DNA hypomethylation can activate *Xist* expression and silence X-linked genes

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***Xist* and other X-linked gene expression was examined by fluorescence in situ hybridization in cells of wild type and DNA methyltranferase (Dnmt) mutant embryos and embryonic stem (ES) cells to determine whether demethylation-induced *Xist* expression leads to inappropriate X chromosome inactivation. In undifferentiated ES cells low-level *Xist* expression was detected from the single active X chromosome (Xa) in male cells and on both Xa's in female cells. Upon differentiation *Xist* expression was detected only in female cells, in which *Xist* RNA colocalized with the entire inactive X chromosome (Xi). Differentiated Dnmt mutant ES cells or cells of mutant postgastrulation embryos showed aberrant patterns of *Xist* expression: *Xist* transcripts colocalized with the single X chromosome in male cells and with both X chromosomes in female cells. X-linked gene expression was not detected from chromosomes coated with *Xist* RNA. These results suggest that ectopic *Xist* expression, induced by DNA hypomethylation, may lead to the inactivation of X-linked genes. We conclude that *Xist*-mediated X chromosome inactivation can occur in the absence of DNA methylation, arguing that DNA methylation may be required to repress *Xist* expression for the maintenance of a transcriptionally active Xa. In differentiated Dnmt mutant ES cells the activation of *Xist* expression correlated with a dramatic increase in apoptotic bodies, suggesting that *Xist*-mediated X chromosome inactivation may result in cell death and contribute to the embryonic lethality of the Dnmt mutation.**

[*Key Words*: DNA methylation; *Xist*; X-chromosome inactivation]

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Mammalian dosage compensation is achieved by the transcriptional silencing of one X chromosome in female somatic cells, a process known as X-inactivation (Lyon 1961). X-inactivation is initiated in the mouse during the blastocyst stage at the time of implantation (Gardner and Lyon 1971; Monk and Harper 1979). Random inactivation of either the maternally or paternally derived X chromosome occurs in somatic lineages, and once established, the inactive state is clonally inherited (Gardner and Lyon 1971; Takagi 1994; Rastan 1982). At least three steps are thought to be involved in X-inactivation: initiation, propagation, and maintenance. Inactivation is initiated at the X-inactivation center (Xic), a *cis*-acting sequence from which the process of inactivation spreads into adjacent chromatin (Russel 1963; Cattanach 1975; Mattei et al. 1981). Propagation and maintenance of X-inactivation are thought to involve heterochromatinization, because the inactive X chromosome (Xi) shows many characteristics of constitutive heterochromatin, including delayed replication timing (Takagi 1974), maintenance of the condensed chromatin throughout interphase (Barr and Carr 1962), lack of acetylation of histone H4 (Jeppesen and Turner 1993), and methylation of CpG islands (Wolf et al. 1984a,b; Lock et al. 1986). Demethylation of CpG dinucleotides by treatment with 5-azacytidine results in the decondensation of the Xi, reactivation of silenced genes, and the replication of the Xi early in S phase, suggesting that methylation of CpG islands may contribute to the transcriptional silencing of genes on the Xi (Mohandas et al. 1981; Jones et al. 1982; Wolf and Migeon 1982; Shafer and Priest 1984).

The *Xist* gene maps to the Xic (Borsani et al. 1991; Brockdorff et al. 1991) and is required in *cis* for X-inactivation (Penny et al. 1996). *Xist/XIST* is transcribed solely from the Xi in female somatic cells (Borsani et al. 1991; Brockdorff et al. 1991; Brown et al. 1991). Activation of *Xist* expression precedes X-inactivation suggesting that *Xist* expression may initiate this process (Kay et al. 1993). In human female somatic cells *XIST* RNA associates with the Xi through an interaction with the nonchromatin nuclear matrix, suggesting that it may be a functional RNA that is required to initiate or maintain the silent state, perhaps by modulating the formation of heterochromatin (Brown et al. 1992; Clemson et al. 1996; Lee et al. 1996). In contrast to silent genes on the Xi, *Xist* is unmethylated and is expressed (Norris et al. 1994). In female somatic cells and embryonic stem (ES) cells that have been differentiated to form embryoid bodies (EB) the methylation status of the 5′ end of *Xist* coding sequences correlates with gene expression (Norris et al. 1994; Beard et al. 1995). Thus, in female somatic cells

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the silent *Xist* gene on the active X chromosome (Xa) is hypermethylated whereas the expressed copy on the Xi is hypomethylated. In differentiated male ES cells the *Xist* gene is methylated and silent, whereas in differentiated female ES cells, which show random X-inactivation, one allele is activated and hypomethylated while the other is methylated and silent (Norris et al. 1994; Beard et al. 1995). These data suggest that *Xist* expression in somatic cells is regulated by DNA methylation. Disruption of the DNA methyltransferase (Dnmt) gene results in hypomethylation of the *Xist* locus and an increase in levels of *Xist* RNA in differentiated male Dnmt mutant ES cells and embryos, indicating that the increase in methylation of the *Xist* gene is crucial for the correct regulation of *Xist* expression during differentiation of murine somatic cells (Beard et al. 1995).

One possible consequence of the activation of *Xist* expression from the single X chromosome in male cells is the silencing of that chromosome. In this report we use fluorescence in situ hybridization (FISH) to assay ES cells and the cells of Dnmt mutant embryos for indications of X-inactivation. We demonstrate that *Xist* RNA is distributed in two distinct patterns that depend upon the sex and differentiation status of the cells examined. The low level of *Xist* RNA in undifferentiated ES cells shows the distribution of a nascent transcript and does not result in silencing of X-linked genes. In murine female somatic cells *Xist* RNA is distributed as has been reported previously for human *XIST* RNA, appearing as the accumulation of many distinct, bright clusters localized to one large contiguous domain in the nucleus, a distribution that correlates with inactivation of the *Xist*-expressing chromosome (Brown et al. 1992; Clemson et al. 1996). We extended these observations to demonstrate that Dnmt mutant cells in postgastrulation embryos and differentiated ES cell cultures show signs of X-inactivation using three criteria: the appearance of an *Xist* RNA signal with the same pattern as that seen in female somatic cells, lack of X-linked gene expression from an *Xist*-expressing chromosome, and colocalization of *Xist* RNA with the X chromosome. These results indicate that the activation of *Xist* expression in Dnmt mutant cells can result in the silencing of the X chromosome in *cis* and suggest that *Xist* expression may be sufficient for X-inactivation in the absence of normal levels of genomic methylation.

