A proximal conserved repeat in the Xist gene is essential as a genomic element for X-inactivation in mouse

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X-inactivation in female mammals is triggered by the association of non-coding Xist RNA in cis with the X chromosome. Although it has been suggested that the A-repeat located in the proximal part of the Xist RNA is required for chromosomal silencing in ES cells, its role in mouse has not yet been addressed. Here, we deleted the A-repeat in mouse and studied its effects on X-inactivation during embryogenesis. The deletion, when paternally transmitted, caused a failure of imprinted X-inactivation in the extraembryonic tissues, demonstrating the essential role of the A-repeat in X-inactivation in the mouse embryo. Unexpectedly, the failure of X-inactivation was caused by a lack of Xist RNA rather than by a defect in the silencing function of the mutated RNA, which we expected to be expressed from the mutated X. Interestingly, the normally silent paternal copy of Tsix, which is an antisense negative regulator of Xist, was ectopically activated in the preimplantation embryo. Furthermore, CpG sites in the promoter region of paternal Xist, which are essentially unmethylated in the extraembryonic tissues of the wild-type female embryo, acquire a significant level of methylation on the mutated paternal X. These findings demonstrate that the DNA sequence deleted on the mutated X, most probably the A-repeat, is essential as a genomic element for the appropriate transcriptional regulation of the Xist/Tsix loci and subsequent X-inactivation in the mouse embryo.

KEY WORDS: X-inactivation, Xist, Tsix, Gene targeting, Mouse embryo

INTRODUCTION

During the early development of female mammals, one of the two X chromosomes undergoes transcriptional silencing along almost the entire region of the chromosome to achieve dosage equivalence with males of the X-linked genes, which females have twice as many of as males do (X-inactivation) (Lyon, 1961). Several lines of evidence indicate that the paternal X chromosome is preferentially inactivated in the early preimplantation stage embryo (Huynh and Lee, 2003; Mak et al., 2004; Okamoto et al., 2004). This imprinted X-inactivation is maintained throughout development of the extraembryonic tissues, such as the placenta and part of the extraembryonic membranes (Takagi and Sasaki, 1975), which originate from the trophectoderm and primitive endoderm of the blastocyst. In the epiblast lineage, a derivative of the inner cell mass of the blastocyst, the previously inactivated paternal X transiently restores transcriptional activity, and subsequently either the maternal or paternal X undergoes inactivation in a basically random fashion as cells differentiate (random X-inactivation).

It is known that the Xist (X-inactive specific transcript) gene located in the X inactivation center (Xic), a cytogenetically identified chromosomal region essential for X-inactivation to occur in cis, plays a crucial role in both imprinted and random X-inactivation. Targeted disruption of Xist renders the mutated X incompetent to undergo inactivation (Marahrens et al., 1997; Penny et al., 1996), and therefore paternal transmission of Xist deficiency results in the failure of imprinted paternal X-inactivation in the extraembryonic tissues and, consequently, a selective loss of female embryos soon after implantation owing to the extremely poor development of the extraembryonic tissues.

Xist encodes long non-coding transcripts as long as 17 kb in length, which are subject to splicing and polyadenylation like common protein-coding RNAs (Brockdorff et al., 1992; Brown et al., 1992). The Xist RNA is peculiar in that it stays in the nucleus and associates with the X chromosome, from which it is transcribed (Brown et al., 1992; Clemson et al., 1996), where it eventually induces chromosomal silencing by unknown mechanisms. Although the overall structure of the Xist gene is relatively conserved among eutherian mammals, its nucleotide sequence diverges greatly (Chureau et al., 2002; Nesterova et al., 2001), which is consistent with the presumed role of the Xist gene product as a functional RNA. It is known, however, that several regions consisting of a series of repeats are conserved between mouse and human (Brockdorff et al., 1992; Brown et al., 1992). Wutz et al. previously showed that one of these repeats, known as the A-repeat, which is located in the proximal part of the Xist RNA, is crucial for the silencing function of the RNA (Wutz et al., 2002). They demonstrated that the RNA transcribed from a single copy of Xist cDNA lacking the A-repeat driven by an inducible promoter can accumulate on the X chromosome in male ES cells upon induction but fails to initiate chromosomal silencing. The A-repeat contains 7.5 copies of a conserved direct repeat unit, which harbors two short inverted repeats that might fold into a secondary structure comprising two stem loops. These findings imply that the stem loop structures might be the modules that interact with the putative protein factors that are involved in chromosome silencing.

