Methylation profiles of DXPas34 during the onset of X-inactivation

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X chromosome inactivation is controlled by the cis-acting X-inactivation centre (Xic). In addition to initiating inactivation, Xic, which includes the Xist gene, is involved in both a counting process that senses the number of X chromosomes and the choice of X chromosome to inactivate. Controlling elements lying 3′ to Xist include the DXPas34 locus. Deletion of DXPas34 in undifferentiated embryonic stem (ES) cells eliminates expression of both Xist and the antisense transcript Tsix, thought to initiate from a CpG island lying close to, but telomeric to, the DXPas34 locus itself. Deletion of DXPas34 leads to non-random inactivation on ES cell differentiation and disrupts imprinted X-inactivation in vivo. In order to investigate the role of methylation at DXPas34 in the initial steps of X-inactivation, we studied its methylation status during pre- and post-implantation embryonic development and ES cell differentiation, using the bisulphite sequencing technique. Analysis of the methylation status of both the DXPas34 locus and the associated downstream CpG island shows that extensive hypermethylation of the DXPas34 locus is a relatively late event in differentiation and embryogenesis. We conclude that methylation of DXPas34 cannot be the X chromosome imprint, nor can it be involved in the parent-of-origin effects associated with deletion of the DXPas34 locus and the neighbouring CpG island.

INTRODUCTION

X-inactivation is a complex epigenetic phenomenon which results in the transcriptional silencing of one of the two X chromosomes in the female diploid cell. During the earliest stages of embryogenesis both the X chromosomes contributed by the sperm and the egg are active. Differentiation of the earliest lineages occurs at the blastocyst stage with the formation of the trophectoderm and primitive endoderm, both of which contribute to the extra-embryonic tissues, and is associated with non-random inactivation of the paternal X chromosome. Random X-inactivation first occurs in the epiblast, which gives rise to the embryo proper in the late blastocyst and egg cylinder stages, at the time of placental implantation. The imprint responsible for non-random X-inactivation in extra-embryonic tissues is no longer operative in these cells and either the paternal or maternal X chromosome can be inactivated (for a review see ref. 1).

The process of X-inactivation is under the control of a unique region called the X-inactivation centre (Xic). The Xic is a complex locus that controls the initiation and propagation of the inactivation process in cis. It also ensures that the correct number of X chromosomes undergo inactivation (counting) and influences which X chromosome becomes inactivated (choice) (2). Several distinct genetic elements lying within the Xic have been shown to play a role in the X-inactivation process. One such element, the Xist gene, which is essential for the initiation of X-inactivation in cis (3), encodes a large nuclear, untranslated RNA, which coats the inactive X chromosome at interphase (4–8). A Xist imprint, the nature of which remains unknown, is moreover likely to be at least partly responsible for imprinted X-inactivation (9,10). Xist is expressed at low levels from every X chromosome in the cell prior to X-inactivation (11). At the onset of inactivation, however, steady-state levels of Xist RNA increase markedly and the Xist transcript becomes stabilized and coats the X chromosome that has been selected to be inactivated (12,13). A series of regulatory elements of X-inactivation have been proposed to lie 3′ to Xist. One such element is the X-controlling element (Xce) which influences, in an as yet undefined manner, the randomness of the X-inactivation process in the mouse (14). Three alleles at the Xce locus have been identified: Xce+, Xce− and Xce0 (15). In Xce+/Xce− heterozygotes, the X chromosome carrying the Xce− allele is more likely to be inactivated than that carrying the Xce+ allele; similarly, in Xce0/Xce− heterozygotes, the X chromosome carrying the Xce0 allele is more likely to be inactivated in most cells. The most extreme non-randomness is seen in Xce+/Xce− heterozygotes. Another regulatory element, situated 15 kb downstream of Xist, is the DXPas34 locus. The DXPas34 locus (Fig. 1) is a CpG-rich region consisting of a Sall site and a neighbouring cluster of HpaII sites lying within a 34mer minisatellite repeat. This locus was first identified as a result of its hypermethylation on the active X chromosome (Xa) and an apparent correlation of its methylation level with allelism at the genetically separable Xce locus (16–18).