Results

Xist RNA colocalizes with the Xi in murine female fibroblasts and appears as a nascent transcript from the Xa in ES cells

Xist RNA was shown previously to be expressed in undifferentiated male and female stem cell lines using RT–PCR (Tai et al. 1994; Beard et al. 1995). To determine the distribution of *Xist* RNA in undifferentiated mouse ES cells, we assayed wild-type female and male and Dnmt mutant male ES cells for *Xist* expression using FISH. In addition, we carried out simultaneous analysis for nascent phosphoglycerate kinase 1 (Pgk-1) transcripts to confirm that the X chromosomes in ES cells were active (Rastan and Robertson 1985; Norris et al. 1994). *Xist* RNA was detected using digoxigenin-labeled p*Xist*3K, a probe containing a 3-kb fragment from *Xist* exon 1. Pgk-1 transcripts were detected using a biotin-labeled mouse 17-kb genomic probe encompassing exons 1–8. *Xist* RNA was detected in two small, discrete domains in female ES cells that were positioned closely to the two sites of Pgk-1 transcription (Fig. 1A), whereas in male ES cells one site of *Xist* RNA accumulation was seen located closely to the single site of Pgk-1 transcription (Fig. 1B). The distribution of *Xist* and Pgk-1 transcripts resembled the pattern reported previously for nascent RNAs (Carter and Lawrence 1991; Xing and Lawrence 1991; Carter et al. 1993; Xing et al. 1993). These results confirm that *Xist* RNA is transcribed from the two Xa's in female stem cells and from the single Xa in male cells. The *Xist* gene in male Dnmt mutant ES cells, although almost completely demethylated, was expressed at a level indistinguishable from that seen from the methylated *Xist* gene in wild-type ES cells (Fig. 1C) confirming previous observations that *Xist* gene expression is not regulated by DNA methylation in undifferentiated ES cells.

In female somatic cells *Xist/XIST* is expressed solely from the Xi (Borsani et al. 1991; Brockdorff et al. 1991; Brown et al. 1991) and it was shown that *XIST* RNA is associated with the entire Xi in human cells (Brown et al. 1992; Clemson et al. 1996). We used FISH to determine if *Xist* RNA in murine female somatic cells showed the same distribution as in human cells. When female embryonic fibroblasts cells were analyzed for Pgk-1 and *Xist* RNA simultaneously, a single site of *Xist* RNA accumulation was detected, which did not overlap with the Pgk-1 signal (Fig. 1D). This *Xist* RNA signal was distinctly different from the small discrete spots observed in undifferentiated ES cells. In female fibroblasts the *Xist* RNA was spread over a large area of the nucleus, a distribution similar to that of the *XIST* RNA associated with the Xi in human female somatic cells. These observations indicate that *Xist* RNA is distributed in two distinct patterns: (1) *Xist* RNA appears at the site of transcription as a single pinpoint signal on Xa's in undifferentiated ES cells, and (2) *Xist* RNA comprises a significant portion of the nuclear volume in female somatic cells, suggestive of its association with the Xi. In the following sections we will use an ES cell-like pattern of *Xist* expression to describe low-level, highly localized *Xist* signals like those seen in undifferentiated ES cells and the female somatic cell-like *Xist* expression pattern to describe large *Xist* RNA signals similar to those seen in female fibroblasts.

Activation of Xist expression in differentiated male Dnmt mutant ES cell cultures results in silencing of X-linked genes

Upon differentiation of wild-type male ES cells, *Xist* transcription ceases, whereas the *Xist* gene in male Dnmt mutant stem cells becomes highly expressed

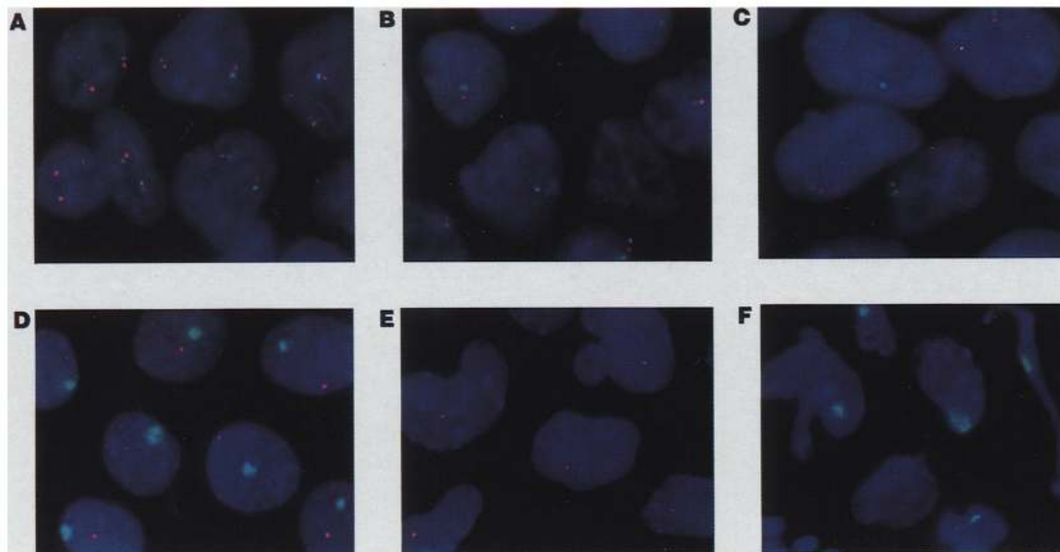

Figure 1. FISH for *Xist* (green) and Pgk-1b (red) RNA in nuclei (blue) of ES cells (*A–C*) or differentiated cells (*D–F*). *Xist* RNA was detected with the digoxigenin-labeled 3-kb exon 1 probe and detected with FITC conjugated anti-digoxigenin antibody. A 17-kb genomic Pgk-1 probe was labeled with biotin and detected with Texas-red conjugated avidin for use in combination with the *Xist* probe. DNA was counterstained with DAPI. ES cells were trypsinized and adhered to glass slides using a cytospin apparatus. Fibroblasts and EB's were grown on glass slides and fixed prior to hybridization. (*A*) Female ES cells; (*B*) male ES cells; (*C*) Dnmt mutant male ES cells; (*D*) female embryonic fibroblasts; (*E*) differentiated male ES cells; (*F*) differentiated male Dnmt mutant ES cells.

(Beard et al. 1995). To determine the distribution of *Xist* RNA in differentiated male Dnmt mutant ES cells and to assess whether *Xist* activation correlated with repression of X-linked genes, we analyzed differentiated wild-type and Dnmt mutant cells for expression of *Xist* and Pgk-1 (Fig. 1E,F), or *Xist* and methyl cytosine binding protein 2 (MeCP2) (not shown). ES cells were aggregated into EB for 4 days in suspension culture and grown in monolayer culture for another 10–12 days prior to analysis. Cells at the periphery of individual EB cultures, which migrated away from the multilayered EB in the center, appeared differentiated as assayed by morphology and by expression of Oct-3/4 and fibronectin (Okamoto et al. 1990; Rosner et al. 1990; Scholer et al. 1990; Shimazaki et al. 1993). These cells acquired a fibroblastic appearance and did not express Oct-3/4, which was highly transcribed in ES cells and silenced upon differentiation, but expressed fibronectin RNA, which was not detected in ES cells (data not shown). The differentiated cells at the periphery of individual EB cultures were scored for *Xist* and Pgk-1 expression (Table 1). No *Xist* RNA was detected in cells from wild-type cultures, 40% of which expressed Pgk-1 RNA , a proportion similar to that seen in male and female embryonic fibroblasts. In contrast, about 90% of the cells in differentiated male Dnmt mutant cultures expressed high levels of *Xist* RNA corresponding to the female somatic cell-like pattern. Coexpression of *Xist* RNA and Pgk-1 RNA was never observed. In the remaining 10% of the cells that did not express *Xist* RNA, only a small proportion expressed Pgk-1 RNA. Similar results were observed with *Xist* and MeCP2: ~90% of the cells showed elevated *Xist* expression and no MeCP2 expression, whereas a small portion of the cells with no detectable *Xist* RNA displayed a MeCP2-specific signal (Table 1). These data indicate that a female somatic cell-like pattern of *Xist* expression in male Dnmt mutant cells is associated with silencing of X-linked genes.

