation. The 5′ end of this gene is fully methylated on the active X chromosome and completely demethylated on the inactive X chromosome, suggesting that DNA methylation may be involved in controlling allele-specific transcription of this gene. To directly investigate the importance of DNA methylation in the control of *Xist* expression, we have examined its methylation patterns and expression in ES cells and embryos that are deficient in DNA methyltransferase activity. We report here that demethylation of the *Xist* locus in male mutant embryos induces *Xist* expression, thus establishing a direct link between demethylation and expression of the *Xist* gene in the postgastrulation embryo. The transcriptional activity of *Xist* in undifferentiated ES cells, however, appears to be independent of its methylation status. These results suggest that methylation may only become essential for *Xist* repression after ES cells have differentiated or after the embryo has undergone gastrulation.

[*Key Words*: *Xist* gene; X chromosome inactivation; DNA methylation; transcriptional activity]

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Dosage compensation in mammals is accomplished by X chromosome inactivation, which ensures that an equal number of X-linked genes is expressed in both sexes (Lyon 1961). In female embryos, X inactivation first occurs in the extraembryonic lineages, where the paternal allele of the X chromosome is preferentially silenced (Takagi and Sasaki 1975; West et al. 1977; Monk and Harper 1979; Papaioannou et al. 1981; Harper et al. 1982). X inactivation is random in the epiblast, resulting in silencing of either the maternal or paternal X chromosome (Gardner and Lyon 1971; Takagi 1974; Rastan 1982). The process of inactivation begins at the X inactivation center (Xic) (Russell 1963; Therman et al. 1974; Cattanach 1975; Mattei et al. 1981) which is required in *cis*, and spreads from the Xic to the adjacent chromosomal regions. The *Xist* gene, which maps to the Xic region in mouse and man, is thought to play a role in the initiation of X inactivation as it is expressed only from the inactive X chromosome and its expression precedes both imprinted and random X chromosome inactivation (Borsani et al. 1991; Brockdorff et al. 1991; Brown et al. 1991; Kay et al. 1993).

DNA methylation has long been proposed as a possible mechanism for controlling allele-specific transcription, as it is stable and heritable through many cell divisions, is known to affect gene expression, and is reversible through the germ line. Several groups have recently presented evidence linking DNA methylation and *Xist* expression. These investigators have shown that CpG sites at the 5′ end of the *Xist* gene become demethylated in the perinatal prospermatogonia and remain demethylated throughout preimplantation development (Norris et al. 1994; Ariel et al. 1995; Zuccotti and Monk 1995). These results are consistent with the hypothesis that male gamete-specific hypomethylation is important for the imprinting of the paternal X chromosome in the extraembryonic ectoderm and trophectoderm, which only express the paternal *Xist* allele, whereas the silent maternal allele is methylated (Norris et al. 1994; Ariel et al. 1995; Zuccotti and Monk 1995). Later in development, when the imprint is erased and X inactivation becomes random, the repressed *Xist* gene on the active X chromosome is fully methylated, whereas the expressed gene on the inactive X chromosome is hypomethylated (Norris et al. 1994). A strong correlation between methylation and *Xist* expression is further supported by the observation that in male embryonic stem (ES) cells, which contain only one X chromosome, the silent *Xist* gene is methylated (Norris et al. 1994). Undifferentiated female ES cells, which contain two active X chromosomes, do not express *Xist*. Upon differentiation, however, one *Xist* allele becomes activated and hypomethylated concomitant with random X inactivation.

The importance of DNA methylation in controlling the differential expression of paternal and maternal alleles of imprinted genes has been demonstrated in our laboratory using embryos that have two different muta-

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tions in the DNA methyltransferase gene (Dnmt) (Li et al. 1992, 1993). One of these mutations, the $Dnmt^N$ allele, results in a partial loss of function (Li et al. 1992). Embryos homozygous for this mutation die at embryonic day 11 (E11) and their DNA, although substantially demethylated, still retains 30% of the wild-type level of 5-methylcytosine (Li et al. 1992). A second, more severe mutation, the $Dnmt^S$ allele, is possibly a null mutation. Embryos homozygous for this mutation die at the 5–10 somite stage and their DNA is more demethylated than that of the $Dnmt^N$ homozygous embryos (Li et al. 1993, and in prep.). We have shown that monoallelic expression of three imprinted genes, *H19*, *Igf2*, and *Igf2r*, is altered in these mutant embryos (Li et al. 1993). Moreover, the extent of DNA hypomethylation, which differs in the two Dnmt mutants, dictates the severity of imprinted gene deregulation. In particular, we observed that expression of the *H19* and *Igf2* genes was altered in the weaker $Dnmt^N$ homozygous embryos, whereas *Igf2r* gene expression was affected only in the more severe $Dnmt^N/Dnmt^S$ compound heterozygous or $Dnmt^S$ homozygous embryos. Furthermore, the CpG island, which is methylated on the expressed maternal allele of the *Igf2r* gene, was completely demethylated only in the latter mutant embryos. We were thus able to establish a causal link between DNA methylation and gene activity and we showed that methylation of the *Igf2r* gene was preferentially maintained under conditions of limiting maintenance methyltransferase.

In this report we have used both $Dnmt^N$ and $Dnmt^S$ homozygous ES cells and embryos to study the effects of demethylation on *Xist* expression. We show that demethylation of the *Xist* locus in male mutant embryos and differentiated male mutant ES cells induces *Xist* expression, thus demonstrating the importance of DNA methylation for maintaining the repression of this gene.

Results

Expression of Xist *in male embryos deficient in DNA methyltransferase*

To determine whether methylation is required for the repression of *Xist* transcription on the active X chromosome, *Xist* expression was examined in embryos deficient for DNA methyltransferase. RNA was isolated from E10.5 embryos homozygous for the $Dnmt^N$ mutation and analyzed for *Xist* expression by reverse transcriptase polymerase chain reaction (RT–PCR) using primers that are specific for mature spliced *Xist* RNA (Kay et al. 1993). Figure 1A demonstrates that as expected, *Xist* RNA was expressed in female wild-type and $Dnmt^N$ homozygous embryos (lanes 3,10). No *Xist* transcripts were seen in RNA isolated from wild-type or mutant male embryos (lanes 2,4,8,9,11), suggesting that the level of overall hypomethylation achieved in $Dnmt^N$ mutant embryos did not alter the expected expression pattern.

