Strain-specific transgene methylation occurs early in mouse development and can be recapitulated in embryonic stem cells

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SUMMARY

A murine transgene, HRD, is methylated only when carried in certain inbred strain backgrounds. A locus on distal chromosome 4, Ssm1 (strain-specific modifier), controls this phenomenon. In order to characterize the activity of Ssm1, we have investigated developmental acquisition of methylation over the transgene. Analysis of postimplantation embryos revealed that strain-specific methylation is initiated prior to embryonic day (E) 6.5. Strain-specific transgene methylation is all-or-none in pattern and occurs exclusively in the primitive ectoderm lineage. A strain-independent pattern of partial methylation occurs in the primitive endoderm and trophectoderm lineages. To examine earlier stages, embryonic stem (ES) cells were derived from E3.5 blastocysts and examined for transgene methylation before and after differentiation. Though the transgene had already acquired some methylation in undifferentiated ES cells, differentiation induced further, de novo methylation in a strain-dependent manner. Analysis of methylation in ES cultures suggests that the transgene and endogenous genes (such as immunoglobulin genes) are synchronously methylated during early development. These results are interpreted in the context of a model in which Ssm1-like modifier genes produce alterations in chromatin structure during and/or shortly after implantation, thereby marking target loci for de novo methylation with the rest of the genome during gastrulation.

Key words: DNA methylation, embryonic stem cells, Ssm1, transgenic mice

INTRODUCTION

DNA methylation affords the vertebrate genome a means of encoding information beyond that contained in nucleotide sequence alone. This epigenetic modification, which occurs exclusively to cytosines residing in CpG dinucleotides, is both heritable and reversible. The initial modification of a site is performed by de novo methyltransferase activity, while it is perpetuated through successive cell generations by maintenance methyltransferase activity. Maintenance activity transfers a methyl group to the unmethylated strand of hemimethylated DNA which results from semi-conservative replication. Although the only cloned methyltransferase possesses both de novo and maintenance activities, the latter is 30-50 times more robust (Bestor et al., 1988; Bestor and Ingram, 1983). Additionally, this enzyme co-localizes with replication foci (Leonhardt et al., 1992), arguing that maintenance methylation is its primary role.

Although egg and sperm contribute DNA with gamete-specific methylation patterns, the zygotic genome is substantially cleared of methylation during early cleavage development. Tissue-specific methylation patterns are established during gastrulation, but the means by which specific loci are targeted is not well understood (Kafri et al., 1992; Monk et al., 1987). Modification of the murine transgene HRD provides a model system for targeting of de novo methylation. HRD is methylated when carried in the C57BL/6 (B6) inbred strain background, but not when carried in the DBA/2 (D2) inbred strain background. A single locus on distal chromosome 4 has been identified to control HRD methylation and has been named Ssm1 for strain-specific modifier (Engler et al., 1991a).

Although we have not yet identified endogenous targets for Ssm1 modification, the dramatic dependence of HRD methylation on Ssm1 suggests that its study will provide insights into the mechanisms that determine how specific loci are targeted for methylation, and ultimately, how methylation patterns are established in the genome during development. We describe here the dynamics of HRD methylation during development and discuss how Ssm1 may produce these changes.

MATERIALS AND METHODS

Transgenic mice

The 342 transgenic mouse line carries seven tandem copies of the HRD construct integrated at an undetermined autosomal location (Engler et al., 1991a). The transgenic line is generally carried by crossing hemizygous transgenic D2- or B6-background males to non-transgenic D2 or B6 females, respectively.
Tissue isolation

Postimplantation embryos were generated by mating hemi- or homozygous transgenic D2-background male mice with non-transgenic D2 or B6 female mice. E6.5 and E7.5 embryos were separated into embryo proper andextraembryonic fractions bysectioning them transversely at the level where the posterior amniotic fold meets embryonic ectoderm in E6.5 mice, or where exocoelomic and amniotic cavities meet in E7.5 mice (Reichert's membrane was included in the extraembryonic fraction). Samples were pooled to obtain sufficient quantities for analysis. For E8.5 through E12.5 conceptions, the fetus was separated from extraembryonic membranes (amnion, yolk sac, chorioallantoic placenta, Reichert's membrane). Although the data presented for E8.5 through E12.5 represent individual samples, no fewer than five individuals were examined for each time point with identical results. Component membranes of E13.5 extraembryonic tissues were isolated as described (Hogan et al., 1986) and samples were pooled for analysis.