Xist RNA in differentiated Dnmt mutant ES cells colocalizes with the single X chromosome

The spatial distribution of the hybridization signal ob-

Table 1. *Proportions of cells expressing various combinations of* Xist *and Pgk-1 or* Xist *and MeCP2 in male and female embryonic fibroblast cultures or cells at the periphery of undifferentiated male wild-type or Dnmt mutant ES cell cultures*

	Fibroblasts[a]		Differentiated ES cells[b]	
	XY	XX	Dnmt +/+	Dnmt −/−
$Xist^{high}$/Pgk-1^{+}	0	40	0	0
$Xist^{high}$/Pgk-1^{-}	0	60	0	88
$Xist^{-}$/Pgk-1^{+}	41	0	38	2
$Xist^{-}$/Pgk-1^{-}	59	0	62	10
$Xist^{high}$/MeCP2^{+}	0	17	0	0
$Xist^{high}$/MeCP2^{-}	0	83	0	86
$Xist^{-}$/MeCP2^{+}	20	0	15	2
$Xist^{-}$/MeCP2^{-}	79	9	75	12

[a]Average percentages calculated by counting at least 1200 nuclei from fibroblasts lines isolated from four E 14.5 embryos.
[b]Average percentages calculated by counting at least 1000 nuclei from the periphery of five ES cell cultures differentiated for 12 to 14 days.

tained with the *Xist* probe in female fibroblasts was similar to that reported for *XIST* in human female somatic cells, suggesting that *Xist* RNA is associated with the Xi in murine female somatic cells. To determine the localization of mouse *Xist* RNA, cytogenetic preparations of female fibroblasts were assayed for *Xist* RNA and X chromosome sequences (Fig. 2A). Undenatured cytogenetic preparations were first probed for *Xist* RNA using the digoxigenin 3-kb exon 1 probe described previously. Subsequently, the slides were fixed, denatured, and reprobed with a biotin-labeled X chromosome paint. In interphase nuclei the *Xist* RNA signal colocalized with a signal from one of the two X chromosomes. Because Pgk-1 transcripts do not colocalize with the *Xist* signal (Fig. 1D), our data suggest that *Xist* RNA is associated with the Xi in mouse female somatic cells.

To determine the localization of *Xist* RNA in differentiated male wild-type and Dnmt mutant ES cells, we carried out FISH for *Xist* RNA and X chromosome sequences on cytogenetic preparations of cells differentiated for 12 days in culture as described above (Fig. 2B,C). As expected, no *Xist* RNA was detected in cytogenetic preparations of male wild-type differentiated ES cells, whereas the single X chromosome was identified with the X chromosome paint. Approximately 15% of cells in differentiated male Dnmt mutant cultures showed a pattern of *Xist* RNA distribution similar to that seen in female somatic cells: a large signal that colocalized with the single X chromosome in interphase nuclei. These data demonstrate that *Xist* RNA in male Dnmt mutant cells is associated with the X chromosome. In addition, because differentiated male Dnmt mutant ES cells that showed the female somatic cell-like distribution of *Xist* RNA failed to express two X-linked genes (Table 1), these data suggest that the association of *Xist* RNA with the X chromosome correlates with silencing of that chromosome.

The activation of Xist *expression in Dnmt mutant cells correlates with a dramatic increase in cell death*

Dnmt mutant ES cells do not differentiate as extensively as wild-type ES cells and undergo significantly more cell death upon differentiation (Lei et al. 1996). It seemed possible that this increase in cell death was attributable to inactivation of the single X chromosome in differentiated male Dnmt mutant ES cells. To determine whether the onset of cell death correlated with the activation of *Xist* expression and the silencing of Pgk-1 expression, we carried out TUNEL assays in combination with FISH for *Xist*, Pgk-1, or fibronectin RNA over a 12-day time course on differentiating wild-type and Dnmt mutant ES cells. As before, cells with a fibroblastic appearance, which migrated to the periphery of individual EB cultures, were analyzed. Typical fields from day 3 and day 12 cultures are shown in Figure 3, as are fields at lower magnification, which show the extent of cell death in Dnmt mutant cultures more clearly.

The percentage of cells expressing *Xist*, Pgk-1, or fibronectin in combination with the average number of

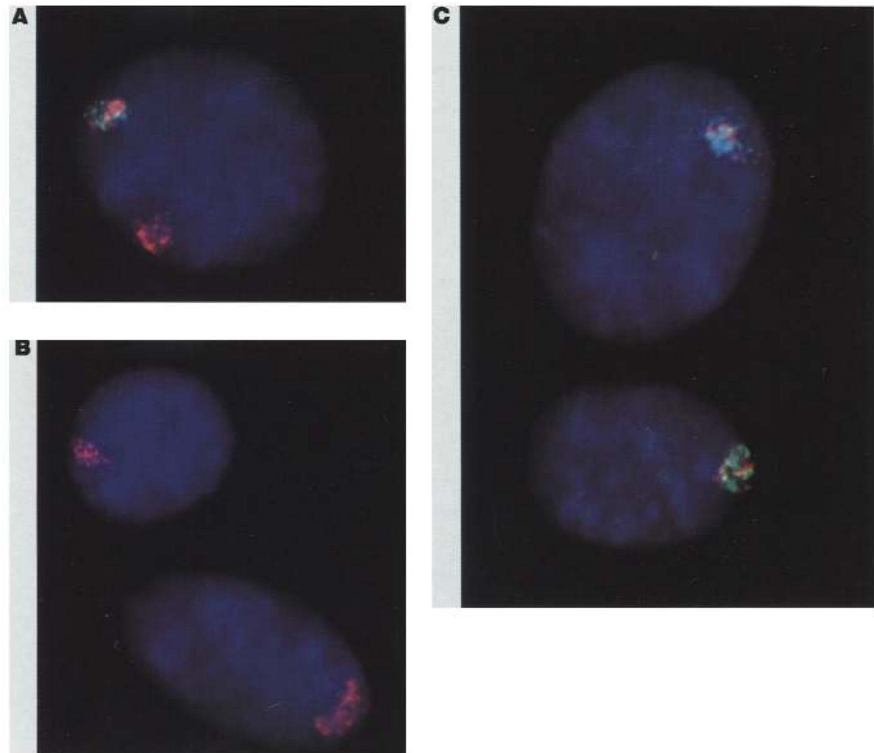

Figure 2. Detection of *Xist* RNA (green) and X chromosome DNA (red). Cytogenetic preparations were produced from (*A*) female embryonic fibroblasts, (*B*) male wild-type, and (*C*) Dnmt mutant differentiated ES cell cultures. *Xist* RNA was detected on undenatured cytogenetic preparations using a digoxigenin-labeled 3-kb *Xist* exon 1 probe. Hybridization of digoxigenin-labeled probe was detected with FITC-coupled anti-digoxigenin antibody. These preparations were then fixed, denatured, and reprobed using a commercially prepared biotin-labeled X chromosome paint. Hybridization of biotin-label was detected using Texas Red coupled avidin. DNA was counterstained with DAPI (blue).