To address whether a further reduction in the overall level of DNA methylation, which is seen in embryos carrying the more severe $Dnmt^S$ allele mutation, would result in *Xist* expression in mutant male embryos, we isolated RNA from E9.5 embryos that were either compound heterozygous $Dnmt^N/Dnmt^S$ or $Dnmt^S$ homozygous mutants. RT–PCR analysis showed very low levels of *Xist* expression in two of six of the $Dnmt^N/Dnmt^S$ male embryos analyzed (see Table 1 and Fig. 1A, lanes 1,6,7; three mutant embryos shown), whereas five of five independently tested $Dnmt^S$ homozygous male embryos showed significant *Xist* expression (Fig. 1B; three mutant embryos shown).

It appeared that in $Dnmt^S$ homozygous embryos the *Xist* gene was expressed. Alternatively, the *Xist* transcription detected by RT–PCR may have been attributable to maternal contamination of the embryo samples. To determine whether the *Xist* expression seen in the $Dnmt^S$ homozygous male embryos was attributable to maternal contamination, cDNA synthesized from the embryonic RNA was also PCR amplified using primers that span the *Sal*I site of the Dnmt gene. A 128-bp product, representative of the wild-type gene, was seen in the RNA samples of heterozygous and wild-type embryos but not in the $Dnmt^S$ mutant embryos (Fig. 1C), indicating that the mutant embryo RNA was not contaminated with maternal tissue. The 600-bp product that was amplified in the mutant embryo RNA samples (Fig. 1C) was cloned and sequenced and found to be attributable to amplification of an unrelated RNA. Because the development of the $Dnmt^S$ mutant embryos is delayed by 1 day when compared to normal littermates (Li et al. 1993), RNA from E8.5 wild-type male embryos was also analyzed by RT–PCR. No *Xist* expression was detected (data not shown). Finally, because the primers specific for *Xist* RNA span the *Hin*dIII restriction enzyme site, digestion of the RT–PCR products with this enzyme allowed us to confirm that the 578-bp product was attributable to the amplification of *Xist* sequences (data not shown). The results summarized in Table 1 indicate that the *Xist* gene was fully repressed in the $Dnmt^N$ homozygous embryos and activated in only some of the $Dnmt^N/Dnmt^S$ embryos. Reproducible activation of the *Xist* gene was seen in all embryos homozygous for the more severe $Dnmt^S$ allele.

Demethylation of the Xist *gene correlates with* Xist *expression*

It has been proposed that methylation at the 5′ end of the *Xist* gene is associated with repression of this gene on the active X chromosome. To assess whether *Xist* expression correlated with *Xist* gene methylation, DNA from the various Dnmt mutant embryos or yolk sacs was digested with *Eco*RV and a methylation-sensitive restriction enzyme, *Hha*I, and analyzed by Southern blot using a 1.5-kb probe that hybridizes to the 5′ end of the *Xist* gene (Fig. 2A). Figure 2B shows that low molecular mass bands (0.8 and 0.2 kb) seen in both wild-type and mutant female yolk sacs were not detectable in DNA from $Dnmt^N$ homozygous male yolk sacs (similar results were

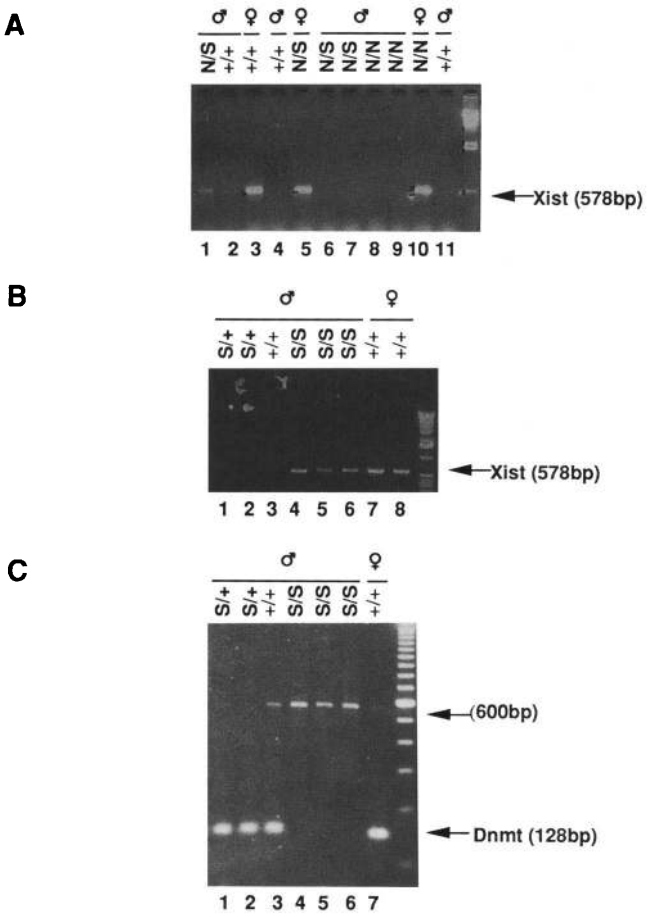

Figure 1. RT–PCR analysis of *Xist* expression in Dnmt mutant embryos. (*A*,*B*) Total cellular RNA was prepared from individual E10.5 ($Dnmt^N/Dnmt^N$) or E9.5 ($Dnmt^N/Dnmt^S$) and ($Dnmt^S/Dnmt^S$) embryos. RNA (2 μg) was reverse transcribed using random hexamers. Half of the first strand product was subjected to 30 cycles of PCR amplification using cDNA primers specific for *Xist* transcription (*A*,*B*) or wild-type Dnmt transcription (*C*). Samples were then subjected to electrophoresis in a 2% agarose gel containing ethidium bromide. Molecular weight size markers λ DNA–*Hin*dIII fragments (*A*, lane *12*) and the BRL 1-kb DNA ladder (*B*, lane *9*; *C*, lane *8*) are shown. The gel was photographed under UV illumination.

