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Regulation of DNA methylation of *Rasgrf1*

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Abstract

In mammals, DNA is methylated at cytosines within CpG dinucleotides. Properly regulated methylation is crucial for normal development^{1,2}. Inappropriate methylation may contribute to tumorigenesis by silencing tumor-suppressor genes^{3–10} or by activating growth-stimulating genes^{11–13}. Although many genes have been identified that acquire methylation and whose expression is methylation-sensitive^{14,15}, little is known about how DNA methylation is controlled¹⁶. We have identified a DNA sequence that regulates establishment of DNA methylation in the male germ line at *Rasgrf1*. In mice, the imprinted *Rasgrf1* locus is methylated on the paternal allele within a differentially methylated domain (DMD) 30 kbp 5' of the promoter. Expression is exclusively from the paternal allele in neonatal brain¹⁷. Methylation is regulated by a repeated sequence, consisting of a 41-mer repeated 40 times, found immediately 3' of the DMD. This sequence is present in organisms in which *Rasgrf1* is imprinted¹⁸. In addition, DMD methylation is required for imprinted *Rasgrf1* expression. Together the DMD and repeat element constitute a binary switch that regulates imprinting at the locus.

We generated mice in which the *Rasgrf1* repeats were replaced by a single loxP site. This allele of *Rasgrf1* was designated *Rasgrf1^{tm1Pds}* (Fig. 1). To evaluate whether the mutation affected methylation of the DMD, we subjected tail DNA from mice inheriting the repeat deletion from their mother or father to Southern-blot analysis using *PstI* and the methylation-sensitive enzyme *NotI*. A single *NotI* site is present in the DMD, and its methylation state in DNAs from all somatic tissues tested is diagnostic of the methylation state of the DMD¹⁷. *PstI*-digested DNA from wildtype mice (*Rasgrf1^{+/+}*) gave rise to an 8.0-kbp band on Southern blots hybridized to the probe shown in Fig. 2a. The unmethylated maternal fragment was further cleaved by *NotI*, generating a 2.8-kbp band (Fig. 2a,b) as previously reported^{17,19}. In heterozygous mice inheriting the mutated allele from the mother (*Rasgrf1^{tm1Pds/+}*), there was no change in the methylation status of either allele. *PstI* digestion generated a 3.0-kbp fragment from the mutated maternal allele and an 8.0-kbp fragment from the wildtype paternal allele. Digestion with both *PstI* and *NotI* further cleaved the 3.0-kbp maternal fragment to 2.8 kbp. In contrast, animals bearing a paternally transmitted repeat deletion (*Rasgrf1^{+/tm1Pds}*) lost methylation of the paternal allele. *PstI* digestion of DNA gave rise to an 8.0-kbp wildtype maternal allele and a 3.0-kbp mutated paternal allele band. Both were reduced to 2.8 kbp after *NotI* digestion, indicating that neither allele was methylated at the *NotI* site (Fig. 2b). Bisulfite sequencing revealed that loss of methylation was extensive throughout the DMD on the mutant

paternal allele (Table 1). This indicated that the *Rasgrfl* repeats are needed for paternal allele-specific DNA methylation patterns seen at the locus.

The loss of paternal allele-specific methylation may have resulted from a failure to establish the normal paternal methylation pattern in the male germ line or failure of the established pattern to be maintained in the somatic tissue of the progeny. To distinguish between these possibilities, we assayed the methylation status of the *Rasgrfl* DMD in sperm DNA from males heterozygous for the repeat mutation, using three separate methods. The first assay was a Southern blot using the same digests and probes used in Fig. 2. Results showed a substantial reduction in methylation of the mutated allele (Fig. 3a). Previous studies have suggested that although the *NotI* site methylation state is diagnostic for paternal allele-specific methylation in somatic tissue, the germline methylation imprint may be more accurately reflected by the methylation of the five *HhaI* sites within the *Rasgrfl* DMD¹⁹. We therefore assessed the methylation status of these sites in sperm DNA using PCR reactions specific for the mutated and wildtype alleles, with primers that spanned all five *HhaI* sites. Fig. 3b shows the mutant and wild-type loci and the location of primer pairs used in this analysis. Sperm DNA was digested with *HhaI* or left undigested, before PCR analysis, using the two allele-specific assays. We carried out a third control PCR reaction using a primer pair that spanned a region of *Rasgrfl* lacking *HhaI* sites. In all DNA samples, the wildtype allele was amplified using primers P1 and P2 (Fig. 3c, upper gel), indicating that the *HhaI* sites were detectable in the methylated state on the wildtype alleles. When we amplified uncut DNA from sperm of wildtype or heterozygous mice using mutated allele-specific primers P3 and P4, a band of the predicted size was generated only in the reactions containing heterozygous DNA, indicating that the product was indeed allele-specific. However, the band was substantially diminished in reactions containing sperm DNA from heterozygotes that had been pre-digested with *HhaI*. This indicated that one or more of the five *HhaI* sites were hypomethylated at a high frequency on the mutated allele. This result was seen regardless of whether the mutated allele was paternally or maternally transmitted (data not shown for maternal transmission). An internal control PCR reaction using primers P5 and P6 showed that band loss was not due to a failure in the amplification reaction. We carried out bisulfite sequencing to evaluate whether the loss of methylation affected just one or more of the five *HhaI* sites and to determine whether CpGs other than those in the *HhaI* and *NotI* sites were affected. The results indicated that loss of methylation on the mutated allele of sperm DNA affected CpGs across the entire DMD and not just those in the *NotI* and *HhaI* sites (Table 1). In addition, the disruption of DMD methylation was comparable in both somatic and sperm DNA templates. These three independent means of analysis showed that loss of methylation caused by the repeat deletion was a common event affecting many CpG residues within the DMD at a time when methylation of the locus was normally established in the male germ line.