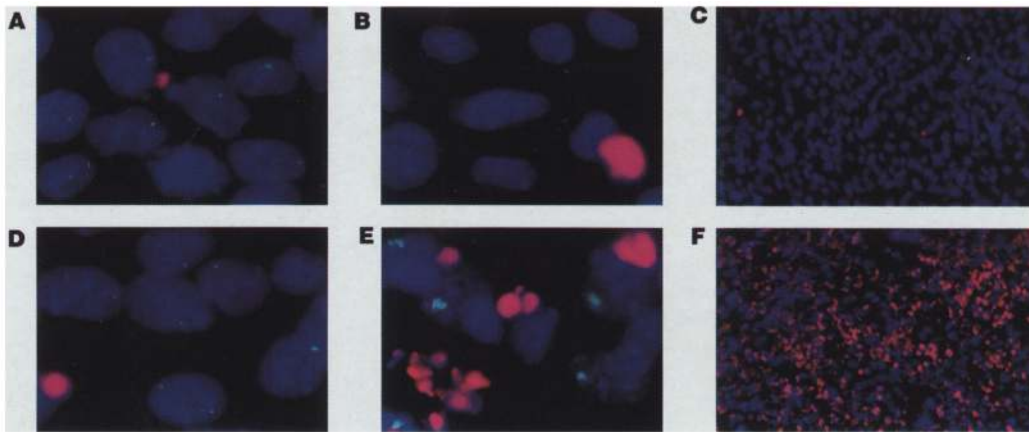

Figure 3. Detection of *Xist* RNA (green) in combination with TUNEL-positive apoptotic bodies (red) in nuclei (blue) of differentiated male wild-type (*A–C*) and Dnmt mutant ES cells (*D–F*). EB's were plated slides and assayed for *Xist* using biotinylated probe 3-kb *Xist* exon 1 probe 3 (*A,D*) and 12 (*B,E*) days after plating. Cultures were subsequently assayed for cell death by incorporation of digoxigenin-labeled dUTP using TdT. Incorporation of labeled TdT was detected with rhodamine conjugated anti-digoxigenin. Nuclei were stained with DAPI. Low power fields of TUNEL and DAPI channels (*C,F*) more clearly demonstrate the extent of cell death in day 12 differentiated ES cell cultures.

TUNEL-positive apoptotic bodies are summarized in Figure 4. The ES cell like low-level expression of *Xist* was observed in ~90% of cells in day 3 wild-type or Dnmt mutant cultures. Although the fraction of low-level *Xist*-expressing cells decreased over time in both wild-type and mutant cultures, the kinetics of this decrease varied. The majority of cells in wild-type cultures did not express *Xist* after 6 days and no *Xist*-expressing cells were detected in day 12 cultures. In contrast, >80% of the cells in day 6 Dnmt mutant cultures still showed low-level expression of *Xist*. Cells showing the female somatic cell pattern of expression first appeared at this time and their numbers increased to almost 90% by day 12 of differentiation. Pgk-1 expression in wild-type cultures showed a decrease over the first 3 days of differentiation and remained constant thereafter. In contrast, Pgk-1-expression in Dnmt mutant cultures continued to decrease, until virtually no Pgk-1-expressing cells could be detected. The decline in Pgk-1-expressing cells coincided with the appearance of cells showing the female somatic cell distribution of *Xist* RNA, providing additional evidence that activation of *Xist* expression in male Dnmt mutant cells results in silencing of the single X chromosome. In contrast, the proportion of fibronectin-

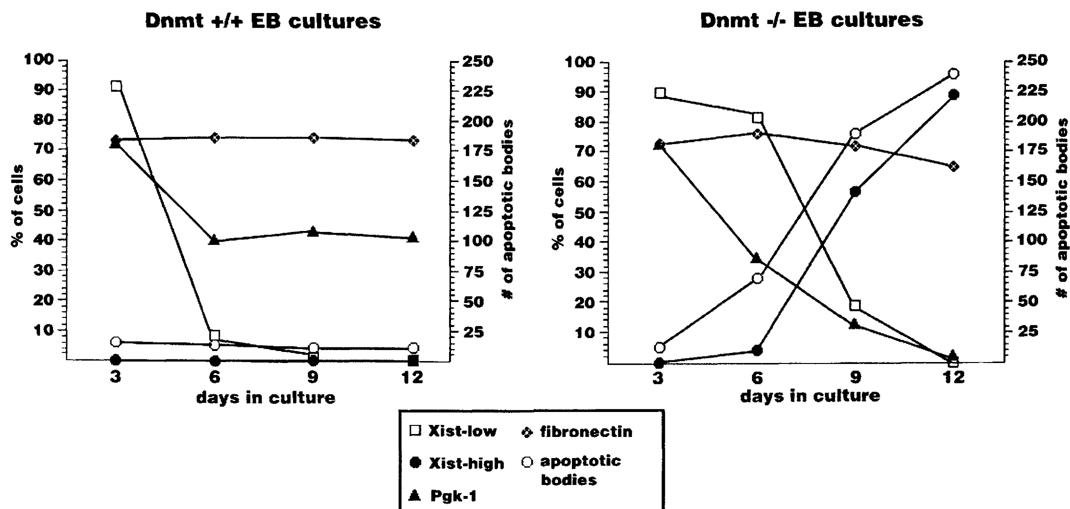

Figure 4. Graphs showing percentage of cells expressing *Xist*, Pgk-1, and fibronectin (*left axis*) and number of apoptotic bodies (*right axis*) detected during a 12-day time course in differentiated wild-type (*left*) and Dnmt mutant (*right*) ES cell cultures. Percentages were calculated on counts of at least 500 cells from the periphery of differentiated ES cell cultures at the days indicated. Numbers of apoptotic bodies were calculated by determining the average number of DAPI intense/TUNEL positive bodies in 10 randomly selected fields, using a 100× lens.

expressing cells remained relatively constant over the 12-day time course in both wild-type and mutant cultures, suggesting that inhibition of X-linked gene expression observed in these cultures is not attributable to nonspecific transcriptional suppression in the Dnmt mutant background.

Quantitation of TUNEL-positive apoptotic bodies over time demonstrated that the onset of the increase in cell death correlated with the appearance of cells with the female somatic cell pattern of *Xist* expression in differentiated Dnmt mutant cultures. The central region of individual wild-type and mutant EB cultures, where the EB originally attached to the plate, consisted mainly of TUNEL-positive cells at all time points, which is consistent with the cell death that occurs during cavitation (Coucouvanis and Martin 1995). The peripheral regions of wild-type and mutant cultures showed similar, low numbers of TUNEL-positive apoptotic bodies at day 3. The number of apoptotic bodies in wild-type cultures did not alter significantly with time, whereas a dramatic increase was seen in Dnmt mutant cultures. By day 12, when nearly 90% of the cells in Dnmt mutant cultures showed the female somatic cell-like pattern of *Xist* expression, the numbers of apoptotic bodies had risen nearly 25-fold. Thus the appearance of cells with female somatic cell-like pattern of *Xist* expression shows a temporal correlation with the silencing of Pgk-1 expression and the increase in cell death, suggesting that *Xist*-mediated inactivation of the single X chromosome may contribute to the cell death in differentiated Dnmt mutant cultures.

