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Differential methylation of a CpG-island concatemer in hemi- and homozygous transgenic mice

Glenn Gundersen, Anne-Brit Kolstø and Hans Prydz

The Biotechnology Centre of Oslo, University of Oslo, Oslo, Norway

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Methylation-free islands (MFIs), clusters of non-methylated CpG-dinucleotides in mammalian genomes, are associated with a majority of studied genes. By which precise mechanism they maintain their unmethylated status is unknown. The behaviour of transgenic MFIs may contribute to unveil this enigma. We have generated a high-copy number transgenic line with the MFI from the murine *Thy*-1.1 allele. A stable, minor fraction of this otherwise non-methylated DNA became completely methylated in all adult tissues tested. Furthermore, individuals homozygous for the transgene showed a significantly higher proportion of methylated copies compared to the hemizygous state. These findings support the hypothesis that a limited pool of *trans*-acting factors are involved in maintaining the hypomethylated state.

Methylation-free island; CpG island; *Thy*-1.1 gene; Transgenic mice

1. INTRODUCTION

Methylation-free islands (MFIs), also referred to as CpG islands or HTF islands, are stretches of DNA (0.5–2.0 kb) which are distinguished from bulk DNA by a high G+C content (>50%), no suppression of the dinucleotide 5′-CpG-3′ (i.e. the occurrence of CpG roughly equals the number of GpC doublets), and that the cytosines in CpG are unmethylated [1,2]. Methyl-sensitive restriction enzymes like *Hpa*II (cleaves 5′-CCGG-3′) are frequently used to diagnose the unmethylated state of island DNA. All housekeeping genes, and an increasing number of genes with a restricted expression pattern are found to be associated with such islands, which usually comprise the proximal end of the genes including sequences for transcription initiation and the first exon/intron [2].

In vitro studies with mammalian DNA methylases (MTase) have suggested that MFIs are refractory to methylation, despite their high density of 'available' CpGs ([3,4], unpublished results). However, methylation of CpG islands has been demonstrated with genes on the inactive X-chromosome in mammalian females and in autosomal genes in cultured cells [5–7], indicating that other factors than island DNA per se must be involved in determining the correct pattern of modification. CpG island DNA adopts an alternative chromatin structure and proteins specifically binding to methylated CpGs have been described [8,9]. DNA-binding factors with specificity towards unmethylated CpGs have, however, not been reported. Previously [10], using mice transgenic for a 9.2-kb mouse–human *Thy*-1.1 construct containing the entire 1.6-kb MFI Kolstø et al. were able to conclude that the island region remained hypomethylated in every tissue of three different lines of mice (except in extra-embryonal fetal tissue), regardless of whether *Thy*-1.1 was expressed or not. If one assumes random insertion of the transgene these observations would make a position effect less likely, i.e. the MFI does not remain unmethylated because of some special location in the genome. The most likely explanation of these observations was that the signal(s) for absence of methylation directly or indirectly (via binding of *trans*-acting factors) had to be present within the transgene. Here we report differential methylation of a multicopy tandem repeat of the well characterized MFI from the murine *Thy*-1.1 allele in hemi- and homozygous transgenic mice. A tenfold increase of methylated copies were seen in homozygous individuals compared to hemizygous genomes - a finding that supports the existence of a limited pool of *trans*-acting factors which may be implicated in maintaining the unmethylated state of CpG islands. This difference cannot be explained by a position effect.

Abbreviations: bp, base pairs; kb, kilobases

Correspondence address: H. Prydz, Biotechnology Centre of Oslo, University of Oslo, P.O. Box 1125, Blindern, N-0316 Oslo 3, Norway. Fax: (47) (2) 694130.

2. MATERIALS AND METHODS

2.1. *Materials*

Highly concentrated restriction enzymes (>40 U/μl) were obtained from Bethesda Research Laboratories (BRL), New England Biolabs and Promega. Isotopes were from Amersham.

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2.2. *Preparation of transgene*

A 1339-bp *Bgl*II-fragment containing the central region of the previously described 1.6-kb MFI associated with the murine *Thy*-1.1 gene was isolated from the 9.2-kb mouse–human transgene construct published by Kolstø et al. [10], and subcloned as a *Hin*dIII-fragment into pUC18 vector. DNA for microinjection was separated on a low melting agarose gel (0.7%), purified on NACS ion-exchange columns (BRL) and recovered in 10 mM Tris-HCl pH 7.4/ 0.1 mM EDTA at 1 μg/ml.

