Resistance of IAPs to Methylation Reprogramming May Provide a Mechanism for Epigenetic Inheritance in the Mouse

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Summary: Genome-wide epigenetic reprogramming by demethylation occurs in early mouse embryos and primordial germ cells. In early embryos many single-copy sequences become demethylated both by active and passive demethylation, whereas imprinted gene methylation remains unaffected. In primordial germ cells single-copy and imprinted sequences are demethylated, presumably by active demethylation. Here we investigated systematically by bisulphite sequencing the methylation profiles of IAP and Line1 repeated sequence families during preimplantation and primordial germ cell development. Whereas Line1 elements were substantially demethylated during both developmental periods, IAP elements were largely resistant to demethylation, particularly during preimplantation development. This may be desirable in order to prevent IAP retrotransposition, which could cause mutations. In turn, this can result in the transgenerational inheritance of epigenetic states of IAPs, which could lead to heritable epimutations of neighbouring genes through influencing their transcriptional states. genesis 35:88–93, 2003.

Key words: DNA methylation; demethylation; IAP; epigenetic inheritance; reprogramming

INTRODUCTION

Epigenetic modifications such as DNA methylation have important roles in genome function and stability (Bird, 2002; Ben-Porath and Cedar, 2001; Rideout et al., 2001; Bestor, 2000; Reik et al., 2001; Surani, 2001). DNA methylation patterns are generally stable in somatic tissues both in embryos and after birth. In mammals there are two developmental periods, however, during which global demethylation occurs, followed by de novo methylation several days later, generating patterns of DNA methylation that are different from the ones before demethylation commenced (Reik et al., 2001). In primordial germ cells, primarily from E11.5–12.5, demethylation occurs in all single-copy and imprinted genes analysed so far (Kafri et al., 1992; Brandeis et al., 1993; Lee et al., 2002; Hajkova et al., 2002). The mechanism of demethylation is likely to be active since there is at most a single round of DNA replication that the germ cells undergo between the two stages. In addition, Dnmt1 the maintenance methyltransferase is present in nuclei at these stages, so passive demethylation is unlikely to occur (Hajkova et al., 2002). In the zygote, the sperm-derived genome undergoes large-scale demethylation before DNA replication commences. This has been identified both by immunofluorescence using an antibody against 5-methyl cytosine (Mayer et al., 2000; Dean et al., 2001; Santos et al., 2002) and by bisulphite analysis of single-copy sequences (Oswald et al., 2000). However, an important difference to demethylation in germ cells is that paternally methylated imprinted genes are exempt from demethylation (Olek et al., 1997; Tremblay et al., 1997). The mechanism of this active demethylation is unknown but does not involve the candidate demethylase gene Mbd2 (Santos et al., 2002). Further demethylation in the preimplantation embryo occurs passively during cleavage divisions and depends on DNA replication (Howlett and Reik, 1991; Rougier et al., 1998). The likely reason for passive demethylation is that Dnmt1 protein is excluded from nuclei of cleavage embryos at most stages (Carlson et al., 1992; Howell et al., 2001). Again, passive demethylation does not occur to imprinted genes and the reasons for this are unknown.

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(Reik and Walter, 2001). De novo methylation begins after the morula stage in the inner cell mass cells of the blastocyst and is presumably dependent upon Dnmt3a and b (Santos et al., 2002; Okano et al., 1999).

The large-scale methylation changes detected with the anti 5-methyl cytosine antibody presumably reflect changes in many different regions in the genome, including in repetitive gene families and transposons. There have been some limited studies of methylation of Line1 elements (10,000–100,000 copies). Intracisternal A Particle elements (IAP, 1,000 copies), and centromeric satellites. High levels of overall methylation in Line1 elements have been found in mature oocytes and in sperm, with evidence of passive demethylation from zygote to blastocyst stage (Sanford et al., 1987; Howlett and Reik, 1991). IAP elements were also highly methylated in the gametes with some demethylation in blastocysts (Howlett and Reik, 1991; Walsh et al., 1998). Some demethylation of IAP elements and Line1 elements was also reported in primordial germ cells (Walsh et al., 1998; Hajkova et al., 2002). However, a precise analysis by bisulphite sequencing of these elements at various critical stages of development is lacking. Such an analysis is particularly important in the light of observations of epigenetic inheritance in the mouse (Roemer et al., 1997), which in one instance has been shown to be mediated by an IAP element inserted in close proximity to the agouti gene (Morgan et al., 1999). The methylation state of this individual element in primordial germ cells and in preimplantation embryos is not known.

