

# Imprint switching for non-random X-chromosome inactivation during mouse oocyte growth

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## SUMMARY

In mammals, X-chromosome inactivation occurs in all female cells, leaving only a single active X chromosome. This serves to equalise the dosage of X-linked genes in male and female cells. In the mouse, the paternally derived X chromosome ( $X^P$ ) is imprinted and preferentially inactivated in the extraembryonic tissues whereas in the embryonic tissues inactivation is random. To investigate how  $X^P$  is chosen as an inactivated X chromosome in the extraembryonic cells, we have produced experimental embryos by serial nuclear transplantation from non-growing (ng) oocytes and fully grown (fg) oocytes, in which the X chromosomes are marked with (1) an X-linked *lacZ* reporter gene to assay X-chromosome activity, or (2) the Rb(X.9)6H translocation as a cytogenetic marker for

studying replication timing. In the extraembryonic tissues of these ng/fg embryos, the maternal X chromosome ( $X^M$ ) derived from the ng oocyte was preferentially inactivated whereas that from the fg oocyte remained active. However, in the embryonic tissues, X inactivation was random. This suggests that (1) a maternal imprint is set on the  $X^M$  during oocyte growth, (2) the maternal imprint serves to render the  $X^M$  resistant to inactivation in the extraembryonic tissues and (3) the  $X^M$  derived from an ng oocyte resembles a normal  $X^P$ .

Key words: X-chromosome inactivation, Imprinting, Reprogramming, Germ cell, Oocyte growth, Nuclear transfer, Mouse

## INTRODUCTION

In mouse embryos, preferential X-chromosome inactivation of paternal X chromosome ( $X^P$ ) is first detected in the extraembryonic lineage at the blastocyst stage (imprinted X-chromosome inactivation) (Takagi and Sasaki, 1975). In contrast to imprinted X-chromosome inactivation, an inactive X chromosome is chosen at random and all X chromosomes except for one are inactivated in each diploid cell of the embryo proper (random X-chromosome inactivation) (Lyon, 1961). The two types of X-chromosome inactivation function to equalize the dosage of X-linked genes in male and female cells.

In the imprinted X-chromosome inactivation, the parental-origin-specific choice event occurs but the counting effect on the basis of X chromosome: autosome set=1:2 ratio is not clearly observed. All maternal X chromosomes ( $X^M$ ) become active and  $X^P$  undergoes inactivation in the extraembryonic cells. Invariable developmental failure of embryos having an additional maternal X chromosome, i.e., 41,  $X^M X^M Y$  and 41,  $X^M X^M X^P$  is due to twice the active X-chromosomal dosage in

the trophectoderm and primitive endoderm cell lineages (Tada et al., 1993; Goto and Takagi, 1998). However, extraembryonic tissues develop extensively in E13.5 experimental female embryos retaining two maternally inherited X chromosomes (Kono et al., 1996). These experimental  $X^M X^M$  embryos were produced by serial nuclear transplantation from non-growing (ng) oocytes at the prolonged diplotene stage of the first meiotic prophase and fully grown (fg) oocytes at the germinal vesicle breakdown stage before the resumption of meiosis. These findings led us to an idea that the primary imprint associated with the choice of X-chromosome inactivation is reprogrammed during the oocyte growth stage.

Two lines of experiments were carried out to analyze reprogramming of X-chromosome imprint in germ cells. First, we produced experimental ng/fg embryos heterozygous for the X-linked *lacZ* transgene (Tam et al., 1994) to examine X-chromosome activity. Expression of *lacZ* derived from ng oocytes but not from fg oocytes was strongly repressed in the extraembryonic tissues. Second, this finding was confirmed by the replication timing of X chromosome in experimental ng/fg

embryos heterozygous for the Rb(X.9)6H translocation (Tease and Fisher, 1991). An X chromosome inherited from ng oocytes but not from fg oocytes replicated allocyclically in the majority of extraembryonic cells examined, whereas random X-chromosome inactivation occurred in the embryonic cells. Our findings clearly indicate that the X chromosome derived from ng oocytes, as for the paternal X chromosome, is fated to be inactivated. It is likely that X-chromosome imprint responsible for resistance to be inactivated is imposed during oocyte growth.