Nearly 90% of the cells in the periphery of intact mutant EB cultures showed the female somatic cell-like distribution of *Xist* RNA (Table 1). This population represented only a subset of cells in these cultures. To determine the total proportion of nonapoptotic, *Xist*-expressing cells in differentiated day 12 wild-type and Dnmt mutant cultures, cells were trypsinized and attached to slides using a cytospin centrifuge and assayed for *Xist* expression using FISH and for cell death using TUNEL assays. Dnmt mutant cultures contained greater than 10 times as many TUNEL-positive apoptotic bodies than wild-type cultures (data not shown), confirming that differentiated Dnmt mutant cultures show an increased amount of cell death. The cells which did not show any labeling using the TUNEL assay were scored for *Xist* expression (Table 2). No cells showing the female somatic cell pattern of *Xist* expression were observed in wild-type cultures, and approximately one percent of cells showed the "ES cell-like" pattern of *Xist* expression. In differentiated Dnmt mutant cultures 4% of the cells showed the ES cell-like pattern of *Xist* expression and nearly 20% showed the female somatic cell-like pattern of *Xist* expression. These data indicate that the majority of nonapoptotic cells in differentiated male Dnmt mutant cultures do not show aberrant *Xist* expression after 12 days of differentiation. This may reflect that only a small fraction of cells undergo *Xist* activation at any given time point following induction of differentiation.

Table 2. *Proportion of non-TUNEL-positive cells in male wild-type or Dnmt mutant differentiated ES cell cultures which showed the "ES cell like" pattern of* Xist *expression* (Xist^{low}) *or the "female somatic cell-like pattern of* Xist *expression"* (Xist^{high})

	Percent of cells[a]	
Xist pattern	Dnmt +/+	Dnmt −/−
Xist^{-}	99	78
Xist^{low}	1	4
Xist^{high}	0	18

[a]Average percentages calculated by counting at least 1000 nuclei from two trypsinized and dispersed ES cell cultures differentiated for 12 or 14 days. Only nuclei which were not TUNEL-positive were scored.

Cells from Dnmt mutant embryos show aberrant Xist *expression*

Homozygous Dnmt mutant embryos die at ~embryonic day 9.5 (E9.5) of gestation (Li et al. 1993a) and previous work has demonstrated that *Xist* becomes inappropriately activated in mutant male embryos (Beard et al. 1995). To assess whether *Xist* activation in vivo correlated with the silencing of X-linked gene expression, as was seen in differentiated ES cells, we performed FISH for *Xist* and Pgk-1 on cells isolated from wild-type and Dnmt mutant embryos. Embryos were trypsinized, affixed to a slide by cytospin centrifugation and simultaneously analyzed for *Xist* and Pgk-1 expression. Mutant embryos were isolated at E9.5 and were compared to cells from E9.5 as well as E8.5 wild-type embryos because mutant embryos appear to be developmentally delayed by 1 day. Figure 5A,B illustrates some of the expression patterns seen in cells of mutant embryos, which are summarized in Table 3. In cells from mutant male embryos three different patterns were seen: (1) The majority of the cells expressed no *Xist* (*Xist*$^{-}$), 36% of which were positive for Pgk-1 and 19% were positive for MeCP2; (2) 10% of the cells expressed low levels of *Xist* (*Xist*^{low}) in the ES cell-like pattern, with a similar fraction coexpressing the other two genes as seen above; (3) 5% of the cells expressed a high level of *Xist* (*Xist*^{high}) in the female somatic cell-like manner, none of which coexpressed Pgk-1 or MeCP2. This contrasted with cells from wild-type males, none of which expressed *Xist*, 37% expressing Pgk-1 and 19% expressing MeCP2, fractions that were similar to those seen in mutant cells with no *Xist* expression or in normal fibroblasts (cf. Table 1). X chromosome inactivation in female somatic lineages occurs progressively between E7.5 and E10.5, and at E8.5 the majority of cells contain two Xa's when assayed for inactivation by the silencing of an X-linked transgene (Tan et al. 1993). When cells from wild-type E8.5 or E9.5 female embryos were analyzed, 97% were found to have the female somatic cell-like *Xist* expression pattern (*Xist*^{high}/*Xist*$^{-}$), 40% and 17% of which expressed also Pgk-1 and MeCP2 from the Xa, respectively, whereas a small fraction of 3% was negative for expression of *Xist* and either X-linked gene. These data indicate

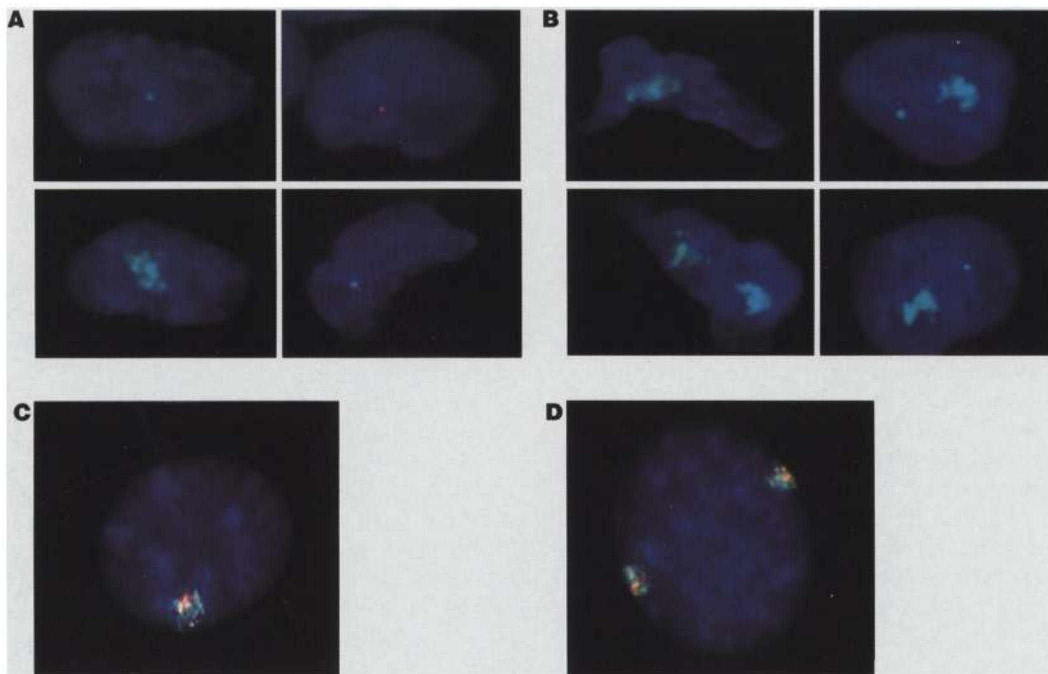

Figure 5. FISH for *Xist* and Pgk-1b RNA in the cells of male (*A*) and female (*B*) Dnmt mutant embryos isolated at E9.5. *Xist* RNA (green) and Pgk-1b RNA (red) were detected, as described in Fig. 1, in the cells of trypsinized, fixed embryos. DNA was stained with DAPI (blue). (*C,D*) Detection of *Xist* RNA (green) and X chromosome DNA (red) in cytogenetic preparations (blue) of male (*C*) and female (*D*) Dnmt mutant embryos. Detection of *Xist* RNA and X chromosome DNA was carried out as described in Fig. 2.

that the activation of *Xist* expression from a single X chromosome has occurred in the majority of female somatic cells by E8.5 and suggest that the inactivation of Pgk-1 and MeCP2 has occurred in these cells. Female mutant embryos showed several expression patterns: (1) 84% of the cells expressed *Xist* in the female somatic cell-like pattern from one locus and did not express *Xist* from the other (*Xist*^{high}/*Xist*$^{-}$), with 38% and 20% of these cells expressing Pgk-1 and MeCP2, from the Xa; (2) 10% of the cells had a *Xist*^{high}/*Xist*^{low} pattern with 35% and 17% of the cells coexpressing Pgk-1 and MeCP2 from the locus showing the *Xist*^{low} expression pattern; (3) 3% of the cells showed a *Xist*^{high}/*Xist*^{high} pattern, none of which expressed the other two genes; (4) 3% of the cells were negative for any signal, a number comparable to wild-type female embryos.