A number of the features displayed by the DXPas34 locus are reminiscent of differentially methylated regions (DMRs) which are frequently found in the vicinity of genes subject to imprinting (19). Like the DXPas34 locus, DMRs show differential
methylation between alleles, occur in or near CpG-rich regions and are often associated with blocks of short tandem repeats. Some DMRs are also known to be associated with the transcription of unusual non-coding RNAs. Likewise, the CpG island lying immediately telomeric to the DXPas34 locus has been proposed to be the major transcription initiation site for Tsix, a novel antisense transcript associated with the Xist locus (20). It has been proposed that the DXPas34 locus might play a role in regulating Xist expression, in chromosome choice and in the initiation of X-inactivation. Deletion of DXPas34 has recently been shown to eliminate both Xist expression and the antisense transcription present in this region in undifferentiated ES cells (21). It also leads to non-random inactivation on differentiation in vitro and impaired imprinting X-inactivation in vivo (21–23). DXPas34 thus appears to be a critical regulator of Xist activity and X-inactivation.

Here we extend our earlier methylation analysis of the DXPas34 locus by a detailed bisulphite analysis of the methylation status of this region. We wished to address in greater depth the pattern of Xa-specific methylation and the associated Xce differences, and also to identify eventual methylation differences in the DXPas34 locus and the associated CpG island occurring between imprinted and random X-inactivation.

Hypermethylation of sites at the Xist proximal (centromeric) end of the DXPas34 minisatellite array was observed in adult tissues and differentiated ES cells, whereas the end of the array containing the Tsix initiation site remained largely unmethylated. Examination of both male and female gametes and pre- and post-implantation embryos revealed that methylation of the DXPas34 locus is a post-implantation event. Our results suggest that methylation of this region does not intervene in the process of imprinted X-inactivation nor in the parent-of-origin effects associated with deletion of DXPas34.

RESULTS

Methylation of adult male DNAs

In order to evaluate the methylation status of all of the CpG sites within the DXPas34 locus associated with the active X chromosome, male spleen DNAs obtained from mice of known Xce genotype were bisulphite treated and sequenced. Use of male DNAs allowed the methylation status of the Xα to be analysed directly. A gradient of methylation was apparent in both the 129/Sv (Xceα) and 129.Pgk1a (Xceβ) strains, with hypermethylation at the Xist proximal end of the DXPas34 minisatellite array and extensive hypomethylation at the Xist distal end of the array (Fig. 2). Overall levels of methylation were clearly lower in the congenic 129.Pgk1a strain. Comparison of the profiles of the 129/Sv and 129.Pgk1a strains suggests that this may, in part, be linked to the absence of a series of potentially methylatable repeat units lying in the 3′ half of the 129/Sv array within the DXPas34 locus of the 129.Pgk1a strain. The DXPas34 locus in the Xceβ strain is much shorter, consisting of only 26 repeat units, as opposed to the Xceα strain which is composed of 39 repeat units. We conclude that the differences in methylation status of the DXPas34 locus may, at least in part, reflect underlying differences in the structure of the locus itself.

Xce and methylation at the DXPas34 locus

We next reconsidered our original interpretation of the Xce genotype–methylation correlation, which had been interpreted as suggesting that Xce was directly controlling the methylation status of the DXPas34 locus (17). In order to clarify the relationship between Xce and the DXPas34 locus we analysed the methylation status of the DXPas34 locus in a subset of
DNAs from male recombinant mice, generated during the course of crosses aimed at localizing the \textit{Xce} locus between an \textit{Xce}\textsuperscript{a} strain and an \textit{Xce}\textsuperscript{c} strain. All the DNAs in question were obtained from animals that had recombined distally to \textit{Xist}, i.e. in the region containing \textit{DXPas34} between the \textit{Xist} and \textit{Atp7a} (mottled) loci. DNA of recombinant strain 4630 that genotyped ‘c’ at the \textit{DXPas34} locus and showed a distinct ‘c’ methylation profile (Fig. 3) was shown to be phenotypically an \textit{Xce}\textsuperscript{a} strain. We conclude that control of the methylation status of the \textit{DXPas34} locus resides at or very close to the locus itself and is unlikely to directly involve \textit{Xce}.