Although the above inducible expression assay in ES cells identified for the first time a likely functional domain in Xist RNA responsible for chromosomal silencing, its significance for X-inactivation taking place in the developing embryo has not yet been addressed. In this study, we introduced a mutant Xist allele lacking the A-repeat into the mouse and examined its effects on X-
inactivation in the embryo. Our results clearly demonstrate that deletion of the A-repeat rendered the mutated X incompetent to undergo inactivation in embryos, which was consistent with the previous ES cell assay. However, the incompetence of the mutated X to undergo inactivation was apparently due to the lack of Xist expression on the mutated X chromosome. This finding suggests an unexpected essential role of the A-repeat as a genomic element for the appropriate regulation of Xist and subsequent X-inactivation in the mouse embryo.

MATERIALS AND METHODS

Targeted deletion of the A-repeat

Targeting vector pXBΔA was constructed so that a floxed HSV-tk and PGK-neo fragment from pGKNeo (a gift from En Li, location?) was flanked by genomic fragments derived from Bac clone 333J22 containing the Xist gene as shown in Fig. 1A (the 5’ arm, nucleotides 97, 704-106, 412 in AJ421479; the 3’ arm, nucleotides 107, 228-113, 284 in AJ421479). J1 ES cells (Li et al., 1992) were electroporated with pXBΔA as previously described (Sado et al., 2005), and selection was started 24 hours later in the presence of 250 μg/ml G418. Of 254 selected colonies, four harbored the expected homologous recombination (XistΔ2281F). Chimeric males were generated and crossed with females heterozygous for a Tsix deficiency (Sado et al., 2001) to facilitate germ-line transmission in the same manner as previously described (Sado et al., 2005). Females heterozygous for XistΔ2281F were crossed with CAG-cre transgenic males to derive pups carrying XistΔA. Excision of the selection marker in ES cells was performed by transient expression of Cre recombinase using pBS185 (Life Technology).

Histology

Decidua dissected out from the uterus were fixed in Bouin’s fixative. Following dehydration, deciduals were embedded in Technovit 7100 (Kulzer), sectioned at 2 μm, and stained with Hematoxylin and Eosin.

Genotyping blastocysts

Blastocysts were flushed from the uterus at E3.5 and the zona pellucida was removed by acid tyrode treatment. Each blastocyst was transferred to 10 μl of water and heated for 3 minutes at 95°C. Five microliters of this solution was used for two-round PCR with semi-nested primer sets for genotyping and sexing. The primers used for the first round amplification were R700P2 (wild-type-specific), dA1F (XistΔA-specific), XistΔ1395R, Zfy1 and Zfy2. Subsequently, the wild-type Xist, XistΔA and Zfy sequences were individually amplified in a second round PCR using R700P2/F1063AS for wild-type Xist, dA1F/F1063AS for XistΔA and Zfy1/Zfy4 for Zfy. Primer sequences used in this study are shown in Table 1. Total RNA was extracted from the remaining 5 μl using Trizol (Invitrogen) in the presence of 10 μg of E. coli tRNA.

RT-PCR

For quantitative RT-PCR analysis of undifferentiated ES cells, cDNA was prepared at 60°C using ThermoScript (Invitrogen) with XistΔ2688R, Tsix2R and GapDR as primers. Real-time PCR was carried out as previously described (Sado et al., 2006), using Xist2281F and Xist2424R as primers for Xist, Tsix2F and P422R for Tsix, and GapF and GapDR2 for Gapd. The expression levels of Xist and Tsix were normalized to Gapd levels as previously described (Sado et al., 2006).