Rasgrfl is expressed predominantly from the paternal allele in many tissues; however, expression in neonatal brain is exclusively from the paternal allele¹⁷. To determine whether the *Rasgrfl*^{tm1Pds} mutation had any effect on imprinted expression of *Rasgrfl*, we analyzed RNA from the brain of neonatal progeny of mutant animals crossed with wildtype C57BL/6 or PWK mates. The cross facilitated separate expression analysis of the two parental *Rasgrfl* alleles because of an exonic polymorphism that exists between 129S4/SvJae, the strain in which the mutation was made, and PWK¹⁷. Maternal transmission of the repeat deletion did not affect the normal expression pattern of the locus—expression was still from the paternal allele only on all strain backgrounds in amounts comparable to those seen in wild-type mice (data not shown). However, paternal transmission of the *Rasgrfl*^{tm1Pds} allele resulted in substantial silencing of the normally active allele in heterozygous progeny of C57BL/6 mothers (Fig. 4, +^{B6}/–). Notably, the silencing of the mutated paternal allele was variable in progeny of PWK mothers (Fig. 4, +^{PWK}/–). In those animals, *Rasgrfl* expression levels ranged from those seen in +^{B6}/– mice (fourth panel from the top) to amounts seen in +^{B6}/+ and +^{PWK}/+

animals (bottom panel). In $+^{PWK}/-$ mice with wildtype levels of expression, the DMD showed extensive methylation despite the presence of a paternally transmitted *Rasgrfl*^{tm1Pds} allele, whereas in animals with substantially silenced *Rasgrfl* expression the paternal allele was unmethylated (Fig. 4, right panel). This indicated that it is the methylation state of the DMD rather than the presence of the repeats that has the most direct influence on expression of the locus, ruling out the trivial explanation that the repeats contain a promoter or enhancer element needed for *Rasgrfl* expression. Moreover, the *Rasgrfl* DMD behaves like a methylation-sensitive enhancer-blocking element, as is the case for the DMD at *H19/Igf2* (refs ^{12,13,20}; B.J. Yoon *et al.*, unpublished data). The variable methylation seen among progeny of PWK mothers also suggested that there are strain-specific modifiers of the methylation phenotype. Analysis of the nine $+^{PWK}/-$ progeny revealed three animals with residual methylation on the mutated paternal allele (data not shown). Methylation analysis of a much larger number of mice on a mixed C57BL/6 $\times$ 129S4/SvJae background revealed no such variability in methylation of the mutated paternal allele. Mice on this background were used in the experiments reported in Fig. 3.

The presence of transgene repeats has been correlated with changes in local DNA methylation and gene expression in diverse systems²¹⁻²³. The repetitive nature of the *Rasgrfl* repeats, the presence of 39 CpGs within the 1,611-nt repeat region, or other structural or sequence motifs may be required for the *Rasgrfl* repeats to regulate establishment of DNA methylation in the male germ line. These motifs may serve as sites of interaction with collaborating *trans*-acting factors that themselves dictate local methylation patterns. The variable loss of methylation and correlation between methylation and expression seen in mutant progeny of PWK mothers was reminiscent of previous studies of *H19* imprinting²⁴ and may be due to strain-specific differences in loci encoding such factors. Expression of these factors must be specific to the male or female germ line for *Rasgrfl* and other imprinted loci to undergo allele-specific methylation.

Studies of methylation regulation at other loci have been reported. The maternally-expressed *H19/Igf2* locus contains a DMD that is hypermethylated at CpGs within and adjacent to the promoter on the paternal allele²⁵. A targeted deletion of the DMD abolished paternal allele-specific DNA methylation and *Igf2* silencing when transmitted paternally²⁶. As is the case for the *Rasgrfl* DMD, the *H19* DMD is an enhancer blocker (refs ^{12,13}; B.J. Yoon *et al.*, unpublished). However, in contrast with the *Rasgrfl* repeat mutation, which caused loss of methylation establishment, the *H19* mutation interfered with the maintenance or persistence of methylation patterns after their normal establishment. Although it is not known how methylation is established at the *H19* locus, repeats seem not to be required. A 461-nt G-rich repetitive element at *H19* was shown to be dispensable for establishment of paternal-allele methylation of an *H19* transgene²⁷. It is possible that regulation of transgene methylation differs from methylation of endogenous loci, that redundant mechanisms regulate methylation at *H19* or that mechanisms regulating methylation at imprinted loci vary. These possibilities are not mutually exclusive.