obtained using DNA isolated from embryos). These lower molecular mass fragments are attributable to demethylation of the expressed *Xist* allele on the inactive X chromosome (Norris et al. 1994). The fragment migrating at ~3.3 kb, which is diagnostic of complete methylation at all *Hha*I sites except *Hha*I(1) or *Hha*I(2), is seen also in wild-type or heterozygous male embryos (Fig. 2D, lanes 3,4). To assess the overall methylation level of mutant and wild-type embryos, the blot was stripped and probed with a Moloney murine leukemia virus (MoMuLV) cDNA. This probe allows detection of multiple endogenous retroviruses that are known to be highly methylated (Stuhlmann et al. 1981; Jahner et al. 1982) and, therefore, serves as an indicator of global genomic methylation levels. In contrast to the results obtained with the *Xist* probe, the MoMuLV probe revealed substantial hypomethylation of the genomic DNA in the $Dnmt^N$ homozygous embryos (Fig. 2C, cf. lane 2 and lanes 1,3–5).

Southern blot analysis of DNA from $Dnmt^N/Dnmt^S$ compound heterozygous male embryos showed partial demethylation of the 5′ region of the *Xist* gene in mutant embryos (Fig. 2D, lanes 1,2). Clearly, substantial levels of methylation were maintained as revealed by the presence of the 4.5-kb full-length band that is generated only when all *Hha*I sites are methylated on the same allele. The prominent band at 3.3 kb and several bands migrating around 2.5 kb were attributable to partial demethylation of some *Hha*I sites within the *Eco*RV fragment. The methylation pattern of the *H19* gene was also analyzed in these mutant embryos to confirm that there was significant demethylation at another independent locus in the genome. Figure 2E shows that the differentially methylated region of the *H19* gene (Bartolomei et al. 1993; Brandeis et al. 1993; Ferguson et al. 1993) was completely demethylated in the $Dnmt^N/Dnmt^S$ mutant embryos.

Finally, when DNAs from male and female $Dnmt^S$ homozygous embryos were analyzed by Southern blot, we observed complete demethylation of *Hha*I sites as demonstrated by the presence of a prominent band at 0.8 kb, which is diagnostic of demethylation of the *Xist* gene on the inactive X chromosome (Fig. 2F, lanes 4–8). These results indicate that the *Xist* gene is more resistant to demethylation than either the *H19* gene or bulk genomic DNA under conditions of limiting levels of methyltransferase as achieved in the Dnmt mutant embryos. Furthermore, our results show a direct correlation between demethylation and expression of the *Xist* gene, suggesting that methylation of the 5′ end of the gene is involved in the control of *Xist* transcription.

Expression of Xist *is increased in male ES cell lines deficient in the DNA methyltransferase gene*

ES cells homozygous for the $Dnmt^N$ or $Dnmt^S$ mutations proliferate normally and are morphologically indistinguishable from wild-type cells (Li et al. 1992, 1993; H. Lei, P.S. Oh, R. Jütterman, R. Jaenisch, K. Goss, and E. Li,

Table 1. *Summary of* Xist *expression in Dnmt mutant male embryos*

Dnmt genotype	*Xist* expression (no. of embryos)		
	++	+	−
wt	0	0	10
N/N	0	0	5
N/S	0	2	4
S/S	5	0	0

The data in this table were derived from individual male embryos. RT–PCR analysis was as described in Fig. 1. (+) A low, but detectable level of *Xist* expression.