2.3. *Transgenic mice*

Thy-1.2 mouse strains, C57Bl/6J (♀) and (C57Bl/6J × DBA/ 2J)F1 (♂) were obtained from Bomholt, Denmark, and were used to produce fertilized eggs for pronuclear microinjection and pseudopregnant recipients for egg implants [11].

2.4. *Southern analysis*

Genomic DNA digested to completion by the use of 10- to 20-fold excess of enzyme (monitored by cleavage of internal lambda DNA controls) was fractionated on 1.0–1.5% agarose gels (TAE-buffer). DNA in gels was denatured and transferred to nylon membranes (Zetaprobe, Bio-Rad) by capillary blotting in 0.4 N NaOH. High specific activity radiolabelled DNA probes were prepared by oligolabelling [12]. Filters were hybridized in a SSC/SPEP solution (equivalent to 1×SSC) [13] at 68°C for 16–20 h, and washed 2–3 h in 1×SSC/ 1% SDS at 68°C. Relative densitometric values were obtained from scanning linearly exposed films at 600 nm using a Shimadzu CS-9000 instrument. Values obtained were critically dependent on the amounts of DNA initially loaded on gels, efficient DNA transfer to filters and hybridization stringency (low background). The methylated fraction of the monomeric transgene (1339 bp *Hin*dIII-fragment) was calculated from the densitometric ratio: [Tg (*Hin*dIII+*Hpa*II)] : [Tg (*Hin*dIII)]. The use of equal amounts of DNA in these experiments was ascertained by using the same preparation of DNA (but different DNA preps for each experiment), and by visually comparing their fluorescence at 260 nm in the presence of EtBr after electrophoresis.

3. RESULTS

3.1. *Transgenic mice*

After subcloning, the 1339-bp MFI alone (without adjacent non-island DNA, Fig. 1a) was injected to produce new lines of transgenic mice to characterize its ability to resist methylation. The endogenous *Thy*-1.2 MFI and the allelic *Thy*-1.1 transgene shared 96% sequence identity [14], allowing a comparison of these bands using a *Thy*-1.1 probe for estimating the copy number. Scanning autoradiograms from Southern blots of genomic DNA, a founder mouse gave a ratio of approximately 2:55 between the endogenous and the transgene (Fig. 1b, lane 2). Further breeding gave homozygous individuals with the expected ratio 1:55 (2:110). Pulsed field gel electrophoresis of leukocyte DNA from this transgenic line revealed one major transgene-specific band of 75 kb (consistent with a tandem of 55 copies), after cleavage with restriction enzymes lacking recognition sites within the MFI — indicating that integration had occurred at a single locus (not shown). A low-copy number founder (3 copies) which failed to transmit the exogenous DNA to its offspring proved to be mosaic for the transgene, and therefore not obtainable in the homozygous state (Fig. 1b, lane 3).