For allelic expression analysis of X-linked genes in the trophectoderm and in E7.5 embryos, cDNA was synthesized from 1 μg of total RNA using an oligo-dT primer, and PCR was carried out using G6pdF4 and G6pdR4 as primers for G6pd, and HprtF4 and HprtR3 for Hprt. The amplified products of G6pd and Hprt were subsequently digested with Drai and Hinfl, respectively (Sugimoto and Abe, 2007).

RESULTS

The mutated Xist allele lacking the A-repeat was introduced into the mouse

To study the importance of the A-repeat in X-inactivation taking place in the mouse embryo, we created a new mutant allele of Xist lacking the A-repeat by a gene targeting strategy (Fig. 1). Following verification by Southern blot analysis, ES cells harboring the expected homologous recombination were serially injected into blastocysts to generate chimeras. We postulated that the presence of a floxed selection marker in the targeted allele (XistΔ2281F) would functionally disrupt the Xist gene, and that the mutated allele, when paternally inherited, would result in a selective loss of female embryos soon after implantation because of the failure of imprinted X-inactivation in the extraembryonic lineages. We previously demonstrated, however, that this female-specific lethality is sometimes rescued by the simultaneous presence of a Tsix deficiency on the maternal X (Ohata et al., 2008; Sado et al., 2005; Sado et al., 2006). Accordingly, the male chimeras were crossed with females heterozygous for the Tsix deficient allele (ΔTsix) (Sado et al., 2001) to facilitate transmission of
the XistΔ A2lox allele from fathers to live female pups, as previously described. Consequently, females carrying the ΔTsix allele and XistΔ A2lox allele on the maternal and paternal X, respectively, were successfully recovered. They were subsequently crossed with males expressing Cre recombinase ubiquitously to derive XistΔ/+ females and XistΔ/Y males. These animals were apparently normal and fertile.

**Basal transcription of Xist was not affected by deletion of the A-repeat in undifferentiated male ES cells**

The targeting strategy was designed to delete the A-repeat without disrupting the endogenous Xist promoter. To confirm that the promoter remained functional in the XistΔ allele, we took advantage of XistΔ/Y ES cells established by transiently expressing Cre recombinase in XistΔ/Y ES cells (data not shown). It is known that the Xist locus is transcribed at a very low level in undifferentiated male ES cells, although this basal transcription is eventually downregulated after differentiation. We examined the basal transcription of Xist to determine whether it was affected by the deletion of the A-repeat in undifferentiated XistΔ/Y ES cells. Quantitative RT-PCR with strand-specifically prepared cDNA revealed that, in XistΔ/Y ES cells, the mutated XistΔ RNA was expressed at a level comparable to wild-type Xist RNA in the parental male ES cells (Fig. 2A). In addition, the XistΔ allele was downregulated in the same manner as the wild-type allele after the induction of differentiation (Fig. 2A). These results demonstrated that neither the function of the Xist promoter per se nor the mechanism for downregulating Xist on the future active X was affected by the deletion.

We further analyzed the stability of XistΔ RNA by treating cells with DRB, an inhibitor of RNA polymerase II. Real-time PCR on cDNA prepared from a series of DRB-treated cells demonstrated that there was no significant difference in stability between wild-type and mutant Xist RNA (Fig. 2B), indicating that the deletion of the A-repeat did not impair the stability of the RNA.

Intriguingly, the expression level of Tsix was increased in the mutant male ES cells. The stability of Tsix RNA in the mutant ES cells was, however, comparable to that in wild-type ES cells (Fig. 2B). It seemed likely therefore that the higher expression of Tsix in the mutant was mediated not by an increased stability of the RNA (Fig. 2A), but by a higher level of transcription. The expression level of Tsix, however, declined once ES cells were induced to differentiate. This suggests that although the genetic alterations we introduced at the Xist locus somehow facilitated the transcription of Tsix on the mutated X, they did not affect the mechanism for downregulating Tsix upon differentiation.