### Methylation of undifferentiated and differentiated embryonic stem (ES) cells

The role of methylation at the \textit{DXPas34} locus and the neighbouring CpG island in X-inactivation might be elucidated through knowledge of its methylation status during ES cell differentiation. We therefore carried out a bisulphite analysis of the methylation status of the \textit{DXPas34} locus in a male ES cell line (D3) before and after differentiation. The 129-derived D3 cell line showed only a low level of methylation over the \textit{DXPas34} minisatellite array prior to differentiation (Fig. 4). The associated CpG island was similarly essentially unmethylated. During differentiation was accompanied by a marked increase in methylation over the minisatellite array. Methylation of the associated CpG island increased during differentiation but remained both limited and heterogeneous in its clonal distribution (Fig. 4). Transcription of the \textit{Tsix} antisense RNA, which is thought to initiate mainly from this CpG island, is down-regulated during ES cell differentiation (20). The antisense transcript is essentially undetectable by RNA fluorescence \textit{in situ} hybridization (FISH) analysis from male ES cells differentiated for 6–7 days (20,21). Both the limited and heterogeneous nature of the methylation present across the CpG island in our 6 day differentiated male ES cells and the very low levels of methylation seen on the CpG island in post-implantation embryos (see below) make it unlikely that this methylation is playing a primary role in regulating antisense transcription from this site.

### Pre-implantation stages of embryogenesis

We wished to examine methylation at the \textit{DXPas34} locus during early embryogenesis, particularly at pre-implantation stages, in which imprinted X-inactivation occurs.

Methylation profiles of the \textit{DXPas34} locus obtained from early embryonic stages are shown in Figure 5. Individual mature sperm and oocytes are almost totally demethylated with relatively little variation in methylation status between individual clones. The globally unmethylated status of the minisatellite array is maintained through the 2-cell embryo stage to the morula and the blastocyst stages, although low levels of methylation (<20% of that seen in adult tissues) were observed at this latter stage. We conclude that methylation at the \textit{DXPas34} locus including the minisatellite array does not represent the \textit{Xist} imprint, which ensures that the maternal \textit{Xist} gene is transcriptionally inactive and that the paternal \textit{Xist} gene is active in extra-embryonic tissues.
Bisulphite analysis of the CpG island adjacent to the DXPas34 locus was also performed in order to assess whether its methylation might be controlling Tsix expression during early embryogenesis. When examined in sperm, oocytes and 2-cell embryos, the CpG island was found to be totally unmethylated (Fig. 5) and this situation persisted in the 7.5 days post-coitum (d.p.c.) embryo.

In pre-implantation female embryos the paternally derived X chromosome (Xp) is coated by Xist RNA from at least the 8-cell stage (13). Such Xist coating of the Xp continues to be observed in extra-embryonic tissues of both pre- and post-implantation embryos (13). Since such mature Xist domains are never associated with an antisense signal (20), our results imply that neither the methylation of the DXPas34 locus itself nor the methylation of the associated CpG island regulates Tsix antisense transcription on the Xp.

Post-implantation stages of embryogenesis

Significant levels of methylation at the DXPas34 locus were observed in 7.5 d.p.c. embryos in both embryonic and extra-embryonic tissues (Fig. 5). Embryonic and extra-embryonic tissues from female embryos at this stage showed lower levels of overall methylation than the corresponding male embryos, as expected given the presence of both a methylated Xa and an unmethylated Xi chromosome in the female. Male embryos at 12.5 d.p.c. also showed hypermethylation at the DXPas34 locus. Significantly, no differences in methylation profile between embryonic and extra-embryonic tissues were observed in either male or female 7.5 d.p.c. embryos. Although methylation of the DXPas34 locus itself is associated with gastrulation, only low and mosaic methylation of the neighbouring CpG island was found in both embryonic and extra-embryonic tissues of male and female 7.5 and 12.5 d.p.c. embryos. For example, less than a quarter of the modificable sites on the Xa were methylated by a third or more when male 7.5 d.p.c. embryos were examined, and even fewer in female embryos (Fig. 5).