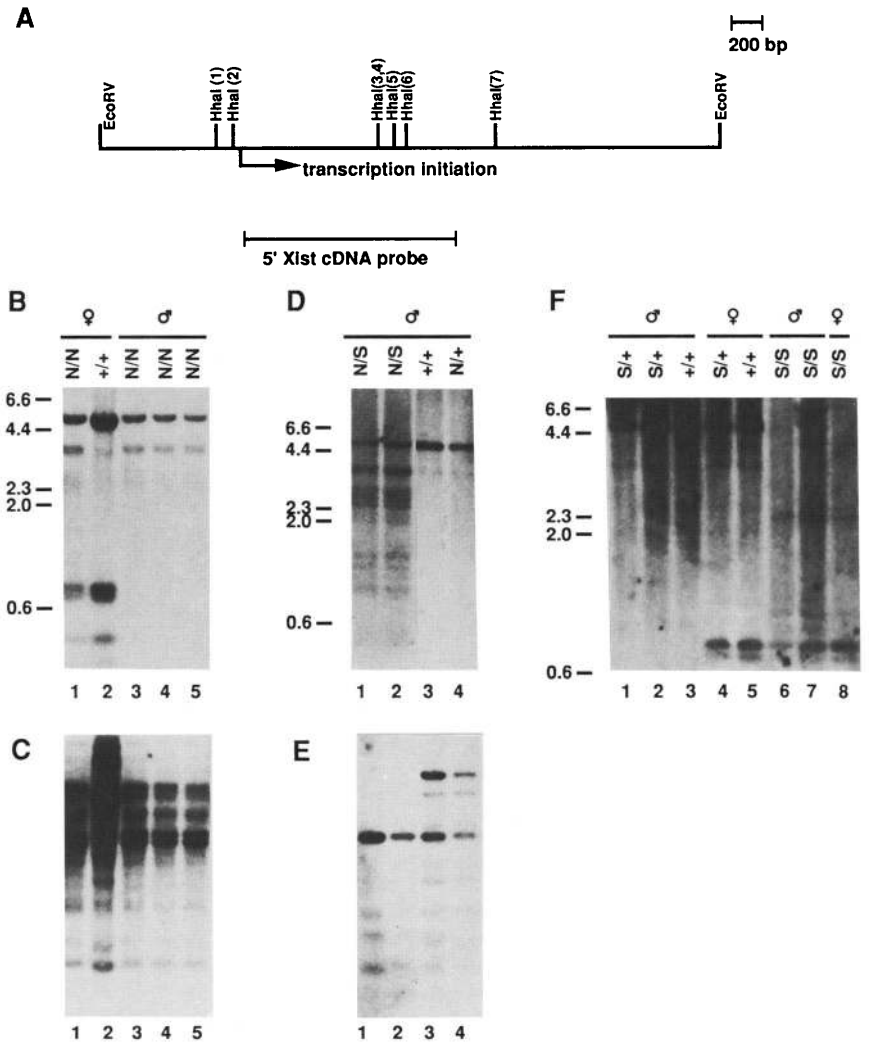

Figure 2. The relative resistance of *Xist* to demethylation in Dnmt mutant embryos correlates with expression of the gene. (*A*) Schematic representation of the 5′ end of the *Xist* gene showing the positions of the methylation-sensitive restriction enzyme, *Hha*I, and the probe used in this analysis. (*B,C*) Southern blot analysis of the $Dnmt^N$ homozygous embryos (N/N). Approximately 5 μg of genomic DNA isolated from individual E10.75 yolk sacs was digested with *Eco*RV and *Hha*I. The filter was hybridized with the 5′ *Xist* cDNA probe (*B*), stripped, and then rehybridized with the MoMuLV probe (*C*). (*D,E*) Southern blot analysis of the $Dnmt^N/Dnmt^S$ embryos (N/S). In each lane ~2 μg of genomic DNA from individual E9.5 yolk sacs was digested with *Eco*RV and *Hha*I and probed with the 5′*Xist* probe (*D*) or digested with *Sac*I and *Hpa*II and probed with the *H19* probe (*E*). (*F*) Southern blot analysis of $Dnmt^S$ homozygous embryos (S/S). Approximately 2 μg of genomic DNA from individual E9.5 embryos plus yolk sac were digested with *Eco*RV and *Hha*I and hybridized with the 5′ *Xist* probe. Molecular mass (in kilobases) is marked at *left*.

in prep.). These cell lines can be induced to differentiate and form embryoid bodies in vitro, although the $Dnmt^S$ mutant ES cells do not differentiate or proliferate to the same extent as the wild-type ES cells. We wished to determine whether *Xist* expression and methylation of the *Xist* gene in mutant male ES cells would mimic what was seen in vivo in the homozygous male embryos. This was important because the paucity of material available from E8.5 or E9.5 embryos hampers the study of methylation and X inactivation in vivo.

Male ES cells, which contain a single X chromosome, do not express *Xist* in the undifferentiated state or after differentiation (Kay et al. 1993). We first analyzed *Xist* expression in undifferentiated wild-type and mutant male ES cells using RT–PCR. To exclude the possibility of *Xist* expression from embryonic fibroblast (EF) feeder cell contamination, ES cells were seeded on male EF cells that do not express the *Xist* gene. We were surprised to see a low but detectable level of *Xist* expression even in wild-type male ES cells (Fig. 3A, lane 1,4) which did not, however, appear to appreciably increase when the cells were induced to differentiate. Undifferentiated $Dnmt^N$ and $Dnmt^S$ mutant ES cells and an early passage wild-type female cell line V44 also showed a low level of *Xist* expression, which, in contrast to the wild-type male ES cells, increased substantially upon differentiation. RT–PCR analysis of the ES cells using primers specific for FGF-5, which is an early marker of differentiation (Hebert et al. 1991; Conover et al. 1993), showed a low level of expression in the undifferentiated ES cells (Fig. 3B), suggesting that the expression of *Xist* seen in the undifferentiated cultures might be attributable to cells that had differentiated or were poised to differentiate.

RNase protection analysis was used to quantitatively measure *Xist* expression to evaluate the degree of activation of this gene upon differentiation of the ES cells. Figure 4 shows that, as expected, high levels of *Xist* specific transcripts were seen in RNA from female but not male adult spleen (lanes 7,8). Although no *Xist*-specific RNA was detected in any of the undifferentiated ES cells, differentiation for 15 days induced a low level of *Xist*- specific RNA transcription in the $Dnmt^N$ mutant

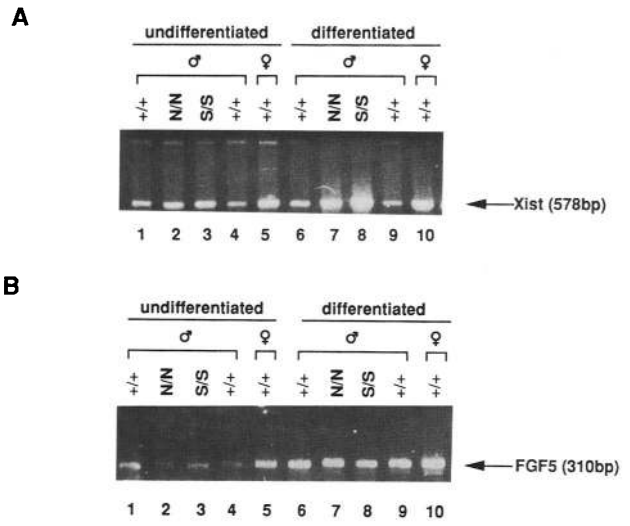

Figure 3. RT–PCR analysis of *Xist* expression in undifferentiated and differentiated Dnmt mutant ES cells. (*A*) Ethidium bromide-stained gel showing *Xist* expression in ES cells, undifferentiated and differentiated for 15 days. The higher molecular weight product, which is visible in lanes *1–5* and *9*, is attributable to amplification of contaminating DNA in these RNA samples. The wild-type male cell line in lanes *1* and *6* is J1 and in lanes *4* and *9* is V18. V44 is the female cell line shown in lanes *5* and *10*. (*B*) Ethidium bromide-stained agarose gel showing FGF-5 expression in ES cells, undifferentiated and 3 days following differentiation as embryoid bodies.

ES cells and a significantly greater level of *Xist* RNA in the Dnmt^S mutant ES cells. The level of expression of *Xist* in the Dnmt^S mutant ES cells was comparable to that seen in RNA isolated from the spleen of an adult female. No *Xist*-specific RNA was detected in the differentiated male wild-type ES cells.