## MATERIALS AND METHODS

### Mice

Female BCF1; (C57BL/6J×CBA)F<sub>1</sub> mice were used as donors of ng and fg oocytes, and oocytes at the second meiosis metaphase stage (MII) as recipients of nuclei. Female H253 transgenic mice carrying the X-linked *HMG-lacZ* transgene (Tam et al., 1994) and female mice homozygous for the Rb(X.9)6H translocation (Tease and Fisher, 1991) were also used as donors of ng and fg oocytes. The *HMG-lacZ* transgene is known to be subject to X-chromosome inactivation. The X chromosome and chromosome 9 are fused at the centromere in the Rb(X.9)6H translocation resulting in the formation of a metacentric chromosome. CD-1 female mice were used as foster mothers.

### Production of ng/fg embryos

Reconstituted ng/fg embryos containing genomes from ng and fg oocytes were produced by serial nuclear transfer as described previously (Kono et al., 1996; Obata et al., 1998). Fully grown germinal vesicle stage oocytes were recovered from ovarian follicles 44–48 hours after PMSG injection. Non-growing primary oocytes were recovered from one-day-old newborn mice. Enucleated fg oocytes received nuclei from ng oocytes in a medium containing 200 mM dbcAMP and 5% calf serum. Manipulated oocytes were allowed to progress into MII by incubation in Waymouth MB752/1 medium (GIBCO/BRL) supplemented with 0.23 mM pyruvic acid, 26.7 mM NaHCO<sub>3</sub> and 5% fetal calf serum, for 14 hours. The chromosomes at MII were transferred into fresh ovulated oocytes with the aid of inactivated Sendai virus (HVJ; 2700 haemagglutinating activity U/ml). After artificial activation with 10 mM SrCl<sub>2</sub> in Ca<sup>2+</sup>-free M16 medium for 1 hour, oocytes with two second polar bodies and two female pronuclei were cultured for 3.5 days. Blastocysts developed from these embryos were transferred into the uterus of 2.5-day pseudopregnant CD-1 mice.

### X-gal staining of embryos

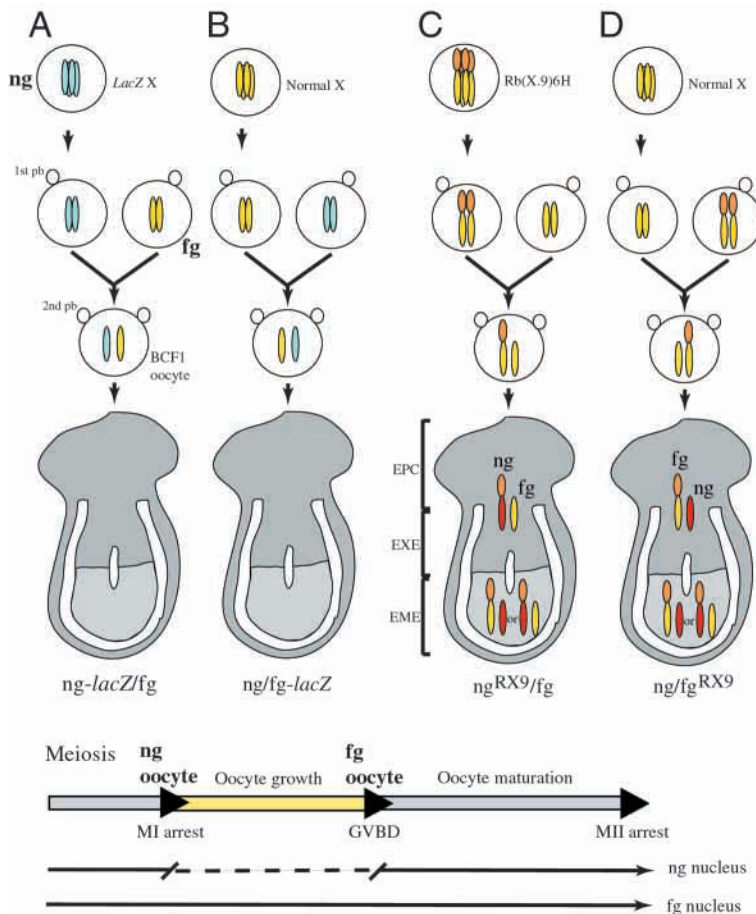
Embryos at E7.5 were fixed in phosphate-buffered saline (PBS) containing 1% formaldehyde, 0.2% glutaraldehyde, 0.02% NP40 and 1 mM MgCl<sub>2</sub> for 1–2 hours at 4°C. They were then washed with PBS and incubated in PBS containing 1 mg/ml 4-Cl-5-Br-indolyl-β-galactosidase (X-gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM MgCl<sub>2</sub> for 24 hours at room temperature. The embryos were then fixed in PBS containing 4% formaldehyde at 4°C overnight. After rinsing with PBS the embryos were stored in 70% ethanol.