Table 3. *Proportion of cells in male or female wild-type and Dnmt mutant embryos with different patterns of* Xist *expression and percentage of those cells which express Pgk-1 or MeCP2*

Xist pattern	Dnmt +/+[a] Percent cells with each *Xist* pattern	Dnmt +/+[a] Percent expressing Pgk-1	Dnmt +/+[a] Percent expressing MeCP2	Dnmt −/−[b] Percent of cells with each *Xist* pattern	Dnmt −/−[b] Percent expressing Pgk-1	Dnmt −/−[b] Percent expressing MeCP2
			Males			
Xist^{-}	100	37	18	85	36	19
Xist^{low}	0			10	34	19
Xist^{high}	0			5	0	0
			Females			
Xist^{-}	3	0	0	3	0	0
Xist^{high}	97	40	17	84	38	20
Xist^{high}/*Xist*^{low}	0			10	35	17
Xist^{high}/*Xist*^{high}	0			3	0	0

[a]Average percentages calculated by counting at least 1500 nuclei from five E8.5 embryos (in males) or at least 1200 nuclei from four E8.5 embryos (in females); Dnmt +/− embryos behave as wild type embryos (data not shown).
[b]Average percentages calculated by counting at least 1200 nuclei from four E9.5 embryos (in males) or at least 600 nuclei from two E9.5 embryos (in females).

To determine the intranuclear localization of the *Xist* RNA present in Dnmt mutant embryos we carried out FISH for *Xist* RNA and X chromosome DNA sequences on cytogenetic preparations of homozygous Dnmt mutant embryos. As was shown above, these embryos consist of a mixed population of cells, of which only a minority have aberrant *Xist* expression. Nuclei of ~5% of cells of male Dnmt mutant embryos showed a female somatic cell-like pattern of *Xist* RNA distribution, which colocalized with the single X chromosome (Fig. 5C). In female Dnmt mutant embryos, ~5% of cells had two sites at which *Xist* RNA accumulated in the female somatic cell pattern and these sites colocalized with the two X chromosomes (Fig. 5D). These results suggest that the association of *Xist* RNA with a single X chromosome in cells of homozygous male Dnmt mutant embryos and both X chromosomes in cells of female Dnmt homozygotes correlates with silencing of X-linked genes. We conclude that the hypomethylation of *Xist* sequences and activation of *Xist* expression reported in male Dnmt mutant embryos correlates with the accumulation of a small number of cells which show signs of X-inactivation.

Discussion

Methylation of *Xist* sequences has been shown previously to regulate *Xist* expression in somatic cells, but not ES cells (Beard et al. 1995). Male wild-type and Dnmt mutant ES cells showed similar low levels of *Xist* expression, though *Xist* sequences in Dnmt mutant ES cells were significantly less methylated. In differentiated male wild-type ES cells and embryos the *Xist* gene was silenced and this correlated with the increase in methylation of *Xist* sequences while in differentiated male Dnmt mutant ES cells and embryos *Xist* expression was activated and the gene remained hypomethylated. The results presented in this paper extend these observations by using FISH to analyze the distribution of *Xist* RNA and other X-linked transcripts to assay for X-inactivation in differentiated Dnmt mutant cells that ectopically express *Xist*. The distribution of *Xist* RNA in wild-type and Dnmt mutant cells prior and after induction of differentiation, as well as the methylation level of the *Xist* gene and the transcriptional state of the X chromosomes are represented diagramatically in Figure 6. The results can be summarized as follows: Wild-type ES cells express low levels of *Xist,* visible as a pinpoint signal and likely representing nascent RNA, from each X chromosome, i.e., one signal in male and two signals in female cells. Hypomethylation in Dnmt mutant ES cells does not change the level or nuclear distribution of *Xist* transcripts. These results confirm our previous conclusion that DNA methylation has no role in *Xist* expression prior to differentiation. Upon differentiation, at a time when male ES cells inactivate the single *Xist* gene and female cells choose to activate one of the two *Xist* genes leading to inactivation of one X chromosome in *cis,* DNA methylation becomes crucial for the control of *Xist* expression because there is ectopic activation of *Xist* expression from the single X chromosome in differentiated male Dnmt mutant ES cells. These results were corroborated in vivo: In some cells of postgastrulation Dnmt mutant embryos *Xist* becomes ectopically activated from the single X chromosome in male cells and from both X chromosomes in female cells. In the cells in which ectopic *Xist* transcripts associate in *cis* with the X chromosomes, in a manner similar to that seen in wild-type female somatic cells, expression of the two X-linked genes, Pgk-1 and MeCP2, was silenced. The association of the ectopically induced *Xist* RNA in *cis* with the X chromosome suggests that the molecular mechanism underlying normal X-inactivation is recapitulated in the Dnmt mutant cells upon differentiation. We conclude that the Dnmt mutation-induced DNA hypomethylation may initiate the process of X chromosome inactivation through activation of the *Xist* gene. In addition, our data indicate that *Xist* transcription is in itself insufficient for X-inactivation, but that *Xist* RNA must be expressed abundantly and colocalize with the X-chromosome to mediate X-inactivation. A similar conclusion was reached with transgenic cells carrying an autosomal Xic (Lee et al. 1996).