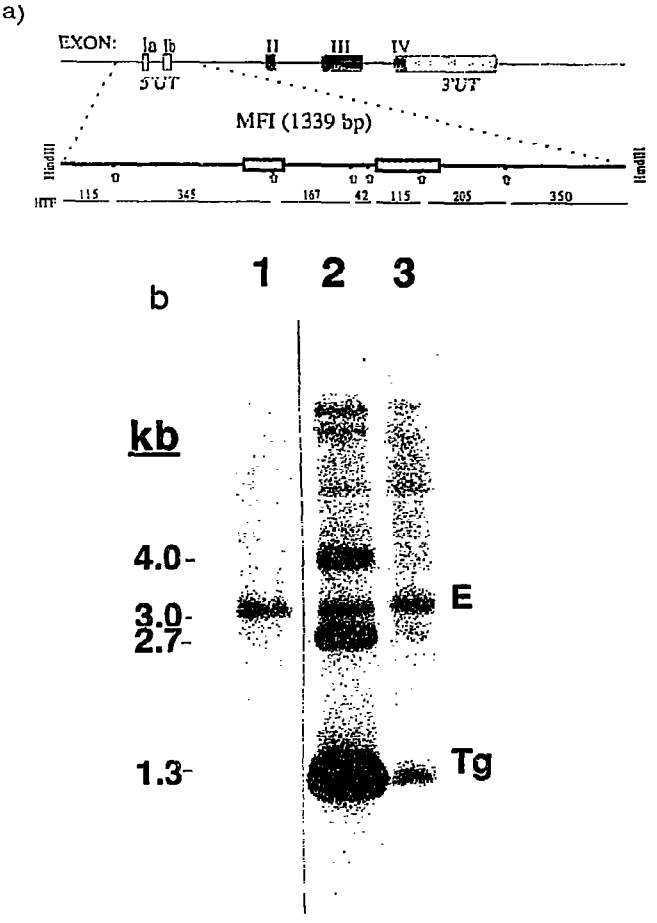

Fig. 1. Southern blot of transgenic mice. (a) Restriction map of the 1339 bp *Hin*dIII fragment from the murine *Thy*-1.1 MFI (EMBL accession no. X 17683) used as a transgene. Boxes designated 1a/1b are the alternative first exons with multiple transcriptional start sites [22]. Filled exons are translated. *Hpa*II sites are marked by arrows, and the resulting *Hpa*II-tiny fragments (HTF) are indicated below (in basepairs). (b) Southern blot of *Hin*dIII-digested genomic tail DNA from a non-transgenic C57Bl/6 mouse (lane 1), male founder transgenic (no. 289) with high copy number (lane 2), female mosaic founder (no. 290) with low copy number (lane 3). E, indicates the 3.0-kb endogenous *Thy*-1.2 fragment. Tg, indicates the 1339-bp MFI-copies with intact *Hin*dIII termini. Bands at 2.7 and 4.0 kb are described in text. The entire MFI was used as a probe.

The transgene-dependent *Hin*dIII-fragments of 2.7 and 4.0 kb (Fig. 1b, lane 2) always appeared (in spite of optimal *Hin*dIII conditions), suggesting that some *Hin*dIII-sites in the concatemer had been destroyed, thus giving rise to dimers (2×1339 bp) and trimers (3×1339 bp) of the transgene.

3.2. *Methylation in hemi- and homozygous mice*

The use of methylation-sensitive restriction enzymes showed that the majority of these transgenic MFI copies were left unmethylated at all sites and in all tissues tested from adult mice. However, analyzing hemi- and homozygous offspring (Fig. 2) we observed that about 20% of the total 110 copies in homozygous off-