Embryonic stem cell lines

Pluripotent ES cell lines were derived essentially as described by Martin (1981) with minor modifications. Transgenic E3.5 blastocystcs were obtained by crossing homozygous transgenic D2-background male mice to non-transgenic D2 or B6 female mice. Inner cell masses were obtained by immunosurgery (Solter and Knowles, 1975) and placed into culture. ES cultures were carried on feeder layers of either irradiated primary mouse embryonic fibroblasts or mitomycin C-treated STO fibroblasts in ES culture medium supplemented with LIF.

For in vitro differentiation, feeder-free ES cells (Robertson, 1987) were plated onto tissue culture-grade plastic dishes without LIF. Three days later, colonies were dislodged from the dish and replated onto bacteriological-grade plastic dishes (Lab-Tek, Nunc) for growth in suspension for 7-10 days. For in vivo differentiation, 5×10⁶ ES cells were injected subcutaneously into the flanks of athymic nude female mice (Harlan Sprague Dawley) and allowed to grow into tumors of about 1 cm diameter (3-5 weeks).

Nucleic acid analysis

DNA was prepared as described by Weng et al. (1995). Epididymal sperm DNA was prepared by homogenization in 4 M guanidine isothiocyanate/0.05 M EDTA/0.14 M NaCl and dialysis against TE, pH 8, prior to proteinase K digestion. E6.5 through E8.5 embryo DNA was prepared as described by Miller et al. (1988).

Southern analysis was carried out as described by Weng et al. (1995). The pgtSV transgene probe was a 2.1 kb Kpnl-PstI fragment from pHRD (Engler and Storb, 1987). The Ig heavy chain constant region (Cμ) probe was a 1.1 kb HindIII-PstI genomic fragment spanning exons 1 and 2.

Quantitation of methylation level

All quantitation was performed using a Phosphorimagher and Image-Quant software (Molecular Dynamics). Methylation level was determined by quantitation of two to five bands in each sample lane. A value for each band was calculated from its signal divided by the sum of signals for all bands in a given lane. These values were then weighted according to the molecular mass of their respective bands and summed. This sum was scaled such that the theoretical minimum of methylation was equal to zero and the theoretical maximum of methylation was equal to 100, giving the 'methylation index'.

RESULTS

Strain-dependent methylation is initiated before E6.5 in the embryo proper

Adult D2-background mice carry the HRD transgene completely unmethylated in all somatic tissues (Engler et al., 1991a) and in sperm (see below). When crossed onto the B6 background, however, HRD becomes completely methylated in all adult somatic tissues (Engler et al., 1991a). Since (B6×D2)F1 animals methylate HRD completely, the B6 background is dominant (Engler et al., 1991a); we shall designate the dominant B6 allele as Ssm1² and the recessive D2 allele as Ssm1d. To determine when during development Ssm1 exerts its modifying effect, we examined transgene methylation in D2- and (B6×D2)F1-background E6.5 through E12.5 postimplantation embryos. Embryo proper andextraembryonic tissues were analyzed separately since these tissues vary with respect to methylation at some loci (Sanford et al., 1985). Genomic DNA was digested with the methyl-sensitive restriction enzyme, HpaII and subjected to Southern blot analysis with a transgenespecific probe (Fig. 1). Hybridization near the bottom of the blot (approx. 2 kb) indicates that the transgene is not methylated, while hybridization near the top (approx. 23 kb) indicates that it is completely methylated. Hybridization between these two extremes indicates intermediate, or partial methylation. Equivalent digestion of all samples was verified by rehybridization with a mitochondrialDNA probe (data not shown).