**Paternal transmission of XistΔ results in a selective loss of female embryos**

The functional significance of the A-repeat in embryonic development was first addressed by examining whether or not the mutated XistΔ allele could be transmitted to female pups from the father. Of 218 pups born to wild-type females crossed with XistΔ/Y males, 216 were male and 2 were female. One of the two females turned out to be XO, where the X chromosome was maternal in origin, and the other female inherited the XistΔ allele (Fig. 3A), suggesting that most female embryos had been lost in utero. In reciprocal crosses, the XistΔ allele was transmitted to apparently healthy female pups from the mothers at the expected ratio (Fig. 3A). Thus, the selective loss of females upon paternal transmission of the mutation was probably due to defects in the imprinted X-inactivation in the extraembryonic lineages. When embryos were dissected out at embryonic day (E) 6.5, +/XistΔ (the maternal allele precedes the paternal one by convention) female...
embryos, although found in a reasonable number, were all stunted with an abnormal morphology that was indistinguishable from that of female embryos carrying a dysfunctional Xist allele derived from the father (Fig. 3B). Histological analysis revealed that the extraembryonic ectoderm was severely affected in the morphologically abnormal embryos, which were most probably females carrying the paternal XistΔA allele (Fig. 3C). These embryos were reminiscent of those carrying an extra-copy of the maternal X (Goto and Takagi, 1998; Tada et al., 1993) or the paternally derived Xist-deficient X (Marahrens et al., 1997). These results strongly suggest that the deletion of the A-repeat severely compromised the function of the Xist gene to initiate X-inactivation.

Fig. 3. XistΔA fails to inactivate the mutated X chromosome. (A) The paternal transmission of XistΔA results in an extreme bias in the sex ratio of live pups born to wild-type females crossed with XΔAY males in favor of males (upper panel). One of the two females turned out to be XO; therefore, the mutated allele was transmitted to only one female (0.46%). By contrast, the mutated allele was transmitted to both male and female pups at the expected ratio from the mothers (lower panel). (B) Gross morphology of an embryo typical of those that inherited the paternal XΔA (XXΔA). Scale bar: 200 μm. (C) Histological sections of the presumptive XXΔA embryos are shown in comparison with a presumptive male litter mate at E6.5. epc, ectoplacental cone; exe, extraembryonic ectoderm; ee, embryonic ectoderm (D) RT-PCR analysis of allelic expression of X-linked G6pd and Hprt in the trophoblast recovered from E6.5 embryos. Xf1 and XΔAYΔAb are maternal and paternal in origin, respectively. Expression of the paternal copy was evident in Xf1XXΔA in both cases. (E) Expression of the GFP transgene on the paternal X in female embryos at E7.5. In contrast to wild-type female embryos (XXGFP), GFP fluorescence was uniformly negative in the embryonic tissues in XΔAXΔA embryos, suggesting that XΔA failed to undergo inactivation even in the embryonic tissues. (F) RT-PCR analysis of the expression of X-linked G6pd and Hprt in the embryonic tissues at E7.5. The maternal copies of both genes on Xf1 were not expressed in XΔAXΔF1 embryos, suggesting that XΔA failed to undergo inactivation.
Genes on the mutated X are not repressed in either the embryonic or extraembryonic tissues