The results presented in Figures 3A and 4 indicate that although the *Xist* gene is expressed at very low levels detectable only by RT–PCR in undifferentiated wild-type and mutant male ES cells, it is activated efficiently only in Dnmt mutant and wild-type female ES cells upon induction of differentiation.

Methylation of the Xist *gene in differentiated ES cells correlates with* Xist *expression*

To determine whether *Xist* expression correlated with demethylation of the *Xist* gene as was seen in vivo in the Dnmt mutant embryos, DNA from male Dnmt-deficient ES cells was analyzed as described in Figure 2. We were surprised to see that the *Xist* gene was partially demethylated in two different undifferentiated wild-type male cell lines, which did not show any *Xist* expression as measured by RNase protection. However, the level of methylation increased as differentiation proceeded (Fig. 5A, cf. lane 1 and 13 with lanes 4 and 16, respectively). Whereas the level of methylation in undifferentiated Dnmt^N mutant ES cells was substantially less than in the wild-type cells, it also increased upon the induction of differentiation (Fig. 5A, lanes 5–8). DNA hypomethylation was, as expected, most pronounced in Dnmt^S mutant ES cells; however, following differentiation an increase in methylation levels was less obvious (lanes 9–12) than in wild-type and Dmnt^N mutant ES cells.

To investigate whether overall genomic methylation levels increased upon differentiation of wild-type and mutant ES cells, we digested samples of genomic DNA with *Hpa*II and hybridized Southern blots with the MoMuLV probe and two repetitive probes, pMR134 and pMR150. pMR134 detects L1 sequences that are interspersed throughout the mouse genome with 30,000 copies of a 5- to 7-kb element (Voliva et al. 1983; Chapman et al. 1984), and pMR150 detects minor satellite sequences that are centromeric, tandemly repetitive elements of 120 bp with 50,000 copies per genome (Chapman et al. 1984). Both of these repetitive elements are highly methylated in adult somatic tissue (Chapman et al. 1984). When the Southern blot was hybridized with the MoMuLV probe or either of the two repetitive probes, we did not observe any significant increase in overall genomic DNA methylation levels upon differentiation of wild-type or mutant cells (Fig. 5B–D). Overall genomic DNA methylation levels in wild-type and Dnmt^N homozygous ES cells as measured using the MoMuLV probe actually decreased after 15 days of differentiation (data not shown). We also noted that overall genomic DNA in undifferentiated wild-type ES cells was highly methylated in contrast to the 5′ end of the *Xist*

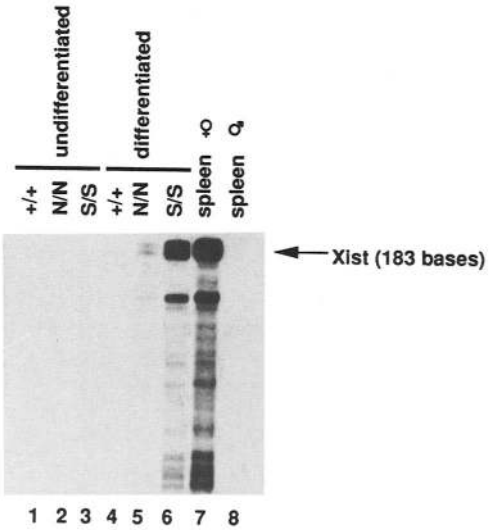

Figure 4. RNase protection analysis of *Xist* expression in undifferentiated and differentiated male ES cells. Samples of total RNA (10 μg) isolated from undifferentiated (lanes *1–3*) ES cells and ES cells differentiated for 15 days (lanes *4–6*) were analyzed by RNase protection using a 254-base antisense RNA probe that hybridizes to sequences contained within exon V and VI of the *Xist* gene. The size of the protected fragment was 183 bases. RNAs from female (lane 7) and male (lane 8) adult spleen represent a positive and negative control, respectively. A cytoplasmic β-actin antisense RNA probe was used as a control to demonstrate the presence of RNA in all samples (data not shown).

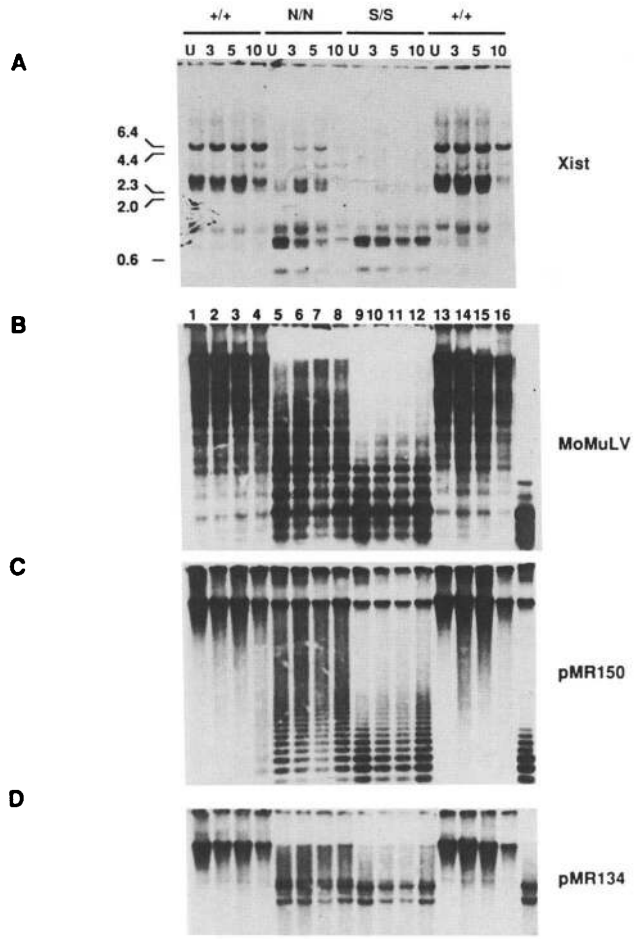

Figure 5. Demethylation of the *Xist* gene correlates with *Xist* expression in differentiated ES cells. Southern blot analysis of wild-type and Dnmt mutant male ES cells. (*A*) Genomic DNAs (10 μg) isolated from undifferentiated ES cells (U) and 3, 5, and 10 days following differentiation were digested with *Eco*RV and *Hha*I and hybridized with the 5′ *Xist* probe. V18 is a low passage male cell line. (*B,C,D*) Ten micrograms of the same DNA as in *A* was digested with *Hpa*II and probed with MoMuLV to measure global methylation (*B*). The blot was stripped and reprobed with either pMR150, which detects tandem centromeric repeat elements (*C*), or pMR134, which detects L1 interspersed repetitive elements (*D*). Lane *17* contains genomic DNA digested with the methylation insensitive restriction enzyme, *Msp*I, as a marker for complete demethylation.

gene, which was only partially methylated (cf. Fig. 5A with B,C, and D, lanes 1 and 13). Similar results were obtained when the Southern blot shown in 5A (an *Eco*RV + *Hha*I digest) was hybridized to the MoMuLV probe (data not shown).