### X-chromosome replication timing

Experimental ng/fg embryos at E7.5 were dissected away from the decidual swellings and Reichardt's membrane and incubated in Eagle's minimum essential medium with 10% fetal bovine serum and 150 μg/ml 5-bromo-2-deoxyuridine (BrdU) at 37°C in an atmosphere of 5% CO<sub>2</sub> in air for 7.5 hours, with the final hour in the presence of 0.5 μg/ml Colcemid. After hypotonic treatment with 1% sodium citrate, embryos were fixed with methanol: acetic acid (3:1). Chromosome slides were prepared by an air-dry method as described previously (Takagi et al., 1982). Slides were stained with freshly prepared acridine orange solution and observed under a fluorescence microscope with the standard B filter.

## RESULTS

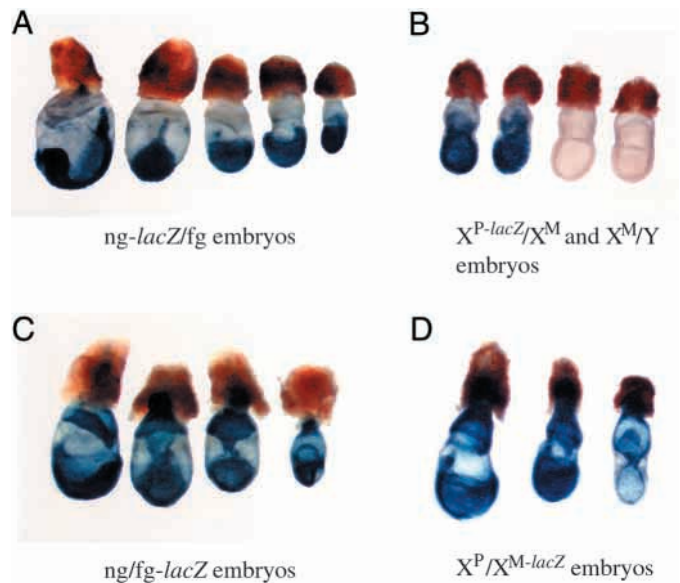
We set out to investigate the imprinting mechanism that is responsible for non-random X-chromosome