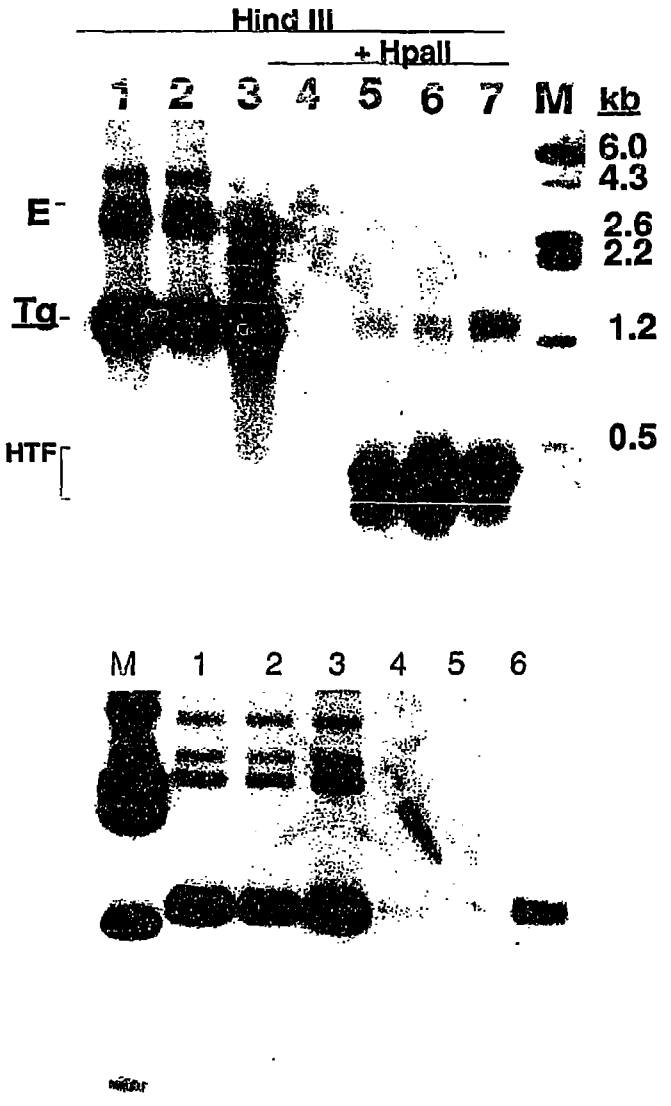

Fig. 2. Methylation analysis of hemi- and homozygous transgenic mice. Southern blot of genomic DNA, digested with enzymes as indicated. Lanes 1,2 and 5,6: 4.0 μg DNA from two hemizygous mice (no. 342 and no. 344). Lanes 3 and 7: 2.0 μg of homozygous DNA (no. 379). Lane 4: 4.0 μg DNA from a non-transgenic C57BL/6 control. M, molecular weight marker; pBR325 DNA digested with *Eco*RI, *Apa*LI, *Pvu*I and *Bgl*I and fragments adjusted to equal amounts of DNA. E, endogenous *Thy*-1.2 MFI-fragment. Tg, excised monomers of transgene. The weak Tg bands after *Hpa*II-digests show the uncleaved methylated copies (lanes 5–7). HTF indicates digested unmethylated island DNA. The gel was 1.2% agarose.

Addendum Fig. 2. Because of suboptimal transfer of DNA during the capillary blotting procedure in the mid-upper section of the picture (Fig. 2), the 3 characteristic bands of 2.7, 3.0 and 4.0 kb (as seen in lanes 1 and 2) do not appear as they should in lane 3. The lower blot of the same DNA shows that this homozygous DNA *does* have these bands (and therefore is not structurally altered compared to the hemizygous state). Lanes 1,2 and 4,5 contain hemizygous DNA (no. 342, 344), while lanes 3 and 6 contain homozygous DNA (no. 379). In this case, however, the amount of DNA in the hemi vs. homozygous lanes were not 2:1 (as in Fig. 2) and the HTF fraction had run out of the gel.

Table I

Methylated fractions of the multi-copy MFI transgene in hemi- and homozygous mice

	Hemizygote	Homozygote
	0.031*	0.176*
	0.040*	0.182
	0.026	0.184
	0.020	0.215
	0.032	0.261
Mean:	0.029	0.207

*Ratios calculated from Fig. 2. The other ratios were calculated from similar blots.

spring were fully methylated de novo as evidenced from resistance to cleavage by *Hpa*II. In hemizygous offspring, carrying 55 copies, the insert was almost completely cleaved by these enzymes and thus had remained (or become) hypomethylated. In repeated experiments on DNA from several transgenic tissues from different individuals, less than 4% (average 2.9%) of the copies in hemizygous mice remained uncleaved (methylated) whereas 18–26% (average 20.7%) were not cleavable in homozygous mice (Table I). We stress that this methylation apparently was an 'all or none' phenomenon in that either a MFI-monomer was methylated at all *Hpa*II-sites and thus uncleaved [showing only *one* band (=1339 bp)] or unmethylated and cleaved accordingly. In other words, the modification apparatus seemed to respect the boundaries of the incorporated transgene. At the resolution of our methods, the island from the endogenous *Thy*-1.2 allele remained unmethylated in all cases.

No further imprinting effect [15] (a gamete-of-origin effect on methylation) was observed when hemizygous offspring from matings between homozygous male transgenics and female Balb/c or DBA/2J, or the opposite crosses between homozygous female transgenics and male Balb/c or DBA/2J, were investigated (not shown).

Interestingly, the transgene in the low-copy mosaic founder mouse with 3 copies in the hemizygous state was fully cleavable, i.e. no methylation at CCGG-sites (*Hpa*II) were detected at the sensitivity of the methods (Fig. 3). The HTF-fraction was seen after prolonged exposure. Thus, in this case the transgene seemed to have been efficiently protected from de novo methylation, or alternatively, demethylated in early development after a prior methylation.

4. DISCUSSION

The three most obvious hypotheses for explaining the fidelity of obtaining and/or maintaining the unmethylated state of MFIs are: (i) the islands are protected by transacting factor(s) preventing the MFI from being an accessible substrate for the MTase [10,16]; (ii) a precise