We conclude that the *Xist* gene, in contrast to other sites in the genome, is partially methylated in undifferentiated wild-type ES cells and that differentiation increases its methylation level. Substantial hypomethylation of the *Xist* gene is seen in ES cells homozygous for the $Dnmt^N$ allele and, more so, in ES cells homozygous for the $Dnmt^S$ allele. The more pronounced hypomethylation seen in the latter cells correlates with increased expression, suggesting that methylation is causally involved in transcriptional repression of the *Xist* gene.

Discussion

The relationship between DNA methylation and *Xist* expression was investigated in embryos and ES cell lines that had differing degrees of hypomethylation produced by two alleles of the DNA methyltransferase gene. Specifically, we have shown that in male embryos homozygous for the weaker $Dnmt^N$ allele, the 5′ end of the *Xist* gene is fully methylated and the gene is not expressed. In contrast, male embryos homozygous for the most severe $Dnmt^S$ mutation consistently expressed *Xist* with the gene being completely demethylated. Partial *Xist* methylation was seen in $Dnmt^N/Dnmt^S$ compound heterozygotes, only a fraction of which produced low levels of *Xist* RNA. Thus, the correlation between DNA methyltransferase levels, *Xist* methylation, and *Xist* expression provide clear in vivo evidence for a methylation mediated repression of *Xist* transcription in the postgastrulation embryo.

The results obtained with embryos in vivo were corroborated in male ES cells carrying the different Dnmt alleles. *Xist* activation was much more pronounced in the ES cells homozygous for the strong $Dnmt^S$ allele than in the cells homozygous for the weaker $Dnmt^N$ allele. Furthermore, the level of expression correlated with the degree of demethylation at the 5′ end of the *Xist* gene: The $Dnmt^S$ mutant ES cells were almost completely demethylated, whereas the $Dnmt^N$ mutant ES cells showed partial demethylation of the gene.

Interestingly, the RT–PCR results suggest that *Xist* is expressed in all ES cells prior to differentiation at a level low enough to escape detection by less sensitive techniques such as RNase protection. Preliminary in situ hybridization data are consistent with the *Xist* gene being expressed in undifferentiated ES cells (B. Panning and R. Jaenisch, in prep.). This expression is detectable in both male and female undifferentiated ES cells, suggesting that regulation of *Xist* expression prior to differentiation relies on a different mechanism that is both methylation and sex independent. What significance *Xist* expression may play at this stage and whether this in vitro result reflects an in vivo situation remains to be determined, but it is intriguing in light of *Xist*'s role in initiating X inactivation.

Our results, in conjunction with results of an earlier study from our laboratory (Li et al. 1993), demonstrate that the methylation of certain sequences is preferentially maintained under conditions of limiting methyltransferase. We have shown that the *Xist* gene is more resistant to demethylation than the *Igf2r* gene which, in turn, is more resistant than the *H19* gene. Complete demethylation at the 5′ end of the *Xist* gene was seen only in the most severe $Dnmt^S$ homozygous embryos, which do not have any detectable methyltransferase activity. The reason for the preferential maintenance of

methylation of the *Igf2r* and *Xist* genes, which is clearly seen in embryos homozygous for the less severe $Dnmt^N$ mutant allele, is not known. It is possible, for example, that specific sequence motifs, present in regions of the *Xist* and *Igf2r* genes, allow for more efficient binding of the DNA methyltransferase resulting in a preferential retention of methylation. In this regard, it has been shown that the human methyltransferase enzyme has an exceptional facility for actively methylating unusual DNA structures (Smith et al. 1991). Furthermore, direct tandem repeats, which are potentially able to form secondary structures, have been found in the putative imprinting box of the *Igf2r* gene (Neumann et al. 1995) and at the 5′ end of the *Xist* gene (Pfeifer and Tilghman 1994). These direct repeats are unique to the *Igf2r* and *Xist* genes and do not show any homology to the highly repeated DNA sequences in the mouse (Pfeifer and Tilghman 1994). The source of the methyltransferase activity that is responsible for the preferential methylation of the *Igf2r* and *Xist* genes is not known. It could be provided for by the low levels of maintenance methyltransferase that are detectable in the $Dnmt^N$ homozygous embryos and that would selectively methylate these sequences. Alternatively, another as yet undefined methyltransferase activity might be responsible for methylating certain sequences such as those present in the *Igf2r* and *Xist* genes. This latter possibility will be discussed below.

Although hypomethylation is essential for *Xist* expression in the postgastrulation embryo, in vitro data suggest that repression of *Xist* transcription in undifferentiated ES cells and, by implication in the pregastrulation embryo, may be independent of the methylation status of the gene. Thus, only a low level of *Xist* expression was seen in undifferentiated male wild-type and Dnmt mutant ES cells, although the gene was highly demethylated in the latter cells. Upon differentiation, however, demethylation of the gene appeared to be crucial for activating *Xist* transcription as *Xist* expression increased significantly only in the differentiated mutant but not in differentiated wild-type ES cells. These results suggest that in addition to demethylation of the *Xist* gene, repressors or activators of *Xist* expression are required for expression, and these factors are only present in differentiated ES cells or in embryos that have undergone gastrulation.