**Fig. 1.** Schematic representation of the method used to produce four types of experimental ng/fg embryos by serial nuclear transplantation. (A) ng-*lacZ*/fg embryos carrying a *lacZ*-bearing X chromosome transferred from ng oocytes and a normal X chromosome from fg oocytes are stained with X-gal for detecting X-chromosome activity. (B) ng/fg-*lacZ* embryos carrying a normal X chromosome derived from ng oocytes and a *lacZ*-bearing X chromosome from fg oocytes, stained as above. (C) ng<sup>RX9</sup>/fg embryos carrying an X chromosome with the Rb(X.9)6H translocation derived from ng oocytes and a normal X chromosome from fg oocytes were made for cytogenetic analysis. (D) ng/fg<sup>RX9</sup> embryos carrying a normal X chromosome derived from ng oocytes and an X chromosome with the Rb(X.9)6H translocation from fg oocytes were produced. Embryos in C and D were carefully dissected into three embryonic parts, EME; embryonic ectoderm, EXE; extraembryonic ectoderm, EPC; ectoplacental cone, for analyzing replication timing of the X chromosomes in each region. Blue; *lacZ*-bearing X chromosome. Yellow; normal X chromosome. Orange; chromosome 9. Red; inactivated X chromosome. MI; division I of meiosis. GVBD; germinal vesicle breakdown. MII; division II of meiosis.

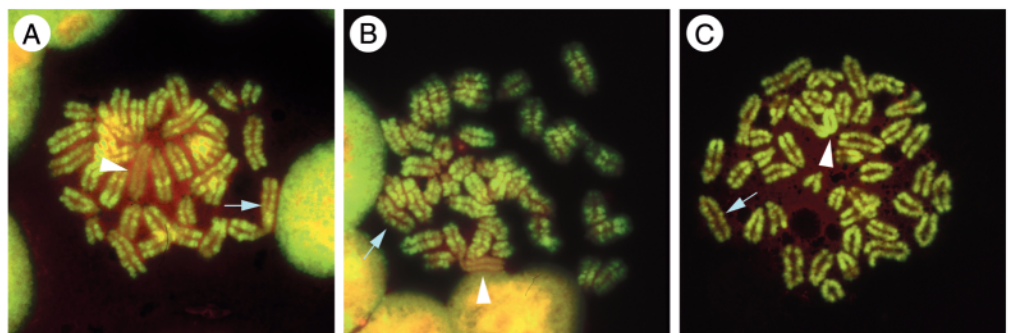
inactivation in the mouse extraembryonic tissues. Experimental ng/fg embryos were produced by serial transplantation of nuclei from ng oocytes (at the prolonged diplotene stage of the first meiotic prophase) and fg oocytes (at the germinal vesicle breakdown stage, prior to resumption of meiosis) (Fig. 1). To visualise the inactivation pattern, we used the H253 transgenic strain which carries an X-linked *HMG-lacZ* reporter gene. Expression of *lacZ* correlates with X-chromosome activity in individual cells in this strain (Tam et al., 1994). It is known that, in normal embryos, X inactivation has been completed in the majority of cells, both embryonic and extraembryonic, by E7.5 (Monk and Harper, 1979). In all 20 ng-*lacZ*/fg embryos at E7.5 examined (Fig. 1A), we found that the chorionic ectoderm and ectoplacental cone of the extraembryonic region had little *lacZ* activity, while *lacZ* activity was high in the embryonic ectoderm, amniotic mesoderm and allantois (Table 1). A similar pattern of *lacZ* activity was seen in E7.5  $X^P\text{-}lacZ/X^M$  control embryos (Fig. 2B; Table 1). Thus, the ng-*lacZ* X chromosome was preferentially inactivated in the extraembryonic tissues of the ng/fg embryos in a similar manner to preferential  $X^P$  inactivation in normal embryos. Analysis of ng/fg-*lacZ* embryos at E7.5 (Fig. 1B) showed strong *lacZ* activity in both the extraembryonic and the embryonic tissues in all five embryos obtained, again consistent with preferential inactivation of the ng X chromosome in the extraembryonic tissues and random inactivation in the embryonic tissues (Fig. 2C; Table 1). This pattern was consistent with that seen in  $X^P/X^M\text{-}lacZ$  control embryos (Fig. 2D; Table 1). No *lacZ* staining was ever observed in the trophoblastic giant cells, although the reason is unclear (Table 1). These results indicate that the maternal imprint is established on the X chromosome late in oogenesis, after the first meiotic prophase, which renders the  $X^M$  resistant to inactivation. Prior to this stage, the maternal X chromosome behaves like a paternally derived X chromosome, being preferentially inactivated in the extraembryonic tissues.