We went on to study the transcriptional status of the mutated paternal X (X\textsuperscript{ΔA}) in the extraembryonic tissues. X-inactivation is imprinted in the trophectoderm, a derivative of the trophectoderm in the blastocyst, in favor of the paternal X. The expression of X-linked genes was analyzed using trophoblasts isolated from E6.5 embryos. Embryos were prepared by crossing females carrying an X chromosome derived from JF1 (Mus m. molossinus) with X\textsuperscript{ΔA} males, so that the parental origin of the X-linked gene transcripts could be addressed by the presence or absence of restriction site polymorphisms between JF1 and the laboratory strains used in this study (C57BL/6 and 129). The regions harboring a polymorphism in the X-linked G6pd and Hprt genes (Sugimoto and Abe, 2007) were amplified by RT-PCR and the products were subsequently digested with DraI and HinfI, respectively. In the trophectoderm of wild-type female embryos (X\textsuperscript{B/F}X\textsuperscript{ΔA}), the expression of G6pd and Hprt was confined to the maternal alleles, consistent with the imprinted paternal X-inactivation in this tissue (Fig. 3D). By contrast, expression of the paternal copy of these genes was evident in X\textsuperscript{B/F}X\textsuperscript{ΔA} females (Fig. 3D). This result demonstrated that genes on the paternally derived X\textsuperscript{ΔA} were, at least in part, misexpressed in the trophectoderm. It seemed likely therefore that the paternal X\textsuperscript{ΔA} failed to undergo inactivation in the extraembryonic lineages, where the paternal X is programmed to be inactivated.

Subsequently, we examined whether the maternal X\textsuperscript{B} could undergo inactivation in the embryonic lineage, where the X chromosome imprint is no longer effective, by using wild-type males carrying EGFP transgenes on the single X (X\textsuperscript{GFP}) (Nakamichi et al., 2002) as the father. It has been shown that the expression of the transgene reflects the activity of X\textsuperscript{GFP} (Ohihata et al., 2004). In wild-type female embryos recovered at E7.5, GFP fluorescence was observed in the embryonic tissue but not in the extraembryonic tissue, as expected (Fig. 3E). This substantiates that the transgene used here serves as a good reporter for addressing the activity of X\textsuperscript{GFP} at this stage of embryo development. Female embryos heterozygous for X\textsuperscript{ΔA}, which were morphologically indistinguishable from their wild-type littermates, were essentially negative for GFP throughout the embryo, suggesting that X\textsuperscript{GFP} was uniformly inactivated in the embryonic lineage, which is normally subject to random X-inactivation (Fig. 3E). Furthermore, allelic expression analysis of G6pd and Hprt revealed that both genes were expressed exclusively from X\textsuperscript{ΔA}, and that the transcripts from X\textsuperscript{B} were barely detectable in X\textsuperscript{ΔA}X\textsuperscript{B} heterozygotes (Fig. 3F). These results indicate that, as is the case with the X carrying the dysfunctional Xist allele, X\textsuperscript{ΔA} is incompetent to undergo inactivation in the embryonic lineage, as well as in the extraembryonic lineages.