At all stages examined, the embryo proper contains transgenes which are substantially methylated in (B6×D2)F1 samples, but not in D2 samples (Fig. 1). Interestingly, the extraembryonic tissues from all stages displayed a rather uniform pattern of partial methylation, regardless of strain background. This partial methylation is also apparent in some of the embryo proper samples, most notably in those from E6.5. It is likely that incomplete separation of the embryo proper from extraembryonic tissues during dissection resulted in cross-contamination of tissue types (especially at E6.5 and E7.5, see Materials and methods), however, and may explain the partial methylation in the embryo proper samples. Alternatively, the trend toward increasing methylation in F1 embryo proper samples may suggest that the transgene is in the process of being methylated during this time. In either event, strain-specific methylation of the transgene (all-or-none pattern) is restricted to the embryo proper and occurs around the time of gastrulation (E6.5). Also, strain-independent methylation (partial pattern) is found in extraembryonic tissues, occurs before E6.5, and is presumably the result of a process unrelated to Ssm1.

Strain-dependent methylation is restricted to the primitive ectoderm lineage

To address whether partial methylation of the transgene is characteristic of all extraembryonic tissues or only of distinct cell lineages, extraembryonic tissues from E13.5 embryos were subdivided into amnion, visceral yolk sac (vys) mesoderm, vys endoderm, placenta, and parietal yolk sac (pys) endoderm. Since the meso- and endodermal layers of the vys required enzymatic treatment and fine dissection to separate (see Materials and methods), we expected to obtain enriched, but not pure populations. Transgene methylation was analyzed by digestion with HpaII (plus HindIII) and Southern blot (Fig. 2). Methylated transgenes hybridized at 3.6 kb, while unmethylated ones hybridized at 2.0 kb. The 5.0 and 4.0 kb bands result from the integration site 3’ of methylated and unmethylated transgene arrays, respectively. The transgene was donated by the male, so any contaminating maternal tissues would not be detected. The all-or-none methylation which is dependent on
strain background is clearly restricted to fetus (embryo proper), amnion, and vys mesoderm, while the partial methylation which is independent of strain background is observed in vys endoderm, placenta, and pys endoderm. This segregation of methylation phenotypes (all-or-none versus partial) is consistent with a restriction of strain-specific methylation to the primitive ectoderm lineage and of strain-independent methylation to the primitive endoderm and trophectoderm lineages (see Fig. 5) (Hogan et al., 1986). Rehybridization of similar blots with probes for endogenous loci (C_m, H19, DHFR) revealed no differences in methylation between the two backgrounds (data not shown).

Transgene methylation in sperm

The germ cell lineage is not a progenitor of either embryonic endo-, ecto-, or mesoderm and differentiates directly from primitive ectoderm at E7.2 (Lawson and Hage, 1994). Since this time period is approximately when Ssm1 may be acting, we attempted to assess Ssm1 modification in the germ line by examining transgene methylation in sperm DNA. DNAs from six different types of mice (relationships between types are diagrammed in Fig. 3A) were analyzed by Southern blot (Fig. 3B). Ssm1 genotypes were determined by PCR-based RFLP analysis of a closely linked marker (zero recombinants in over 502 backcross progeny; P. Engler, unpublished data). The blot was rehybridized with probes for C_m and DHFR, demonstrating equivalent methylation of endogenous loci in all sample DNAs (data not shown). Quantitation of transgene (Tg) and endogenous gene (C_m) methylation is given below each lane as the ‘methylation index’ (see Materials and methods).

HRD methylation in the germ line is clearly dependent on Ssm1 genotype. It does not, however, parallel that observed in somatic tissues. In sperm, HRD is completely methylated in Ssm1^bb^- mice, partially methylated in Ssm1^bd^- mice, and completely unmethylated in Ssm1^dd^- mice. In somatic cells, HRD is completely methylated in the presence of Ssm1^b^, and completely unmethylated in the absence of Ssm1^b^, except in the case of mouse type 3 (see Discussion). Thus, HRD methylation level differs between somatic and germ tissues only in heterozygous Ssm1^bd^- mice, indicating that Ssm1^-mediated methylation is subject to a tissue-specific dosage effect. Interpretation of this dosage effect in terms of potential mechanisms of Ssm1 action is presented in the Discussion.