It is not known, however, whether lack of *lacZ* activity from the H253 transgene is an accurate representation of X-chromosome inactivation. To investigate this further, we studied the well-characterised phenomenon of X-chromosome replication asynchrony, using a replication (R) banding technique (Sugawara et al., 1983). After continuous incorporation of BrdU through the second half of S phase and staining with acridine orange, the active X chromosome and the autosomes are seen as banded red and green elements. The inactive X chromosome is uniformly dull red or bright green. The dull red staining is due to delayed replication of the inactive X chromosome, whereas bright green staining



**Fig. 2.** X-linked *lacZ* expression in E7.5 experimental ng/fg embryos. (A) ng-*lacZ*/fg embryos. The embryonic tissue is mostly X-gal positive, whereas the extraembryonic tissue is negative. (B) Control embryos from the cross of male H253 and female BCF1 mice. In two female embryos, the embryonic region stained with X-gal as a consequence of random XCI, whereas the extraembryonic region is totally negative substantiating preferential XCI of  $X^P$ . The two male embryos were totally negative for X-gal staining. (C) ng/fg-*lacZ* embryos. In contrast to the ng-*lacZ*/fg embryos, both the embryonic and extraembryonic tissues are positively stained with X-gal. (D) Control embryos produced by mating female H253 with male BCF1 mice. Both the extraembryonic and embryonic tissues are positively stained by X-gal as seen in ng/fg-*lacZ* embryos.

is due to precocious replication of the inactive X chromosome, seen mainly in extraembryonic cells at early postimplantation stages. In order to distinguish the X chromosomes within a cell, we used the Rb(X.9)6H (RX9) chromosomal translocation,



**Fig. 3.** Replication asynchrony of X chromosomes in extraembryonic cells of E7.5 experimental ng/fg embryos. (A) An ng/fg<sup>RX9</sup> extraembryonic metaphase cell showing a late-replicating normal X chromosome derived from the ng oocyte (arrowhead), seen as a dull red staining chromosome, and an Rb(X.9)6H translocation X chromosome derived from the fg oocyte, banded red and green (arrow), which is replicating in synchrony with the autosomes. (B) In ng<sup>RX9</sup>/fg extraembryonic metaphase cells, it is the translocation X chromosome derived from the ng oocyte (arrowhead) that is late replicating, seen as dull red chromosome arms, whereas the normal X chromosome from the fg oocyte (arrow) replicates in synchrony with the autosomes. (C) In some ng<sup>RX9</sup>/fg extraembryonic cells, the RX9 translocation X chromosome replicates precociously, seen as bright-green-labelled chromosome arm (arrowhead) whereas the normal fg-derived X chromosome (arrow) replicates in synchrony with the autosomes.



**Table 1. *LacZ* expression in E7.5 experimental ng/fg embryos**

Embryo	Embryonic tissue			Extraembryonic tissue		
	Embryonic ectoderm	Amniotic mesoderm	Allantois	Chorionic ectoderm	Ectoplacental cone	Trophoblastic giant cells
ng- <i>LacZ</i> /fg	+	+	+	-	-	-
X <sup>P</sup> - <i>LacZ</i> /X <sup>M</sup>	+	+	+	-	-	-
X <sup>M</sup> /Y	-	-	-	-	-	-
ng/fg- <i>LacZ</i>	+	+	+	+	+	-
X <sup>P</sup> /X <sup>M</sup> - <i>LacZ</i>	+	+	+	+	+	-