Our results are relevant for the role of DNA methylation in initiation as well as maintenance of X-inactivation. It is thought that methylation of CpG islands associated with genes on the Xi may be a relatively late event in X-inactivation, which functions as part of a secondary, and perhaps tissue specific, mechanism for maintaining the silent state (Lock et al. 1987). Previous work using the drug 5-aza-dC to induce DNA hypomethylation led to the hypothesis that DNA methylation was required to keep the Xi inactive (for review, see Grant and Chapman 1988; Singer-Sam and Riggs 1993). This conclusion was based upon the patchy reactivation of some X-linked genes such as hypoxanthine phosphoribosyl transferase (HPRT) but not of other genes following drug treatment of somatic cell hybrids containing a human Xi (Mohandas et al. 1981). Reactivation of HPRT correlated with demethylation of the CpG island associated with the HPRT gene (Yen et al. 1984; Wolf et al. 1984b). It is important to point out here that treatment of cells with 5-aza-dC achieves a moderate level of genomic DNA hypomethylation (Flatau et al. 1984; Taylor et al. 1984; Michalowsky and Jones 1989) that may be insufficient for the activation of *Xist*. This can be inferred from our previous observation that the *Xist* gene, under conditions of limited Dnmt, is more resistant to demethylation than other genes (Beard et al. 1995). These results demonstrated that *Xist* is activated only in cells or embryos carrying a Dnmt null mutation in contrast to the H19 gene that is activated in cells carrying a partial loss-of-function mutation of the Dnmt gene (Li et al. 1993b; Beard et al. 1995). Our results indicate that X chromosome inactivation can occur in the absence of appreciable genomic DNA methylation arguing that the methylation of the CpG islands on the inactive X chromosome is not important for the inactivation process per se. This is consistent with the observation that X chromosome inactivation can occur in cells with low levels

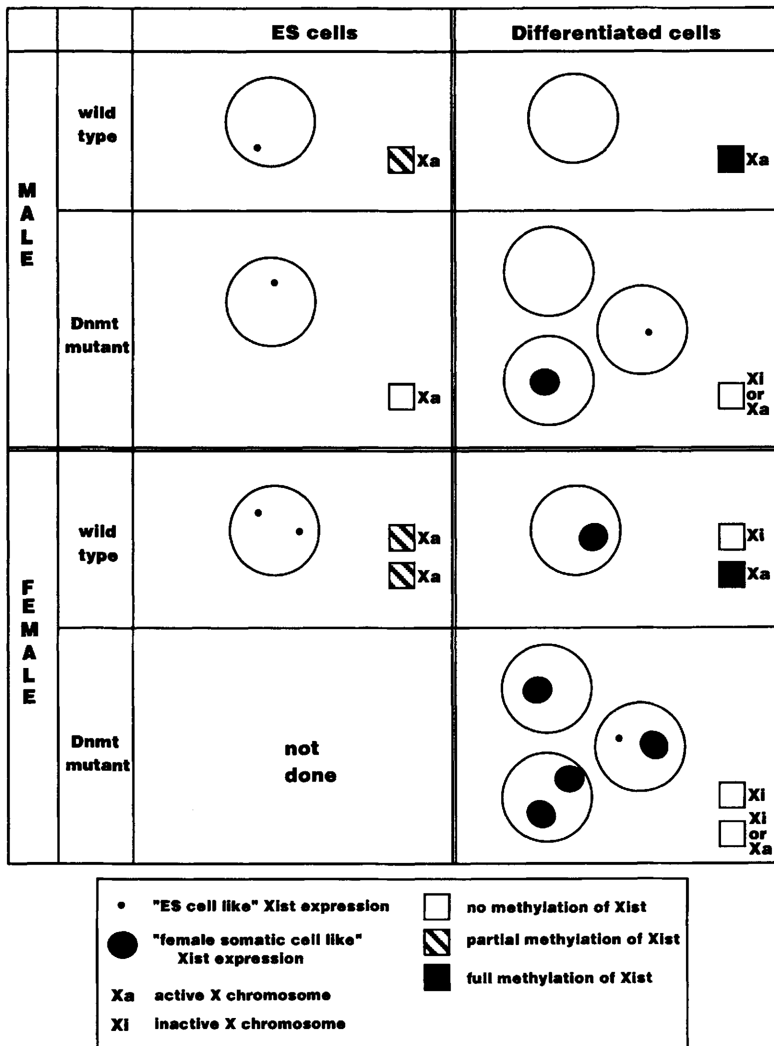

Figure 6. Diagrammatic representation of the correlation between the methylation of *Xist* sequences, the distribution of *Xist* RNA and the state of X chromosomes in wild-type and Dnmt mutant ES cells and differentiated cells (ES cells and embryos). Methylation data are compiled from Norris et al. (1994) and Beard et al. (1995) and refer to the *Eco*RV fragment spanning the 5′ end of the *Xist* gene. Wild-type ES cells showed coexpression of *Xist* and Pgk-1 RNA from the single Xa in male cells and both Xa's in female cells, indicating that the low-level *Xist* expression in ES cells does not result in X-inactivation. Loss of Dnmt activity did not affect *Xist* expression in stem cells when assayed by FISH, confirming that methylation does not regulate *Xist* in ES cells. *Xist* RNA in cells of mouse blastocysts shows the same distribution as in ES cells when assayed by FISH (B. Panning, unpubl.), indicating that early embryonic cells show low-level *Xist* expression in vivo. In differentiated wild-type male and female cells *Xist* sequences were methylated and expression was repressed on the Xa. In female cells the *Xist* gene was unmethylated on the Xi and expression was activated resulting in the colocalization of *Xist* RNA with the entire Xi. In contrast to ES cells, in which loss of Dnmt activity did not affect *Xist* expression, differentiated Dnmt mutant cells showed aberrant patterns of *Xist* expression. Male Dnmt mutant embryos and differentiated ES cell cultures contained two types of cells with unusual patterns of *Xist* RNA distribution: cells that showed activated *Xist* expression, in which *Xist* RNA colocalized with the single X chromosome and expression of Pgk-1 and MeCP2 was not detected, and cells that showed low-level *Xist* expression and normal X-linked gene expression. Similarly, female Dnmt mutant embryos contained two types of cells with aberrant patterns of *Xist* expression: those that showed activated *Xist* expression from both X chromosomes and did not express Pgk-1 and MeCP2 and those which showed activated *Xist* expression and no Pgk-1 or MeCP2 expression from the one X chromosome and low-level *Xist* expression and normal X-linked gene expression from the other X chromosome.

of genomic DNA methylation in some lineages in vivo, such as extraembryonic tissues (Kratzer et al. 1983; Lock et al. 1987). We conclude that in some cells DNA methylation is required to keep the Xa active, presumably by preventing *Xist* transcription. In addition, our results support the hypothesis that X-inactivation in early embryogenesis can occur without substantial genomic methylation.

The majority of cells in Dnmt mutant embryos and differentiated ES cell cultures showed normal levels of *Xist* expression, despite demethylation of the *Xist* gene. The results from differentiated male Dnmt mutant ES cell cultures suggest that cell death may occur as a result of ectopic *Xist* expression in male cells because accumulation of cells with activated *Xist* expression correlated with a dramatic increase in the number of TUNEL-positive apoptotic bodies. If ectopic, demethylation-induced *Xist* expression resulted in embryonic cell death, then cells with aberrant *Xist* expression may be underrepresented in embryos. The following possibilities may account for the normal patterns of *Xist* expression seen in many cells of Dnmt mutant embryos and differentiated ES cell cultures. First, it is possible that maternal stores of Dnmt (Carlson et al. 1992) are sufficient for maintenance of genomic methylation patterns allowing cells of the pregastrulation embryo to methylate and inactivate the sole *Xist* gene in male and one of the two *Xist* genes in female embryos at the time of normal X-inactivation. Once maternal stores of Dnmt are exhausted, correct methylation patterns cannot be maintained, resulting in the demethylation of *Xist* sequences and activation of *Xist* expression from the Xa in male and female mutant embryos. If this process is asynchronous then analysis at any single time point would reveal cells in various stages of *Xist* reactivation. Embryos should therefore consist of cells that have not yet activated *Xist* expression, cells with partially activated, low-level expression and cells with fully reactivated *Xist* expression, as was observed.