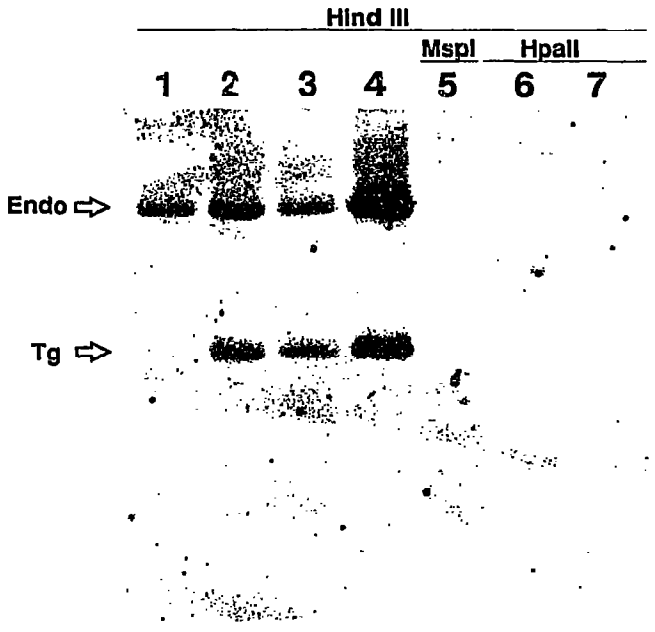

Fig. 3. Methylation analysis of transgenic tissues from a low-copy mosaic founder (no. 290). Genomic DNA from various tissues was digested with the enzymes indicated. Lane 1, liver (no transgene); lanes 2,5 and 6, lung; lanes 3 and 7, adipose tissue; and lane 4, striated muscle. 5 μg DNA for each lane was loaded on a 1% gel.

and well regulated demethylating activity exists to erase modified cytosines in CpG islands [17,18]; (iii) the G+C and CpG rich MFIs assume a conformation that renders them less accessible to the MTase. Such *cis*-acting protection might be due to the MFI assuming a Z-DNA conformation, an altered access/binding of the enzyme in the minor groove caused by the high frequency of G+C [3,4], or the possibility to adopt higher order complex structures (e.g. H-DNA) [19]. This latter hypothesis predicts that *cis*-acting sequences may be expected to be constitutively active in all cases, which is not the case for CpG islands on the inactive X-chromosome [5,6]. Since evidence supporting each of these potential mechanisms has been described (the latter only in vitro), a complex interplay between them may well be the situation.

A prediction from the two former hypotheses would be that it may be possible to exhaust the pool of protective transacting factor(s) which normally lead(s) to either permanent protection or subsequent demethylation. Our results support this presumption: (1) a mosaic founder with 3 copies of the MFI-transgene was unmethylated at all *Hpa*II-sites in the transgene; (2) doubling the number of copies from the hemizygous to the homozygous state (55 to 110) increased the number of methylated copies of the transgene tenfold from 1–3 to 20–30 respectively, indicating that the island DNA indeed had become methylated in vivo and therefore is not totally resistant per se. The fact that doubling the copy number from 55 to 110 only leads to an increase from 3% to 21% of methylated islands (one might expect >50% given a protective threshold <55 copies), may be explained by the ability of the cells to increase their level of protective agent(s) when required.

Methali et al. have reported similar observations using the promoter region (MFI) from the murine housekeeping gene 3-hydroxy 3-methylglutaryl CoA-reductase (HMGCoA-reductase) fused to the chloramphenicol acetyltransferase reporter gene [20]. As in its endogenous counterpart, no methylation was detected at copy number=2. However, with increasing copy number of the transgene the unmethylated island became smaller (at 10 copies) and progressively became methylated internally in the concatemer (at 22, 45 and 260 copies). A *cis*-acting effect via structural alterations based on different insert lengths cannot be entirely excluded in these experiments. Our results definitely exclude such effects, since the physical structure of the concatemer must be assumed to be identical on the two allelic chromosomes. The apparent 'all-or-none' protection seen in our study may reflect a highly cooperative action of nuclear factors, while a different mechanism might exist for the CpG island associated with the HMGCoA-reductase gene. The dosage-dependent degree of methylation suggests very strongly the existence of factor(s) acting in *trans* to prevent MFI methylation.

Several intact (transcribable) CpG island-containing transgenes have been shown to adopt the same methylation pattern as their endogenous counterparts in the animal [10,17]. The 'minimal' MFI used in the present study lacks its normal flanking DNA sequences. Their absence may be of importance for the decreased fidelity of protection.

Since there are approximately 30000 CpG islands in a haploid mouse genome [21], it appears likely that such *trans*-acting protective factors would be to a certain degree sequence-dependent. If the factor(s) were totally indiscriminate with regard to MFI specificity, it is difficult to imagine that the pool of such factors should be exhausted by increasing the number of islands, e.g. from 60000 to 60110. On the other hand, a new family of about 30000 MFI-specific binding proteins seems equally unlikely; the solution may lie in a combinatorial approach. This may also explain why the search for such proteins so far has been unsuccessful.

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