which can be identified cytologically (Tease and Fisher, 1991). Experimental ng<sup>RX9</sup>/fg (Fig. 1C) and ng/fg<sup>RX9</sup> (Fig. 1D) embryos were generated by nuclear transplantation and dissected into embryonic ectoderm (EME), extraembryonic ectoderm (EXE) and ectoplacental cone (EPC) at E7.5. The cells were air-dried onto slides and subjected to R-banding analysis. Of 221 EME metaphase cells examined, from a total of seven ng/fg<sup>RX9</sup> embryos, 103 (46.6%) had a late replicating normal X chromosome (ng-derived), and 118 (53.4%) had a late replicating fg-derived RX9 chromosome (Table 2). A similar pattern of random inactivation was observed in the EME of ng<sup>RX9</sup>/fg embryos, where 143 metaphase cells (47.5%) had a late replicating ng-derived RX9 chromosome and the remaining 158 (52.5%) carried a late replicating fg-derived normal X chromosome (Table 2). Therefore, random X inactivation occurred in the epiblast cell lineages in the presence of the RX9 chromosome, indicating that the RX9 translocation exerted no distortion effect on the choice of which X chromosome is to be inactivated. In contrast to the pattern seen in the embryonic tissues, the extraembryonic tissues showed a clear preference for inactivation of the ng-derived X chromosome in cells from both ng/fg<sup>RX9</sup> and ng<sup>RX9</sup>/fg embryos. The normal X chromosome was dull red or bright green in 117 (98.3%) of 119 cells examined from ng/fg<sup>RX9</sup> embryos (Fig. 3A; Table 2), whereas the RX9 chromosome was dull red or bright green in 153 (98.7%) out of 155 cells of ng<sup>RX9</sup>/fg embryos (Fig. 3B; Table 2). It is noteworthy that a considerable proportion of inactive X chromosome was the precociously replicating X chromosome (Fig. 3C; Table 2). The precociously replicating X chromosomes are mainly observed in cells derived from the extraembryonic tissues in normal female embryos. Indeed, the late replicating and the precociously replicating X chromosomes were entirely dull red or bright green, implying that the entire X chromosome was silenced. It is evident, therefore, that the X chromosome derived from an ng oocyte is comparable to a normal X<sup>P</sup>, being preferentially inactivated.

The fg-derived X chromosome preferentially escapes this imprinted inactivation, comparable to a normal X<sup>M</sup>.

## DISCUSSION

The maternal imprint is so robust that a second X<sup>M</sup> is not inactivated in the trophoectoderm and primitive endoderm cell lineages of embryos such as 41, X<sup>M</sup>X<sup>M</sup>X<sup>P</sup> and 41, X<sup>M</sup>X<sup>M</sup>Y (Tada et al., 1993; Goto and Takagi, 1998). Thus, X<sup>M</sup> appears to be marked to remain active, whereas X<sup>P</sup> can either be inactivated or remain active in response to the imprinted inactivation cue, as seen in X<sup>P</sup>X<sup>M</sup> and X<sup>P</sup>0 embryos (Takagi and Sasaki, 1975; Tada et al., 1993). However, the X<sup>M</sup> derived from ng oocytes in ng/fg X<sup>M</sup>X<sup>M</sup> embryos was preferentially inactivated in the extraembryonic cells. This suggests that acquisition of the maternal imprint during oocyte growth is responsible for the imprinted inactivation. It is likely that preferential X<sup>P</sup> inactivation is a direct consequence of robust X<sup>M</sup> resistance to inactivation.

It was recently demonstrated that many imprinted genes, including *Peg1/Mest*, *Peg3*, *Snrpn*, *Igf2r* and *p57Kip2*, also acquire a maternal imprint during the period of oocyte growth between the first meiotic prophase (ng stage) and germinal vesicle breakdown (fg stage) (Obata et al., 1998). Whilst this imprint establishment is consistent with the maternal imprint on X chromosome identified in this study, the X chromosome differs from the autosomal imprinted genes in the way that the imprint is erased. Imprinting of the X chromosome is lost prior to X inactivation in the epiblast, which is random. Autosomal imprinted genes, on the contrary, retain their imprints throughout development, being erased (prior to re-establishment) only in the primordial germ cells of the E11.5-12.5 embryo (Obata et al., 1998; Tada et al., 1998). One possibility is that the X<sup>M</sup> becomes epigenetically equivalent to the imprint-free X<sup>P</sup> in the epiblast, resulting in random X inactivation. This could be due to instability of the maternal