We designed primers for bisulphite analysis in the 5’LTR of the IAP genome in order to amplify a 258-bp fragment containing up to 11 CpG dinucleotides spanning the IAP promoter, which is known to be methylation sensitive (Walsh et al., 1998). For the Line1 genome, we amplified a 5’ fragment of 239 bp containing up to 9 CpGs, and a 3’ fragment of 485 bp containing up to 7 CpGs (Fig. 1a). We analysed mature oocytes, sperm, zygotes, blastocysts, and primordial germ cells of both sexes from E11.5–E13.5, during which time dramatic demethylation occurs in imprinted genes and single-copy sequences (Lee et al., 2002; Hajkova et al., 2002). ES cells and primary embryo fibroblasts (PEFs) were analysed as representatives of early epiblast cells and foetal somatic cells, respectively. The primordial germ cells were isolated using an Oct4-GFP transgene and fluorescence activated cell sorting (Hajkova et al., 2002). Line1 elements were highly (5’, 98%) and moderately (3’, 54%) methylated in sperm and methylation patterns were homogeneous between different genomic copies (each sequenced clone is assumed to have arisen from a separate element in the genome because of the high complexity of these elements; this is supported by many sequence differences between individual clones; Fig. 1b, and data not shown). Mature oocytes were moderately (5’, 29%) or scarcely (3’, 4%) methylated, and the 5’ clones showed heterogeneity, suggesting different methylation patterns depending on individual sequence or location in the genome (Fig. 1b). Zygotes and blastocysts showed similar low methylation levels and patterns at both the 5’ (25%, 27%) and the 3’ end (23%, 15%) which were similar to those of the oocyte (29%, 4%). This suggests that active demethylation of most of the sperm copies has occurred in the zygote, consistent with the large-scale demethylation in the zygote seen by immunofluorescence (Mayer et al., 2000; Dean et al., 2001; Santos et al., 2002). The notion of active demethylation is further supported by the fact that while most sperm copies of Line1-5’ were fully methylated, none of the zygote copies were (Fig. 1b). Not much further passive demethylation appears to occur between the zygote and blastocyst stages at the CpGs analysed here.

The kinetics of IAP methylation were very different from those of the Line1 elements (Fig. 1b). As with the Line1 elements, each sequenced clone is assumed to have arisen from a separate element in the genome; this is confirmed by the many sequence differences detected in our analysis, reflecting the fact that 5’ and 3’ LTR sequences vary between most IAP copies (Christy et al., 1985). IAPs were highly methylated in oocytes, sperm, and notably remained at the same level in zygotes. Thus, most of the IAP copies in the genome do not undergo active demethylation in the zygote. In this respect IAPs are unusual and behave unlike single copy and Line1 sequences (which are actively demethylated) but like the paternally methylated H19 gene (Olek et al., 1997; Tremblay et al., 1997). IAPs and imprinted genes may, therefore, share sequence elements or epigenetic modifications that protect them from active demethylation in the zygote. There may be further similarities with imprinted genes which are also resistant to passive demethylation, since only a moderate decrease in methylation of IAPs was found in blastocysts (Fig. 1b). Some demethylation had occurred in most individual copies of the IAPs, but there were very few individual copies that were substantially demethylated. This pattern is consistent with active demethylation, but we cannot exclude the possibility that Dnmt1 acts in a highly localised fashion to maintain methylation. However a combination of passive demethylation and de novo methylation could also give rise to these patterns. The relatively homogeneous demethylation between individual IAP copies makes it less likely that there are substantial differences between inner cell mass and trophectoderm cells at this stage. Indeed, ES cells (which are derived from inner cell mass cells) had a very similar level of IAP methylation as blastocysts (Fig. 1b). The same is true of Line1 sequences (Howlett and Reik, 1991).