We and others (Norris et al. 1994) have noted a lower level of overall methylation of the *Hha*I sites at the 5′ end of the *Xist* gene in wild-type undifferentiated ES cells relative to the level seen in somatic tissues. This partial methylation of the *Xist* gene in undifferentiated male ES cells stands in contrast to centromeric and interspersed repetitive sequences that are completely methylated. Upon differentiation, the level of 5′ *Xist* methylation, in contrast to the stable and even reduced level of methylation of endogenous retroviral sequences, appears to increase, consistent with methylation of the *Xist* gene playing an important role in the transcriptional repression of this gene in differentiated ES cells. We consider three possibilities to explain the apparent increase of *Xist* methylation.

Possibility 1 If *Xist* is responsible for X inactivation, its expression would result in cell lethality because of the silencing of essential genes on the single X chromosome. The apparent increase in methylation upon differentiation would, in this case, simply represent a selection for those cells that have a correctly methylated and, therefore, silent *Xist* gene. Prior to differentiation there would be no selective advantage to maintaining the methylation of the *Xist* gene, as repression of *Xist* transcription appears to be independent of the methylation status of the gene in undifferentiated cells.

Consistent with this possibility is the observation that undifferentiated $Dnmt^S$ mutant ES cells proliferate normally; however, upon induction of differentiation the $Dnmt^S$ cells do not appear to differentiate to the same extent as $Dnmt^N$ or wild-type ES cells, but rather they appear to die (H. Lei, P.S. Oh, R. Jütterman, R. Jaenisch, K. Goss, and E. Li, in prep.). Also, undifferentiated $Dnmt^S$ mutant ES cells, in contrast to wild-type or $Dnmt^N$ mutant ES cells, injected subcutaneously into mice do not form teratomas (C. Beard, unpubl.). It is possible that the poor survival of differentiating $Dnmt^S$ mutant ES cells is attributable to the initiation of X chromosome inactivation induced by the expression of the *Xist* gene.

Possibility 2 A demethylating activity, responsible for the hypomethylation of the *Xist* gene, is present in undifferentiated ES cells. Upon differentiation, silencing of this demethylating activity would allow increased methylation of the *Xist* gene. As argued above, the increase in methylation of the *Xist* gene upon differentiation may simply reflect the ratio of differentiated and undifferentiated ES cells present in the culture.

Possibility 3 A distinct de novo methyltransferase activity, which specifically methylates *Xist*, may be activated only upon differentiation, and it is this activity that is responsible for random X inactivation in cells of the inner cell mass (ICM)/epiblast lineage as well as differentiating ES cells. Such an activity would result in increased methylation of the *Xist* gene upon differentiation of wild-type and $Dnmt^N$ mutant ES cells. However, there would be no or only a slight increase in methylation detectable in differentiated $Dnmt^S$ mutant ES cells lacking the maintenance methyltransferase, as these cells would be unable to maintain the de novo methylation patterns through subsequent generations.

It is well established that hypomethylation, induced by treatment of tissue culture cells with the cytosine analog 5-aza dC, results in the activation of X-linked genes on the inactive X chromosome (Mohandas et al. 1981; Graves 1982; Paterno et al. 1985). If DNA demethylation also activated the inactive X chromosome in mutant embryos, mutant females with both X chromosomes activated would be genetically imbalanced and might be expected to die earlier than mutant males. This has not been observed, as male and female mutant embryos appear to die at the same time. Therefore, an im-

portant issue raised by our results is whether demethylation-dependent expression of the *Xist* gene initiates the process of X inactivation, which would lead to silencing of the active X chromosome in both males and females. Experiments to distinguish between these two possibilities are technically difficult due to the early embryonic lethality of the Dnmt mutant embryos and the rapid cell death associated with differentiating $Dnmt^S$ mutant ES cells. Preliminary RT–PCR analysis of the X-linked Pgk-1 gene in male $Dnmt^S$ mutant embryos and differentiated ES cells showed that Pgk-1 was being transcribed (data not shown). To test whether expression of this gene was decreased in differentiated $Dnmt^S$ ES cells relative to wild-type ES cells, we measured Pgk-1 RNA levels by RNase protection and found no significant difference in expression. However, because only a fraction of the cells in these cultures are differentiated at any time, we may not expect to see a reduction in Pgk-1 expression in the mutant ES cells if X chromosome inactivation, triggered by activation of *Xist*, contributes to rapid cell death. We are using other experimental approaches such as in situ analysis to clarify on the single cell level whether demethylation-induced *Xist* activation leads to silencing of other genes on the X chromosome.

Materials and methods

Mice

Mice were maintained in the facilities of the Whitehead Institute for Biomedical Research. $Dnmt^N$ and $Dnmt^S$ (formally called $MTase^n$ and $MTase^s$, respectively) have been described (Li et al. 1992, 1993; H. Lei, P.S. Oh, R. Jütterman, R. Jaenisch, K. Goss, and E. Li, in prep.).

Preparation of embryo tissues

Decidua were dissected from the uterus and washed two times in ice-cold HEPES buffer to remove maternal blood. Embryos and yolk sac were removed from individual decidua and washed with three changes of ice-cold HEPES buffer before being quick frozen in liquid nitrogen. During the dissection care was taken to make sure that the yolk sac was free of any contaminating maternal tissue. Either the placenta or the yolk sac was used for genotyping and sexing of the embryos.

Culture and differentiation of ES cells

Male ES cell lines J1 (wild-type for the Dnmt allele), $Dnmt^N$ (homozygous for the $Dnmt^N$ allele), and $Dnmt^S$(homozygous for the $Dnmt^S$ allele) have been described (Li et al. 1992; H. Lei, P.S. Oh, R. Jütterman, R. Jaenisch, K. Goss, and E. Li, in prep.). V18, a male ES cell line derived from 129/SV mice, and V44, a female ES cell line derived from a BALB/c×(129/SV×BALB/c)F_1, were isolated in this laboratory by Martina Klemm (The Whitehead Institute, Cambridge, MA). Cell lines were maintained in the undifferentiated state by culture on FVB-derived γ-irradiated male EF cells in HEPES-buffered Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal calf serum, 0.1 mM nonessential amino acids (GIBCO), 0.1 mM β-mercaptoethanol, antibiotics, and 500 units of leukemia inhibitory factor (LIF)/ml. ES cells were passaged two times without feeders onto gelatinized tissue culture plates to ensure that there was no feeder contamination in the samples. ES cells were induced to differentiate into embryoid bodies by suspension culture in bacteriological petri dishes as described (Robertson 1987). Samples were collected at various times for isolation of RNA and DNA. Cell pellets were washed with HEPES and stored at −80°C.