ES cells methylate the transgene in a lineage- and strain-specific manner upon differentiation

To examine transgene methylation at developmental stages prior to E6.5, we derived ES lines from transgenic E3.5 blastocysts. Pluripotent ES cells are thought to resemble the E4.5-E5.5 stage and have been demonstrated to recapitulate several events of early development upon differentiation in vitro (Doetschman et al., 1985). Three independent D2-background (awD1-3) and five independent F1-background (awB1-5) lines were derived and analyzed for transgene methylation before and after differentiation. ES cells were differentiated in vitro by aggregation and culture in suspension to form embryoid...
somatic DNA and sperm DNA samples were digested with individual mice representative of types depicted in A. Kidney or tail non-transgenic B6 females. (B) Southern blot of samples from female mice. Mouse type 1 was from an HRD transgenic stock DBA/2) inside circles indicate genetic background of non-transgenic 6) of transgenic male mice; strain designations (B6, C57BL/6; D2, collection of DNA. Numbers inside squares indicate classes (types 1-

We had observed that levels of transgene methylation varied among the five F1-background ES lines before differentiation (Fig. 4A, awB1-5 es lanes). We feel that this instance of partial methylation is distinct from that observed in primitive endoderm lineage tissues since it is strain-specific and changes upon differentiation. Interestingly, we observed this variation to be mirrored exactly at all endogenous loci examined. That is, ES lines which were less methylated for the transgene were less methylated for endogenous loci as well (bottom of Fig. 4A and data not shown). Furthermore, a time course analysis of methyl-

bodies and in vivo by subcutaneous injection into nude mice to form teratomas (Holdener et al., 1994; Robertson, 1987).

We found the transgene to be essentially unmethylated in all three D2-background ES lines before differentiation and to undergo slight de novo methylation upon differentiation in vitro; differentiation in vivo resulted in complete methylation of the transgene (Fig. 4). Quantitation of transgene (Tg) and endogenous gene (Cμ) methylation in Fig. 4 is given below each lane as the ‘methylation index’. As the observed methylation patterns were stable for extended periods in undifferentiated cell cultures (>10 passages, data not shown), we conclude that the HRD transgene undergoes strain-specific de novo methylation in ES cells upon induction of differentiation.

The strain-dependence of transgene methylation was clearly demonstrated after differentiation in vivo. The partial methylation that occurred after differentiation in vitro was unexpected, but can be attributed at least in part to the generation of primitive endoderm lineage tissues. Primitive endoderm gives rise to two derivative lineages, visceral and parietal endoderm (Hogan et al., 1986). Histological analysis of embryoid bodies produced upon differentiation in vitro revealed the presence of substantial amounts of parietal endoderm in both D2- and F1-background cultures, but most prominently in the former (data not shown). Molecular analysis of lineage-specific expression markers (SPARC for parietal endoderm (Mason et al., 1986), α-fetoprotein for visceral endoderm (Becker et al., 1992), and Fgf-5 for primitive ectoderm-derived lineages (Hébert et al., 1991)) in cultures differentiated in vitro revealed copious expression of primitive endoderm lineage markers in all D2 lines, but generally much lower levels in (B6×D2)F1 lines (data not shown). The only (B6×D2)F1 line, awB4, to show substantial expression of a primitive endoderm lineage marker, α-fetoprotein, also demonstrated the most persistent partial methylation after differentiation in vitro (Fig. 4A). These observations support our explanation for partial methylation after differentiation in vitro as being due to primitive endoderm lineage tissues. De novo transgene methylation in ES cultures is therefore likely strictly dependent on strain background in primitive ectoderm lineage tissues.