It has also been suggested that maintenance and de novo methyltransferase activities are required to regulate X chromosome inactivation (Mise et al. 1996). Therefore a second possibility is that a de novo methyltransferase activity, which is present in Dnmt mutant ES cells (Lei et al. 1996) is sufficient to down-regulate *Xist* expression upon differentiation. Asynchronous reactivation of *Xist* expression would be observed because differentiation in ES cell cultures is highly variable. A third possibility is that the methylation of the *Xist* locus is not required initially to repress *Xist* expression from the Xa, but it may be required to lock in repression. Again, in this scenario the reactivation of *Xist* expression might be asynchronous between different cells in Dnmt mutant embryos and differentiated ES cell cultures. It is also possible that *Xist* expression occurred in all cells of Dnmt mutant embryos and in differentiated ES cell cultures, but was not always detected, perhaps because the RNA diffused throughout the nucleus or cytoplasm and was present at a uniform, low, and undetectable (by FISH) concentration. A final possibility is that, in some differentiated cells, factors other than methylation may regulate *Xist* expression from the Xa, which implies that methylation of *Xist* sequences may be a secondary, or perhaps even nonessential, step in the inactivation of *Xist* expression from the Xa in these cells.

The data reported in this study do not address whether demethylation-induced activation of *Xist* in terminally differentiated cells would result in initiation of X-inactivation. Deletion of *XIST* from the Xi in somatic cell hybrids does not result in reactivation of the Xi, suggesting that the inactive state of the X chromosome is not dependent on continuous expression of *XIST* (Brown and Willard 1994). Conditional mutations of the Dnmt or the *Xist* gene may provide direct information on the role of DNA methylation and *Xist* expression in initiation and maintenance of the inactive state in terminally differentiated cells. Finally, the hypothesis that loss of X-linked gene expression may contribute to the embryonic lethality of Dnmt mutants makes a testable prediction: The disruption of the *Xist* gene should partially rescue the lethal phenotype. Experiments to address these issues are in progress.

Materials and methods

Cell culture

Embryonic fibroblasts were isolated from 14-day embryos using standard procedures and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum. ES cells were maintained in DMEM with 15% fetal bovine serum, essential amino acids, 0.1 mM β-mercaptoethanol, and 500 units/ml leukemia inhibitory factor (ESGRO), plated on gelatinized tissue culture plates without feeders. Two lines of ES cells were used, J1, a wild-type male ES line and B, which carries two copies of the Dnmt^s mutation of DNA methyltransferase (Li et al. 1992, 1993a; Lei et al. 1996).

ES cells were differentiated in hanging drop cultures in DMEM with 15% fetal calf serum and 0.1 mM β-mercaptoethanol. Briefly, cells were diluted to a density of 10^4 cells/ml and suspended from the cover of a bacterial dish in 10 to 20 μl drops for 2 days. The cells were then transferred to suspension culture in bacterial dishes at a density of ~60 hanging drops in 10 ml of media. After 2 days in suspension culture, EBs were plated onto a tissue culture-coated substrate, which varied depending on the eventual use of the cells. For FISH, EBs were plated onto 10 well slides (Roboz), at a density of 1 EB per well. For batch cultures to isolate chromosomes approximately 30 EBs were plated into a 150 mm tissue culture dish. Cells were fed every day with fresh media.

Isolation of embryos

Dnmt^s mice have been described previously (Li et al. 1993a). To obtain embryos, decidua were removed from the uterus and washed extensively in HEPES buffer. Embryos and yolk sacs were dissected from the decidua and washed in HEPES buffer. Yolk sacs were then carefully removed and used for genotyping and embryos were used for FISH. Embryos were genotyped by Southern blot analysis and sexed by RT–PCR for Zfy sequences, as described previously (Beard et al. 1995).

FISH

Xist RNA was detected using p*Xist*3K, a plasmid containing a 3-kb *Bam*HI fragment from exon 1 of the *Xist* gene. Pgk-1 RNA was detected using pCAB17, a genomic clone containing 17 kb of Pgk-1 sequences, spanning exons 1–8. MeCP2 RNA was detected using pBam7.6, a genomic clone containing most of the MeCP2 gene. Fibronectin RNA was detected using pTgB + A + V + (1 − 1), a plasmid containing ~10 kb of rat fibronectin cDNA sequences. Probes for FISH were prepared as has been described previously (Johnson et al. 1991). Briefly, double-stranded DNA was labeled with biotin or digoxigenin nucleotides by nick translation or random priming. Labeled probes were precipitated from 0.3 M NaAcetate with the addition of sheared salmon sperm DNA and yeast tRNA. Mouse COT-1 DNA was also added if genomic probes were used. After precipitation, probe DNA was washed extensively with 100% ethanol and dried. The DNA was then resuspended in recrystallized, deionized formamide and denatured at 70°C for 10 min. An equal volume of 2× hybridization mixture (Johnson et al. 1991) was added to the probe DNA in formamide. If COT DNA was being used to squelch repetitive sequences then the probe mixture was incubated at 37°C for at least 1 hr prior to hybridization. X chromosomes were detected using a biotin-labeled mouse X chromosome paint (Applied Genetics).

Cytogenetic preparations were made using standard techniques, with the alteration that freshly prepared slides that were not used immediately for RNA–FISH were fixed further in 4% paraformaldehyde for 10 min and then stored at 4°C in 70% ethanol. Adherent cells were grown on 10 well slides (Roboz) and nonadherent cells were affixed to slides using a cytospin apparatus (Shandon). Cells were then washed in PBS, permeabilized on ice in cytoskeletal buffer (Fey et al. 1986) with 0.5% triton X-100 for 1 min prior to fixation in 4% paraformaldehyde/1× PBS for 10 min and stored in 70% ethanol at 4°C for up to 2 weeks before use. For RNA–FISH hybridization was carried out at 37°C overnight using a probe concentration of 2.5 μg/ml. Controls to ensure that RNA hybridization was detected included treating cells with RNase A or NaOH prior to hybridization. DNA–FISH was carried out in a similar manner, except that probe concentration was 5- to 10-fold lower and cells were denatured prior to hybridization. After hybridization slides were washed extensively and hybridization detected using an anti-digoxigenin conjugated to FITC or rhodamine (Boehringer

Mannheim) or avidin conjugated to fluoroscein (FITC) or Texas Red dye (Vector) using standard techniques. DNA was counterstained with DAPI at a concentration of 10 ng/ml for 10 min and slides were coverslipped with antifade medium (Vectashield). Fluorescent signals were captured using a Sony Videomax CCD camera mounted on a Nikon Photoskop and color channels merged in Adobe Photoshop. Alternatively, some images were captured using a 35 mm camera mounted on a Zeiss Axioskop and Kodak Ektachrome 1600 slide film. Slides were converted to digital images using the Kodak Sprintscan 35 slide scanner and color channels merged in Adobe Photoshop.

TUNEL assays with FISH

RNA FISH was carried out using biotin labeled probes as described above. Following detection slides were fixed for 10 min in 4% paraformaldehyde in PBS. TUNEL assays were then carried out as described previously (Tornusciolo et al. 1995) using digoxigenin-labeled nucleotides. Incorporation of digoxigenin-labeled nucleotide was detected as described above.

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