Expression of Xist is diminished on the mutated X in the preimplantation embryo

The above finding demonstrates that the A-repeat plays an essential role in X-inactivation during mouse development. This is consistent with the previous report by Wutz et al. that Xist RNAs lacking the A-repeat fail to initiate X-inactivation in transgenic ES cells (Wutz et al., 2002). In particular, one of the mutated Xist RNAs tested by Wutz et al. (ASX), which lacks almost the same region as the X\textsuperscript{ΔA} RNA expressed from X\textsuperscript{ΔA}, is defective in silencing despite its accumulation on the X chromosome. This observation predicts that X\textsuperscript{ΔA} RNA coats X\textsuperscript{ΔA} but fails to induce chromosomal silencing at the onset of X-inactivation in the embryo. To examine whether this was the case, RNA-FISH was carried out using an Xist-specific RNA probe in the preimplantation embryo, in which only the paternal copy of Xist is expressed and accumulated in cis (Kay et al., 1993; Sheardown et al., 1997). Embryos were recovered from wild-type females crossed with Xist\textsuperscript{ΔA}/Y males at the eight-cell and blastocyst stages. All the female embryos should inherit the X\textsuperscript{ΔA} allele in this cross. The sex of each embryo was identified by painting with X- and Y-specific probes afterwards (data not shown). RNA-FISH demonstrated that, although the expression of Xist\textsuperscript{ΔA} was detected in XX\textsuperscript{ΔA} eight-cell embryos, the hybridization signal was very faint, essentially like a pinpoint (Fig. 4), and eventually disappeared at the blastocyst stage (Fig. 4). In control female embryos, accumulation of Xist RNA was detected in XX\textsuperscript{ΔA} blastocysts (Fig. 4). In agreement with these observations, real-time RT-PCR on total RNA of individual blastocysts demonstrated that the level of Xist RNA in XX\textsuperscript{ΔA} was much lower than that in XX. The level in XX\textsuperscript{ΔA} was, in fact, almost the same as that in XY, which was nearly below the detection limit (Fig. 5A). This excluded the possibility that Xist\textsuperscript{ΔA} RNA, although expressed in the blastocyst, failed to coat the mutated paternal X chromosome, and is in contrast to human XIST RNA lacking the A-repeat expressed in tumor cells by an inducible promoter, which does not coat the X chromosome (Chow et al., 2007). These results raised the unexpected possibility that the failure of X\textsuperscript{ΔA} to undergo inactivation was primarily due to the lack of Xist\textsuperscript{ΔA} RNA coating the mutated X.


**Tsix is ectopically activated on the paternal X<sup>AA</sup> in the blastocyst**

Available evidence suggests that **Tsix**, the expression of which becomes detectable as early as the eight-cell to morula stage (Y.H. and T.S., unpublished) and is confined to the maternal allele in the preimplantation embryo, prevents the upregulation of **Xist** on the maternal X during the process of imprinted X-inactivation (Lee, 2000; Sado et al., 2001). Given this negative effect of **Tsix** on **Xist** expression, it was of interest to explore whether the expression of **Tsix** was affected on the paternal X<sup>AA</sup> at the blastocyst stage. Accordingly, RNA fractions of single genotyped blastocysts recovered from X<sup>F1</sup>X<sup>F1</sup> females crossed with either XY or X<sup>AA</sup>Y males were individually converted into cDNA using gene-specific primers and subjected to two-round PCR. The parental origin of the cDNA using gene-specific primers and subjected to two-round PCR. (Sugimoto and Abe, 2007). Intriguingly, the paternal copy of **Tsix** that is normally silent on the laboratory strain-derived X chromosome (Sugimoto and Abe, 2007). Intriguingly, the paternal copy of **Tsix** is ectopically activated on the paternal X<sup>AA</sup> in XX blastocysts. (Fig. 5C). It seems possible therefore that the ectopic expression of **Tsix** facilitates CpG methylation at the **Xist** promoter on the paternal X<sup>AA</sup> in the trophoblast.

**CpG sites in the **Xist** promoter on X<sup>AA</sup> are not methylated in sperm**

The methylation profile of the **Xist** promoter was further analyzed in sperm to see whether the aberrant methylation of the paternal allele in the trophoblast was derived from sperm or acquired after fertilization. It has been shown previously that the methylation level of the **Xist** promoter is relatively low in oocytes, sperm and the preimplantation embryo (McDonald et al., 1998). Bisulfite sequencing revealed that whereas the paternal allele in X<sup>F1</sup>X<sup>AA</sup> embryos at E6.5 was analyzed by bisulfite sequencing. It was evident that the methylation level was significantly higher in XX blastocysts. (B) Normally, a silent copy of paternal **Tsix** was ectopically expressed in X<sup>F1</sup>X<sup>AA</sup> blastocysts upon paternal transmission of the X<sup>AA</sup> allel. Following two-round PCR on cDNA prepared from single F1 blastocysts, the amplified products were digested with BsmAI, the recognition site of which is present only in the laboratory strain. (C) Methylation profile of the **Xist** promoter region on the paternal X in the blastocyst isolated from XX and X<sup>AA</sup> embryos at E6.5 was analyzed by bisulfite sequencing. It was evident that the methylation level was significantly higher in XX blastocysts than that in XX, suggesting that the expression of normally silent **Tsix** on the paternal X<sup>AA</sup> induces aberrant methylation of the **Xist** promoter region in cis. An arrow indicates the position of the transcription start site of **Xist**.