DISCUSSION

Our analysis suggests that methylation of the DXPas34 region is absent from both female and male gametes and the earliest...
Figure 5. Analysis of CpG methylation at the DXPas34 region during early embryogenesis. The level of methylation for each CpG (↑) is represented as indicated in the legend to Figure 2. Analysis of both the DXPas34 locus and the neighbouring CpG island was undertaken in sperm, oocytes, 2-cell stage embryos, morula, blastocyst, E7.5 ectoderm and extra-embryonic tissues from male and female embryos, and embryonic tissues from a male E12.5 embryo.
stages of embryogenesis. Methylation of the DXPas34 locus on the active X chromosome is essentially a post-implantation event and our results clearly indicate that methylation of this locus does not precede the onset of imprinted X-inactivation in the trophectoderm. Our results lead us to hypothesize a methylation step occurring after the blastocyst stage and prior to gastrulation, to explain the hypermethylation of the DXPas34 allele on the active X chromosome observed in somatic cells and differentiated ES cells. McDonald et al. (24) similarly concluded that the 5′ promoter region of the Xist gene is subject to post-implantation methylation. It may well be that late onset of methylation of the Xc characterizes much of the central span of the Xic, which includes Xist.

We found no evidence in support of the notion that a methylation-mediated Xist imprint localizes to the DXPas34 locus. This possibility was raised when the methylation status of the 5′ end of the Xist gene had made it unlikely that differentiable methylation status of the maternal and paternal Xist promoters was acting as the primary imprint (24). Although hypermethylation of the Xist promoter region is seen in sperm and not in the oocyte, this hypermethylation is subsequently lost prior to or during the earliest stages of embryogenesis. Interestingly, the possibility that methylation-independent mechanisms of genomic imprinting might be specifically associated with the extra-embryonic lineages has been suggested by Sado et al. (10) to explain observations made in Dmnt1-deficient embryos.

Our earlier analysis showing hypermethylation of this region in adult male cells had led us to suggest that methylation of the DXPas34 region might precede X-inactivation itself and, more specifically, might affect the choice of X chromosome to be inactivated, given that hypermethylation of this locus varied in different Xce strains (17). In order to further dissect whether Xce controls the pattern of methylation at the DXPas34 locus, we have examined DNAs from several of the critical recombinant animals that have enabled the candidate region for Xce to be refined. The methylation profiles obtained suggest that methylation status is controlled by sequences at or immediately surrounding the DXPas34 locus, although an interactive effect with the Xce locus cannot be totally excluded. Such interactive effects would be expected to be mediated through long-range chromatin interactions, which seem to be involved in the onset of X-inactivation. It is interesting to note, in this context and in the context of a postulated locus control region (LCR)-type function for DXPas34, that long-range, rather than short-range, function of the immunoglobulin intronic κ promoters might be specifically associated with the extra-embryonic lineages.

Recent results suggest that DXPas34 may contribute to at least four activities which may or may not be linked: regulation of antisense transcription prior to inactivation, regulation of Xist expression prior to inactivation, the capacity to induce random X-inactivation on differentiation and imprinted X-inactivation (21–23; C. Morey, D. Arnaud, P. Avner and P. Clerc, personal communication). It has recently been suggested by Lee et al. (20) that the CpG island associated with the DXPas34 locus is the initiation site of transcription for the antisense transcript. Other evidence, however, suggests that there may be more widespread antisense transcription extending telomeric to the DXPas34 locus, making it unlikely that there is a single transcriptional origin in undifferentiated ES cells (21). Such antisense transcripts are detected on the maternal but not on the paternal X chromosome in pre-implantation embryos (21,23). The absence of any methylation associated with the DXPas34 locus or the associated CpG island in sperm, oocytes and the early embryo precludes the possibility that the antisense activity initiating at this site in the early embryo is controlled by the methylation status of DXPas34.

Debrand et al. (21) also reported evidence that in adult somatic cells and differentiated ES cells, there are at least two domains of relatively low-level antisense transcription, one centred over Xist and the other, again, centred over the DXPas34 locus. The polarity of DXPas34 methylation on the Xc observed in adult cells, with more pronounced hypermethylation towards the centromeric side of the DXPas34 locus at the opposite end of the array to the CpG island, argues against a functional role for methylation in the control of initiation of this antisense transcription from the CpG island.