In primordial germ cells on E11.5, both IAP and 5’ Line1 methylation was fairly high (74%, 65%), which for 5’ Line1s indicate de novo methylation in germ cell precursors after the blastocyst stage (Fig. 2). For 5’ Line1s this was followed by dramatic demethylation by E12.5 and E13.5 (to 32% and 17% for combined male and female cells, respectively). While there was also considerable demethylation from E11.5 to E12.5 in IAPs (40% male, 34% female), no further demethylation occurred to E13.5, and in fact there was an indication of de novo
FIG. 1. Dynamics of methylation changes at repeat loci in germ cells and preimplantation embryos. a: The regions analysed by bisulphite sequencing in Line1 and IAP elements are shown. Filled circles represent CpG dinucleotides present in the regions analysed in Line1 repeats (accession D84391) and IAP long terminal repeats (LTRs) (accession M17551). b: Bisulphite sequencing profiles of Line1 5', Line1 3', and IAP LTR sequences. Individual DNA methylation profiles are shown, with filled (methylated) and open (unmethylated) circles, following bisulphite treatment and amplification of sperm, oocyte, zygote, and blastocyst DNA. Gaps in the methylation profiles represent mutated or missing CpG sites, indicating sequence differences between individual elements. Combined results from a minimum of two independent bisulphite treatments are shown. The overall percentage of methylated CpGs is shown above each group of clones.
methylation at least in the female PGCs (61%). Previous analyses using Southern blotting found considerable de-methylation of IAP genomes in E13.5 (Walsh et al., 1998) and E17.5 female germ cells (Sanford et al., 1987). These results cannot be quantitatively compared to ours because a different technique was used and the probes used also analysed CpGs within the IAP gene body, whereas our analysis focusses exclusively on the LTR, whose methylation state is crucial for IAP expression. The demethylation events from E11.5–E12.5 parallel those observed in single-copy and imprinted genes, but are less extensive. They are likely to occur by active demethylation because at most one round of DNA replication occurs and even that is in the presence of Dnmt1 (Hajkova et al., 2002). The absence of de novo methyltransferases Dnmt3a and b from PGCs (Hajkova et al., 2002) could also contribute to the loss of methylation in the Line1 elements (Liang et al., 2002). The de novo methylation of IAPs on E13.5 may indicate that reprogramming is incomplete and brief, whereas Line1 sequences continue to be demethylated. De novo methylation of Line1s occurs much later during male and female gametogenesis (Howlett and Reik, 1991).

The results of our study on Line1 and IAP methylation at critical developmental stages are summarised in Figure 3. The most significant finding of our study is that IAP elements are substantially resistant to epigenetic reprogramming, in contrast to many other regions in the genome, including other retrotransposons such as Line1 elements, which are substantially demethylated during PGC and preimplantation development. The overall resistance to demethylation of IAPs might be beneficial to the host organism since many of these elements are capable of retrotransposition, which would have detrimental consequences in the form of mutations. Indeed, high-level transcriptional activation of IAP genomes has been seen in Dnmt1 mutant mouse embryos in which IAP genomes are demethylated (Walsh et al., 1998). In some respects IAPs are similar to imprinted genes, in that there is almost no reprogramming in preimplantation embryos, whereas there is some in primordial germ cells. This may explain the parental imprinting effects observed with some endogenous genes whose expression is influenced by nearby IAP insertions (Morgan et al., 1999; Rakyan et al., 2002). There are, perhaps, individual IAP copies that become reprogrammed in primordial germ cells, followed by remethylation in one but not the other germline. However, a very significant fraction of all the IAP genomes remains substantially non-reprogrammed in primordial germ cells. This may explain the inheritance of epigenetic states of genes such as agouti viable yellow (A^vy) in which an IAP LTR insertion leads to ectopic expression of the agouti gene, thus leading to the mutant phenotype. Methylation of the IAP LTR suppresses the mutant phenotype and epigenetic inheritance of state of expression of the A^vy allele has been reported through the female germline. Thus, partial resistance to reprogramming, which could differ between male and female germline and maternal and paternal genomes immediately after fertilisation, is likely to un-
derlie epigenetic inheritance in the case of Avy and perhaps of other alleles as well. In general, partial resistance to reprogramming of IAP genomes would contribute both to epigenetic inheritance and to epigenetic variability between individuals, as proposed previously (Whitelaw and Martin, 2001). It will thus be important to isolate additional individual IAP loci that are inserted close to endogenous genes and study their interactions.