To examine the methylation status of endogenous loci while the transgene was undergoing lineage- and strain-specific methylation, we reprobed Southern blots shown in Fig. 4. We chose representatives from each of four classes of endogenous loci: DHFR (housekeeping genes), Cμ (tissue-specific genes), H19 and Igf2 (imprinted genes), and L1 (repetitive elements). Methylation over all endogenous loci examined was partial before differentiation and increased after differentiation (data not shown). This de novo methylation occurred in both D2- and F1-background ES lines, demonstrating that the lack of high level transgene methylation in D2-background lines was due to Ssm1 genotype and not to a generalized methylation defect.

Transgene methylation occurs in synchrony with methylation of the rest of the genome

We found the transgene to be essentially unmethylated in all three D2-background ES lines before differentiation and to undergo slight de novo methylation upon differentiation in vitro; differentiation in vivo resulted in no increase in methylation level (Fig. 4). We found the transgene methylation level to vary somewhat between the five (B6×D2)F1-background ES lines, but to be partially in degree in all cases prior to differentiation. After differentiation in vitro, the F1 ES lines all increased their level of transgene methylation substantially, but incompletely; differentiation in vivo resulted in complete methylation of the transgene (Fig. 4). Quantitation of transgene (Tg) and endogenous gene (Cμ) methylation in Fig. 4 is given below each lane as the ‘methylation index’. As the observed methylation patterns were stable for extended periods in undifferentiated cell cultures (>10 passages, data not shown), we conclude that the HRD transgene undergoes strain-specific de novo methylation in ES cells upon induction of differentiation.

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In ES line awB4 during in vitro differentiation revealed that methylation increased progressively and coordinately at all loci, endogenous and transgenic (data not shown). Although these data do not address the question of whether or not Ssm1 plays a role in controlling the methylation of any endogenous loci, the observed synchrony of transgene and endogenous gene methylation suggests that Ssm1-mediated methylation occurs as part of the global de novo methylation of the genome which accompanies gastrulation (Monk et al., 1987).

**DISCUSSION**

Previous work has identified Ssm1, a locus on mouse chromosome 4 that is responsible for directing strain-specific methyl-
ation of the HRD transgene construct (Engler et al., 1991a). In postimplantation embryos it was demonstrated that strain-specific methylation (all-or-none pattern) initiates before E6.5 and is restricted to the primitive ectoderm lineage; strain-independent methylation (partial pattern) occurs in the primitive endoderm and trophectoderm lineages. Analysis of transgene methylation in ES cell cultures revealed that substantial de novo methylation occurs upon induction of differentiation in the (B6×D2)F1-background, but not in the D2 background. While we obtained unambiguous results following differentiation in vivo, interpretation of results following differentiation in vitro required concomitant analysis of tissue lineage representation. Other investigators have similarly observed discrepancies between methylation results obtained after in vivo and in vitro differentiation of ES cells (Feil et al., 1994, 1995). Because we had previously established that the partial methylation phenotype was distinctive of primitive endoderm-lineage tissues, we were able to attribute this discrepancy to differential lineage representation. This lineage analysis also allowed us to determine that endogenous loci underwent de novo methylation with approximately the same kinetics as did the transgene in the F1-background ES cells. These results support our conclusion that Ssm1-directed modification occurs immediately postimplantation in primitive ectoderm, but that subsequent methylation occurs with the rest of the genome during gastrulation.

The similarity of transgene and endogenous gene methylation in (B6×D2)F1-background ES cultures suggests that the embryonic genome was in the process of widespread de novo methylation when the ES lines were derived. Establishment in culture presumably suspended this process, while induction of differentiation allowed it to resume. As genome-wide methylation apparently increases progressively following implantation (Monk et al., 1987), the observed variation in degree of partial methylation implies that less methylated lines are also less differentiated. This idea is consistent with the general observation that some ES lines have broader developmental potential than others, and examination of HRD or endogenous gene methylation may provide a useful means for identifying promising candidate clones for production of high-level contribution chimaeras.