What then is the likely role of the methylation patterns observed at the DXPas34 locus? The presence of methylation at the DXPas34 locus in the 7.5 d.p.c. embryo leaves open the possibility that DXPas34 methylation might play a role in the counting and choice mechanisms occurring during random X-inactivation in epiblast-derived tissues rather than a maintenance role. Neither the finding of DXPas34 hypermethylation in both extra-embryonic and embryonic tissues of the 7.5 d.p.c. embryo, nor the recent finding that random X-inactivation in the embryo is initially normal in Dmnt1-deficient embryos but is associated with increased instability, invalidates this possibility. The examination of Dmnt3a- and Dmnt3b-deficient embryos unable to carry out de novo methylation may allow elucidation of this point (26).

**MATERIALS AND METHODS**

**Mice**

The origin of the 129.Pgk1a congenic strain was described by Courtier et al. (17). Male Xce recombinant animals 3116, 4033 and 4630 were obtained by Cattanach and colleagues from crosses of Fl Xce+Xce+ heterozygote females to Xce+ males. Recombinants for the Xce region were obtained by selecting animals, having recombinated the Ta and Aip7a flanking coat markers used to mark the parental stocks. These flanking markers define an ∼4–6 cM region of the mouse X chromosome (27). Phenotypic analysis for the Xce locus was as described by Cattanach and Williams (14).

**Cell lines and culture conditions**

The male ES cell line D3 was obtained from R. Kemler and was described by Doetschman et al. (28). ES cells were grown on irradiated mouse fibroblast feeder layers in medium containing 15% fetal calf serum (FCS), 10⁻³ U/ml lymphocyte inhibitory factor (LIF), 0.1 mM 2-mercaptoethanol and 2 mM glutamine. Differentiated ES cultures were obtained by forming aggregates using standard techniques and replated for 6 days prior to harvesting (29).

**Methylation studies by Southern blot analysis**

Double restriction digests were carried out on 20 μg of spleen DNAs with EcoRI and HpaII. At least a 10-fold excess of enzyme was added to the DNA digests in three installments.
Digested DNA was separated by electrophoresis on standard 0.8% agarose gels overnight at 1.25 V/cm in 89 mM Tris–borate (pH 8.3), 2 mM EDTA and transferred to Hybond-N+ membrane (Amersham). The unique sequence probe Pas34 described by Courtier et al. (17) was labelled using the standard Megaprime DNA labelling system (Amersham).

Collection and preparation of oocytes, sperm and embryos

Oocytes were collected from 3-week-old (C57Bl/6J × DBA/2J) F1 female mice following superovulation by intraperitoneal injection of 5 IU of pregnant mare’s serum (Foligon; Intervet) and of 5 IU of human chorionic gonadotropin (Chorulon; Intervet), 44–46 h later. The next morning, ampoules were injected into a drop of M2 medium containing 0.5 mg/ml hyaluronidase (Sigma) for 5 min at room temperature to dissected into a drop of M2 medium containing 0.5 mg/ml hyaluronidase (Sigma) for 5 min at room temperature to remove cumulus cells. Oocytes were collected, washed three times in M2 medium and pooled in a 5 µl volume (for ~80 oocytes). To collect embryos, F1 females were mated overnight with (C57Bl/6J × DBA/2J) F1 males. Females were checked for the presence of a vaginal plug and 2 days later [embryonic day (E) 1.5] 2-cell stage embryos were removed by flushing the oviduct with 0.5 ml of M2 medium. Embryos were pooled in a 5 µl volume containing 40 embryos. Embryos at E2.5 (morulae) were removed by the same method. Four or five morulae (of 8–32 cells) were pooled in a volume of 5 µl. E3.5 blastocysts were collected by flushing the uterus with 0.5 ml of M2 medium and two or three blastocysts pooled in a volume of 5 µl. Sperm DNA was prepared as described by Gill et al. (30). Embryonic and extra-embryonic tissues from E7.5 and E12.5 embryos were separated as described by Beddington (31). Embryo sexing was carried out as described by Kay et al. (11).