**Fig. 5. **Xist** and **Tsix** are aberrantly regulated in XX<sup>AA</sup> embryos. (A) Quantitative RT-PCR analysis of individual F1 blastocysts recovered from X<sup>F1</sup>X<sup>F1</sup> females crossed with either XY or X<sup>AA</sup>Y males. The level of **Xist<sup>AA</sup>** RNA in X<sup>F1</sup>X<sup>AA</sup> was much lower than that of wild-type **Xist** in XX blastocysts. (B) Normally, a silent copy of paternal **Tsix** was ectopically expressed in X<sup>F1</sup>X<sup>AA</sup> blastocysts upon paternal transmission of the **Xist<sup>AA</sup>** allele. Following two-round PCR on cDNA prepared from single F1 blastocysts, the amplified products were digested with BsmAI, the recognition site of which is present only in the laboratory strain. (C) Methylation profile of the **Xist** promoter region on the paternal X in the blastocyst isolated from XX and X<sup>AA</sup> embryos at E6.5 was analyzed by bisulfite sequencing. It was evident that the methylation level was significantly higher in XX blastocysts than that in XX, suggesting that the expression of normally silent **Tsix** on the paternal X<sup>AA</sup> induces aberrant methylation of the **Xist** promoter region in cis. An arrow indicates the position of the transcription start site of **Xist**.**
The significance of this difference in the methylation levels is not known at present, but this result indicates that the aberrant methylation found in the Xist promoter on the paternal XΔA in the trophoblast arises during embryogenesis.

It has been proposed that the differentially methylated domains in Tsix and Xite found between oocytes and sperm might constitute primary marks for the imprinted expression of Tsix in the zygote (Boumil et al., 2006). One of these domains, HS6, in Xite has been relatively well characterized by bisulfite sequencing and has been shown to be heavily methylated in sperm. It was postulated that if the methylation of this region in sperm was causally involved in the repression of paternal Tsix in the preimplantation embryo, it might be abolished in sperm isolated from XΔA males. We therefore assessed the methylation status of this region in sperm of the mutant males. As shown in Fig. 6B, this region was heavily methylated on XΔA as on the wild-type X, suggesting that the methylation status of HS6 in the Xite region was not directly involved in the ectopic activation of Bx6 on the paternal XΔA chromosome.

**DISCUSSION**

By taking advantage of an inducible expression system of a single copy Xist cDNA with various deletions from the single X chromosome in male ES cells, Wutz et al. showed that the silencing function of Xist RNA is dramatically compromised if the A-repeat is deleted, even though the mutant forms of the RNA accumulate in cis on the X chromosome (Wutz et al., 2002). This finding was further corroborated by the functional assay of Xist RNA lacking the A-repeat expressed from the endogenous locus by the inducible promoter in male ES cells (Wutz et al., 2002). Although this study highlighted for the first time the possible functional domain of Xist RNA that is crucial for inducing chromosome-wide silencing, the importance of the A-repeat for X-inactivation occurring in the mouse embryo had not been addressed until this study. The XistΔA allele was created so that it would produce a transcript nearly the same as the one lacking the A-repeat expressed from the endogenous Xist locus upon induction in the study by Wutz et al. (Wutz et al., 2002). Genetic and molecular analyses of female embryos heterozygous for XistΔA clearly demonstrated that the deletion of the A-repeat rendered the mutated X incompetent to undergo inactivation, indicating the crucial role of the A-repeat in X-inactivation during mouse development. The presence or absence of the A-repeat, however, does not seem to be directly involved in the primary choice of X-inactivation in the embryonic lineage, as different deletions in the Xist gene, although they retain the A-repeat, have resulted in primary non-random X-inactivation similar to that observed in this study (Marahrens et al., 1998; Sado et al., 2005).