**Table 2. Replication timing of X chromosome in E7.5 experimental ng/fg embryos**

Experimental embryos	No. embryo examined	X chromosome	Embryonic tissue		Extraembryonic tissue			Total	
			EME		EXE	EPC			
			LRX	PRX	LRX	PRX	LRX	PRX	
ng/fg <sup>RX9</sup>	7	ng	103	-	50	32	23	12	117
		fg <sup>RX9</sup>	118	-	1	0	1	0	2
ng <sup>RX9</sup> /fg	6	ng <sup>RX9</sup>	143	-	80	43	16	14	153
		fg	158	-	2	0	0	0	2

ng, X chromosome derived from ng oocytes; fg, X chromosome derived from fg oocytes; RX9, X chromosome carrying the Rb(X.9)6H translocation; EME, embryonic ectoderm; EXE, extraembryonic ectoderm; EPC, ectoplacental cone; LRX, late replicating X chromosome; PRX, precociously replicating X chromosome.

imprint on the X chromosome, which may be lost during the period of global demethylation (Monk et al., 1987; Kafri et al., 1992). Alternatively, it is possible that there is a delay in establishment of secondary epigenetic modification on the X<sup>P</sup> but, in the epiblast, the modification is fully established making X<sup>P</sup> indistinguishable from X<sup>M</sup>.

The *Xist* gene, located within the X-chromosome inactivation centre (Xic), has been shown to be essential for X inactivation by gene targeting in vivo (Marahrens et al., 1998), in vitro (Penny et al., 1996) and by YAC transgenesis (Lee et al., 1996). A hypothesis has been proposed that initially *Xist* is transcribed from an upstream promoter (P<sub>0</sub>) which produces an unstable transcript, but transcription is then switched to a second promoter (P<sub>1</sub>) producing a more stable RNA species (Johnston et al., 1998). However, recent detail analysis using strand-specific RT-PCR techniques indicated that stable and unstable *Xist* RNA expression is initiated at the P<sub>1</sub> but not at P<sub>0</sub> (Warshawsky et al., 1999). Accumulation of this stable transcript in *cis* triggers X inactivation (Clemson et al., 1996). Thus, *Xist* is a part of elements responsible for X-chromosome inactivation. In addition to *Xist*, the X-chromosome controlling element (*Xce*) locus may be involved in determining X-chromosome imprinting. This locus maps immediately 3' to *Xist* and is known to be involved in determining which X chromosome might be inactivated (Simmler et al., 1993). Deletion of a 65 kb region downstream of *Xist* was shown to disrupt the regulation of random X inactivation, since *Xist* was stably transcribed from the mutated X chromosome in both differentiating XX and XO embryonic stem cells (Clerc and Avner, 1998). It was recently suggested that expression of the *Tsix* gene, antisense to *Xist*, may be involved in the mechanism of switching between the *Xist* promoters (Lee et al., 1999). Indeed, deletion of a 3.7 kb fragment of 5' region of *Tsix* containing CpG island, transcriptional starting sites and putative promoter resulted in nonrandom inactivation of the mutant X chromosome in heterozygous female cells but no sign of X-chromosome inactivation in male cells, implying that this region is responsible for choice but not for counting in random X inactivation (Lee and Lu, 1999). X-chromosome counting and choice are genetically separable. Thus, a candidate element responsible for nonrandom inactivation is the CpG island of *Tsix*. It is possible that the CpG island of *Tsix* is hypermethylated in X chromosome of ng and hypomethylated in X chromosome of fg as seen in inverse correlation between methylation status of the CpG island and *Xce* strength (Simmler et al., 1993; Courtier et al., 1995). For further analysis of orchestrated regulation of imprinted X-chromosome inactivation, choice of the mutated X chromosome after passing through spermatogenesis or oogenesis should be tested in vivo.

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