Bisulphite treatment

Bisulphite treatment of chromosomal DNA was carried out as described by Olek et al. (32), using 100 ng of DNA to form agarose beads. For embryos, 7 µl of 1× phosphate-buffered saline (PBS), 2% molten low melting point agarose (Sea Plaque; FMC) was mixed with 5 µl of M2 medium containing the embryos and pipetted into 300 µl of cold heavy mineral oil (Sigma) laid over 600 µl of lysis solution (10 mM Tris–HCl pH 7.2, 10 mM EDTA pH 8.0, 1% SDS, 20 µg/ml Proteinase K). The drop containing the agarose and embryos solidified in the oil and the agarose beads were mechanically pushed into the lysis solution. After overnight incubation at 50°C, the Proteinase K was inactivated by PMSF [40 µg/ml in 1× TE (0.1 mM Tris–HCl pH 7.2, 1 mM EDTA pH 8.0), twice for 30 min], followed by several rinses in TE (once for 30 min at 50°C, twice for 15 min at room temperature). The beads were then equilibrated with restriction buffer (100 µl, twice for 15 min) and digested overnight with 30 U of EcoRI enzyme (Boehringer) at 37°C. After a 1× TE wash, the beads were overlaid with 200 µl of mineral oil and heated to 100°C for 10 min in order to melt the agarose and fully separate individual DNA strands. The DNA–agarose solution was resolidified to agarose beads by chilling the reaction tube on ice. Aliquots (200 µl) of a 5 M bisulphite solution [2.5 M sodium metabisulphite (Merck), 125 mM hydroquinone (Sigma) pH 5.0] were added to each reaction tube containing a single bead. The reaction mixtures were then incubated for 4 h at 50°C in the dark. Treatment was stopped by rinsing with 1 ml of 1× TE (twice for 1 min, three times for 10 min), followed by desulphonation in 1 ml of 0.2 M NaOH (twice for 15 min). Beads were washed with 1 ml of 1× TE (twice for 10 min) and kept overnight at 4°C.

PCR amplification of bisulphite-treated DNA

Beads were first dialysed for 30 min against 1 ml of H2O and polymerase chain reaction (PCR) amplification was then performed on individual beads. A nested PCR amplification approach to DXPas34 was used in order to increase the sensitivity and specificity of PCR product detection. The DXPas34 minisatellite and the neighbouring Cpg island were amplified by two rounds of PCR using the following primers: for the minisatellite region in the first round, primers F1 (5'-GGT GAG GTA GTA TTG AAT TG-T3') and R1 (5'-ACA AAA AAA TTC CTA AAA TAC TTA CCA AC-3') and, in the second, primers F2 (5'-AGG TAG TAT TGT TGA ATT TGG TTT GAG-3') and R2 (5'-CCA AAA TTT AAA ATA TCT ATC CCT TAC-3'); for the neighbouring Cpg island in the first round, primers F1 (5'-TTT TAT TGT TTT AGT ATT TAG TTA T3') and R1 (5'-ACC ATT ATC TTT TCT TAC TAT AAC TCC CAC A-3') and, in the second, F2T (5'-TTA GTT ATT TTT TTA TTT TAA TAA GGA-3') and R2T (5'-AAA TAA TCA AAT ATA CTA ACC ACA CCT TCT-3'). PCR amplification reactions were carried out using Taq DNA polymerase (Gibco BRL) in a 100 µl reaction for the first round containing 30 pmol of each primer, 1.5 mM MgCl2, 0.25 mM dNTPs, 1× PCR buffer, 5 U of Taq DNA polymerase, and in a 50 µl reaction for the second round containing 20 pmol of each primer, 1.5 mM MgCl2, 0.25 mM dNTPs, 1× PCR buffer, 2.5 U of Taq DNA polymerase. The first round PCR product (3 µl) was used as a template for the second round. PCR cycles were 5 min of denaturation at 94°C, followed by 35 cycles: 30 s of denaturation at 94°C, 30 s of annealing at 61°C, 2 min of elongation at 72°C, and a final 10 min elongation step. After the second round of PCR, 5 U of Taq DNA polymerase was added to the reaction tube for 15 min at 72°C in order to improve the fraction of products with an A overhang.

Cloning and sequencing

PCR products amplified from bisulphite-modified DNA were gel-purified using the QIAquick Gel extraction kit (Qiagen), and cloned into the pGEM-T Easy TA plasmid vector (Promega). Sequencing of plasmids containing bisulphite PCR products was carried out using the ABI PRISM Dye Terminator cycle sequencing ready reaction kit incorporating the –21 and Reverse sequencing primers, and the product was analysed on an ABI automated sequencer.

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REFERENCES