We initially postulated that the failure of X-inactivation could be ascribed to the defect in XistΔA RNA, which should be capable of coating the mutated X based on the inducible expression assay in ES cells (Wutz et al., 2002). Intriguingly, it was found that the level of XistΔA RNA was greatly reduced in XXΔA preimplantation embryos compared with that of wild-type Xist RNA in XX embryos. This could be due to a reduction either in the stability of the mutated RNA or in the expression level per se. Quantitative RT-PCR demonstrated, however, that the stability of Xist RNA detected in undifferentiated male ES cells was comparable regardless of the presence or absence of the A-repeat, suggesting that the latter possibility was more favorable. It is likely therefore that the reduction in the level of Xist RNA in preimplantation embryos is primarily due to the

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**Table 1. Primer sequences used for PCR**

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<th>Primer</th>
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<td>TGCTTTTTGAGTGCTGATG</td>
</tr>
</tbody>
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**Fig. 6. Methylation profile of the Xist promoter region and Xite HS6 in sperm recovered from XY and XΔA males.**

(A) Although the Xist promoter region was partially methylated in sperm recovered from XY as previously reported (McDonald et al., 1998), it exhibited even lower methylation in the sperm of XΔA males. (B) The Xite HS6 region, the methylation of which in sperm was suggested to be an imprint responsible for the repression of paternal Tsix in the tissues or cells showing imprinted X-inactivation, was heavily methylated in sperm of both XY and XΔA males.
transcriptional silencing of Xist. Our result demonstrates that the region encoding the A-repeat plays a crucial role as a regulatory element in the appropriate regulation of Xist in vivo.

Intriguingly, the lack of Xist RNA in the blastocyst was accompanied by an ectopic activation of the normally silent paternal copy of Tsix on the same X chromosome. Furthermore, the Xist promoter on the mutated paternal X in the trophotroph at E6.5 was aberrantly methylated at CpG sites that are normally unmethylated on the paternal X. These findings raised an interesting possibility that the transcriptional silencing of Xist in preimplantation embryos is triggered by ectopically expressed Tsix, which subsequently promotes CpG methylation in the Xist promoter region on the mutated paternal X. In this scenario, the deleted region in the XistΔA allele, most probably the A-repeat, is crucial for the appropriate repression of Tsix on the paternal X at the onset of imprinted X-inactivation. Given the fact that the transcription of Tsix is initiated 40 kb downstream from the A-repeat, it is tempting to speculate that the A-repeat exerts its effect on Tsix through a long-range chromatin conformation. However, the opposite scenario is also possible: the upregulation of Tsix was somehow caused by the primary silencing of Xist that resulted from the loss of some crucial regulatory element located within the deleted region. Because the aberrant methylation of the Xist promoter appears to be established during embryogenesis, it might be expected that the mutated XistΔA RNA would be transcribed from the paternal XΔA in the early preimplantation embryo. This was not the case, however, and the expression of paternal XistΔA was diminished from the very early stages. This observation may favor the later scenario that the silencing of Xist is the primary event. These two possibilities cannot be distinguished between on the basis of current data and the further experimentation is certainly required. The simplest way to address this issue is to terminate Tsix on XΔA and see whether XistΔA is expressed or not. We are currently trying to produce mice carrying a Tsix deficiency on XΔA through second gene targeting in ΔA210x111 ES cells.

Although the targeted deletion of the A-repeat did not allow us to address the functional significance of the A-repeat as an element in the Xist RNA because of the unexpected lack of expression from the mutated X, this study clearly demonstrates that the region encoding the A-repeat is essential as a genomic element for X-inactivation in the mouse embryo. Further attempts to identify the factors that interact with the DNA sequence harboring the A-repeat should provide further insight into the molecular mechanisms of Xist/Tsix regulation and the random choice of X-inactivation.

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