Isolation of RNA and DNA from embryos and ES cells

RNA was prepared from embryos and ES cells by the guanidinium isothiocyanate/phenol/chloroform method (Chomczynski and Sacchi 1987). For preparation of RNA from individually frozen embryos or yolk sacs, 10 μg of yeast transfer RNA was added as carrier. Purified RNA was treated with 2 units of RNase-free DNase (BRL) for 45 min at 37°C. The RNA was phenol extracted and ethanol precipitated.

DNA was prepared from individual embryos or yolk sacs by a modification of the method of Laird et al. (1991). Samples were incubated at 37°C overnight in 200 μl of buffer (100 mM Tris at pH 8.0, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 200 μg of proteinase K/ml) containing 10 μg of yeast tRNA. DNA from ES cells was processed by the same procedure but without the addition of yeast tRNA as carrier. Samples were extracted with phenol and precipitated with an equal volume of isopropanol.

Southern blot analysis

Genotypes were established by Southern blot analysis of DNA isolated from the yolk sac or placenta of individual embryos as described above. Purified DNA was digested with restriction enzymes, fractionated by electrophoresis through 0.8% agarose gels, blotted with 0.4 M NaOH onto Gene Screen Plus (DuPont–NEN), and hybridized with either the pBB probe for $Dnmt^N$ (Li et al. 1992) or the pHH probe for $Dnmt^S$ (H. Lei, P.S. Oh, R. Jütterman, R. Jaenisch, K. Goss, and E. Li, in prep.). These blots were then stripped by treatment of the filters with 0.4 M NaOH for 30 min at 37°C and then hybridized with a Y-specific probe (Lamar and Palmer 1984) for sexing individual embryos. Probes for Southern hybridization were gel purified and labeled with [^{32}P]dCTP by random hexamer priming (Feinberg and Vogelstein 1984).

To examine DNA methylation at CCGG sites, DNA was digested with *Eco*RV + *Hha*I (*Xist* analysis), *Sac*I + *Hpa*II (*H19* analysis), or *Hpa*II (MoMuLV, pMR150, and pMR134 analyses) according to the manufacturer's recommendations, electrophoresed, and blotted as described above. The following probes were used: 5′*Xist* plasmid (kindly provided by Huntington Willard, Case Western Reserve University, Cleveland, OH) contains a 1.5-kb mouse cDNA fragment that includes the start site of transcription and 1.5 kb of exon 1. The *H19* plasmid (kindly provided by Shirley Tilghman) contains a 4.0-kb *Eco*RI genomic fragment that lies upstream of the 5′ end of the gene (Bartolomei et al. 1993). The MoMuLV probe is a 5.5-kb cDNA fragment (Jahner et al. 1982). pMR150 and pMR134 are plasmids (kindly provided by Verne Chapman, Roswell Park Cancer Institute, Buffalo, NY) that represent the minor satellite and dispersed L1 repeat sequences, respectively (Voliva et al. 1983).

First-strand synthesis

Approximately 1–2 μg of RNA (DNase treated) was annealed to 1 μg of random hexamers at 65°C for 5 min in a volume of 12 μl. Samples were quick cooled on ice and 8 μl of reverse transcriptase mix, which contained 1.25 mM dNTPs, 25 mM DTT, 20 units of RNasin (Promega), 100 units of M-MLV reverse tran-

scriptase (BRL) and 4 μl of 5xM-MLV reaction buffer (BRL), were added. Reactions were incubated at 37°C for 1 hr.

PCR reactions

First strand synthesis (1–10 μl) was amplified by PCR in a total volume of 20–30 μl per reaction. PCR reactions contained 50 mM KCl, 10 mM Tris at pH 9.0, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.6 μM each of forward and reverse primers, and 0.05 units of *Taq* polymerase (Promega)/μl of reaction. The amplifications consisted of denaturation at 95°C for 5 min, followed by 30 cycles of PCR amplification at 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and a 10-min extension at 72°C. Products were analyzed by electrophoresis in a 2% agarose gel and staining with ethidium bromide.

The primers used for *Xist* amplification were MIX 20 and MX 23 (Kay et al. 1993). FGF-5 forward and reverse primers amplify a 310-bp sequence from exon 3. The sequence of FGF-F is 5′-CGGATGACTGTAAGTTCAGG-3′ and the FGF-R is 5′-TCAGTCTGTACTTCACTGGG-3′. Primers used for detecting DNA methyltransferase were Sal5′ (5′-AGTCGCGGGCACCTGTGTCC-3′) and Sal3′ (5′-CCACTGATTGATTGGCCCGAGC-3′). These primers amplify a sequence that includes the *Sal*I site (position 1198) in the cDNA.

RNase protection

Total RNA (~10 μg/lane) was hybridized with 2×10^5 cpm of antisense *Xist* RNA probe in a volume of 20 μl containing 80% deionized formamide, 40 mM HEPES at pH 7.9, and 400 mM NaCl. The RNA probe, which was 254 bases and included polylinker sequence and positions 9604–10181 of the mouse *Xist* gene, was gel purified on a denaturing 7% acrylamide, 7 M urea gel and eluted in 400 μl buffer containing 10 mM Tris at pH 8.0, 0.1% SDS, and 10 μg of yeast tRNA. The samples were denatured at 65°C for 5 min and hybridized overnight at 50°C. Samples were processed using the Guardian RNase Protection Assay Kit (Clontech) according to the manufacturer's instructions. RNase was diluted 1:25, and digestions were carried out at 30°C for 30 min. Products were analyzed on a denaturing 7% acrylamide gel containing 7 M urea.

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Loss of methylation activates Xist in somatic but not in embryonic cells.

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