Examination of HRD methylation in sperm revealed a dosage effect of the Ssm1 modifier in sperm, but not in somatic DNA (Fig. 3). Mouse type 3 presented an exceptional case in that it carried no copies of Ssm1P, yet HRD was partially methylated in its somatic DNA (Fig. 3). The representatives of mouse types 3 and 4, however, are actually littermates and both progeny of a mouse of type 2. Since it is clear that HRD methylation is variable in mouse type 2 sperm, we suspect that a hypermethylated transgenic sperm gave rise to mouse type 3 while a hypomethylated transgenic sperm gave rise to mouse type 4. This apparent ability of a methylated transgene in sperm to affect its methylation status in the next generation may liken the Ssm1-mediated modification to those that occur over endogenous imprinted loci (Efstratiadis, 1994).

Several observations allow us to pinpoint Ssm1 activity to a relatively discrete window of development. First, the restriction of strain-dependent transgene methylation to the primitive ectoderm lineage places the modification event after the primitive ectoderm and primitive endoderm lineages have split. Since these lineages are probably distinct by the blastocyst stage (Hogan et al., 1986), we can limit Ssm1 activity to after formation of the blastocyst at E3.5. Second, the fact that Ssm1 genotype can affect transgene methylation in the germ line suggests that Ssm1 is expressed prior to separation of the germ cell lineage from primitive ectoderm at E7.2 (Lawson and Hage, 1994; see below). Third, we have attempted to assess Ssm1 activity in adult tissues by analyzing methylation of the HRD construct after transfection into immortalized B lymphoid cell lines of a genetic background which is capable of methylating the transgene during development (Engler et al., 1991a). In these transfected cells, we have never observed methylation of HRD (Engler et al., 1991b), implying that Ssm1 activity does not persist in the adult. Strain-specific methylation can occur over a transfected target, however, as we have observed differentiation-induced methylation of HRD after it was transfected into embryonal carcinoma cells of appropriate genetic background (data not shown). Ssm1 activity may therefore be limited to a short period of time during peri-implantation development. These conclusions are summarized in Fig. 5.

The observed dosage effect which is unique to the germ line may have arisen via several possible routes. First, modification could have been completed prior to germ allocation, but subsequently lost in some cells during germ cell proliferation. Since the onset of de novo methylation in the germ cells may occur as late as E15.5 (Kafri et al., 1992; Tam et al., 1994), transgene methylation would only be observed in those cells in which the Ssm1 modification had survived. Alternatively, methylation could have occurred soon after allocation with incomplete demethylation occurring later during gametogenesis. Second, the germ line may have departed the primitive ectoderm lineage prior to completion of modification, such that the extent of modification varied among different germ cell precursor clones. The complete methylation observed in sperm from Ssm1P/P mice could have been accomplished by the increased dosage of modifier producing higher density, more stable, or more complete modification prior to germ allocation. The third and only scenario in which Ssm1 activity may not precede germ allocation involves activity in the germ line during gametogenesis independently from its activity in the somatic lineages. While formally possible, we do not favor this scenario as it would require different activities (dosage dependence and independence) from the same genetic locus.

The all-or-none nature of Ssm1-mediated methylation may imply that the determining mechanism utilizes a ‘switch’ whereby only two states exist (methylated versus not methylated) and that a threshold defines the boundary between them. Implementation of such a binary system could be facilitated more appropriately by chromatin structure, which is often characterized as open versus closed, than by methylation density, which can present many intermediate levels given multiple sites. Furthermore, the partial methylation evident in primitive endoderm and trophectoderm (where Ssm1 is presumably not expressed) suggests that methylation level itself cannot serve as a threshold. Mechanistically, chromatin condensation is amenable to thresholds in that chromatin proteins may exhibit cooperative binding to DNA. A detailed model describing the ability of a single locus to regulate large scale changes in chromatin structure has been proposed with regard to modifiers of position effect variegation (PEV) in Drosophila (Tartof and Bremer, 1990). We believe that Ssm1 could likely function as
such a modifier in regulating chromatin structure over the HRD transgene locus. Our observation of dosage effect of the modifier in the germ line lends further support to this analogy (Henikoff, 1992). We believe therefore that chromatin modification may be the primary result of Ssm1 activity. Current efforts toward identification of the Ssm1 gene product and its natural targets in the genome may provide further insight into the mechanism of DNA modification and the role of methylation in development.

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REFERENCES


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