MATERIALS AND METHODS

Sample Preparation
Sperm were collected from C57BL6/JxCBA/Ca (F1) mice and DNA was immediately extracted. Samples of 50–100 oocytes were collected from super-ovulated F1 females. F1 zygotes (50–100 per sample) and F1 blastocysts (3–6 per sample) were collected from super-ovulated females 9–10 h postfertilisation and on embryonic (E) day 4, respectively, following in vivo fertilisation (Hogan et al., 1986). Samples were stored in 1× PBS at −70°C.

Primordial germ cells (PGCs) were obtained from whole embryonic genital ridges at different stages of development by the use of expression from an Oct-4-GFP reporter transgene, as described previously (Hajkova et al., 2002). PGCs were sorted from pooled genital ridges using a MoFlo (Cytomation Bioinstruments, Freiberg im Breisgau, Germany) into batches of 200 cells and stored in 1× PBS at −70°C. Embryonic stages were designated as E, where E0.5 is noon on the day of plug detection.

Bisulphite Treatment
Isolated sperm DNA and cell samples were embedded in agarose (1.5%) and subjected to the bisulphite treatment followed by sequence specific PCR amplifications (Oswald et al., 2000). The PCR products were gel purified using QiaexII (Qiagen, Chatsworth, CA), ligated into pCR-TOPO 2.1 cloning vector (TOPO cloning kit; Invitrogen, La Jolla, CA) and transformed into TOP10 (Invitrogen) Escherichia coli cells. Positive clones were verified by restriction analysis and the products were sequenced using standard methods.

Experiment Validation and Clone Selection
All PCR primers and conditions were tested for unbiased amplification of methylated and unmethylated alleles by comparison to Southern blots (data not shown). Sequenced clones were selected for inclusion in the analysis only if there were no more than three non-CpG located cytosines per sequenced clone that remained unconverted (to uracil) by the bisulphite reaction. This equates to a conversion efficiency of at least 94% for clones included in the analysis. In our experiments it was generally found that no sequences had to be rejected on this basis and the level of bisulphite conversion was extremely high. A few whole treatments were rejected due to there being an overall low level of conversion, or poor PCR amplification that could have been due to a number of causes, including a poor preparation of sodium bisulphite solution or excessive DNA template degradation during the bisulphite treatment. Additionally, those clones that had the same pattern of nonconversion and an identical sequence were rejected because they could have originated clonally. The results shown for each cell type are a combination of at least two individual bisulphite treatments.

Amplification of Line1 5’ Regions (Accession D84391)

Primer sequences. F1: GTTAGAGAAATTGATAGTTTTTGGAATAGG, R1: CCAAAACAAAACCTTTCTCAAACACTATATTACTTTAACAATT.

FIG. 3. Methylation reprogramming of repeat loci during mouse development. The top diagram represents the overall methylation changes that occur during mouse development. Below are representations of the methylation level of Line1 5’ and 3’ regions and IAP LTRs at several developmental time-points. The methylation percentages shown were obtained by combining the methylation profiles from different treatments in bisulphite sequencing experiments and calculating an overall methylation percentage. Methylation percentages are also shown for ES cells and primary embry fibroblasts (PEF); these were obtained by bisulphite sequencing but are not shown in Figs. 1 and 2.
CCCA; 1st PCR (30 cycles): F1/R1; 2nd PCR (30 cycles): F2/R2. PCR conditions: 94°C 3 min, 94°C 1 min, 56°C 1 min (2nd PCR 56°C), 72°C 1 min, 72°C 5 min.

Amplification of Line1 3’ Regions (Accession D84391)

**Primers and conditions.**
- F1: GGAGTTGAGATGAGAGTATTATTTTGAGTG, R1: TTCCAATACTATACCATGATTATGTAG, R2: AATACTATACCAAAAATC
- Primers and conditions.
- F1: TTCCTTACCCAC; 1st PCR (30 cycles): F1/R1; 2nd PCR: F2/R2. PCR conditions: 94°C 3 min, 94°C 1 min, 56°C 1 min, 56°C 1 min (2nd PCR 58°C), 72°C 1 min, 72°C 5 min.

Amplification of IAP LTRs (Accession M17551)

**Primers and conditions.**
- F1: TTGATGGATTATGTAG, R2: AATACTATACCAAAAATC
- Amplification of IAP LTRs (Accession M17551)
- F1: TTGATGGATTATGTAG, R2: AATACTATACCAAAAATC
- Amplification of IAP LTRs (Accession M17551)
- Amplification of Line1 3’ Regions (Accession D84391)

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**LITERATURE CITED**