At the *Igf2r* locus, a 3-kbp intronic sequence regulates methylation of the maternal allele before fertilization²⁸. Transient transgenesis experiments identified a 6-nt allele-discrimination sequence (ADS) that protects the paternal allele from methylation and an 8-nt *de novo* methylation sequence (DNS) that facilitates methylation of the unprotected maternal allele. The ADS and DNS sequences interact with factors present in androgenetic and gynogenetic ES cells, respectively¹⁶. Dot-plot analysis shows that the *Igf2r* imprinting control region contains some repeated elements; however, it is not clear if these are needed for the ADS or DNS to function. Although a single copy of the 6-nt ADS is found within the *Rasgrfl* repeats, it is not likely to be functionally significant, as in *Igf2r* transgenes the ADS sequence protects

the paternal allele from methylation. Because the paternal allele is methylated at *Rasgrfl*, the ADS cannot be protecting the locus from methylation.

Methods

Generation of mutant mice

We prepared the *Rasgrfl*-targeting construct (pBJR3) which replaced a 2-kbp repeat-containing fragment with a *PGKneo^r* cassette flanked by *loxP* sites as follows. The 5' vector arm consisted of a 2-kbp fragment immediately 5' of the repeat that we PCR-amplified using *Rasgrfl* reverse and forward primers derived from the plasmid pSPL3, which contained the 5' *Rasgrfl* fragment. The reverse primer modified four nucleotides of the wildtype *Rasgrfl* sequence to generate new *PstI* and *HindIII* sites immediately 5' to the repeat. We cloned the *PstI* fragment from the amplification into the *PstI* site of pBluescriptII (Stratagene). The 5' arm, isolated as a *SacII*-*HindIII* fragment from the resulting clone, was inserted into the *SacII* and *HindIII* sites of pTK-NEOF which carried the *loxP-neo^r-loxP* and *HSVTK* markers (D. Lam and P. Aplan, National Cancer Institute). We inserted the 3-kb homologous arm, consisting of an *EcoRV*-*SmaI* fragment located 3' of the repeats, into the *SaI* site of pTKNEOF to generate the pBJR3 targeting vector. We carried out ES cell culture and blastocyst microinjections according to the standard protocols. We excised the *neo^r* selectable marker by crossing germline progeny with *Zp3Cre* transgenic mice²⁹.

Sperm DNA isolation

We prepared sperm DNA from 8-wk or older heterozygous male mice inheriting a mutated allele from their fathers, mothers or wildtype control littermates by allowing sperm to swim out from the cauda epididymis into 500 μ l phosphate-buffered saline (PBS). We added SDS (1.5 μ l of 10% w/v) and incubated the sperm at room temperature for 15 min. Cells were centrifuged (3 min, 16,000 $\times$ g), resuspended in 100 μ l PBS, diluted with an additional 900 μ l PBS and 100 μ l β -mercaptoethanol (1 M), and then incubated at 37 °C for 1 h. Cells were centrifuged (3 min, 16,000 $\times$ g), resuspended in 100 μ l PBS, centrifuged again (5 min, 16,000 $\times$ g), resuspended in 200 μ l PBS, diluted with an equal volume of 2 $\times$ lysis buffer (200 mM Tris pH 8.5, 200 mM EDTA, 400 mM NaCl, 2% SDS) and proteinase K added (400 μ g/ml⁻¹), followed by an overnight incubation at 55 °C with shaking. We then isolated DNA by phenol chloroform extraction and ethanol precipitation.

Methylation analysis

We carried out assays for *NotI* site methylation using Southern-blot hybridization as described¹⁷ and assays for *HhaI* site methylation in sperm as described¹⁹, with modifications. Using sperm DNA that was digested with *HhaI* or left uncut, we carried out PCR amplification. PCR product sizes were as follows: P1–P2, 263 bp; P3–P4, 228 bp; P5–P6, 148 bp.

We carried out bisulfite sequencing as described³⁰ with some modifications, using freshly prepared solutions. Briefly, 1.0 μ g of sheared DNA in 50 μ l water was denatured by addition of 5 μ l fresh 3 M NaOH, then incubated at 37 °C for 10 min. After adding 30 μ l 10 mM hydroquinone and 520 μ l 3 M sodium bisulfite, we allowed deamination to proceed at 50 °C for 16 h. We purified treated DNA using a Qiaquick gel extraction kit according to the manufacturer's instructions (Qiagen) and eluted DNA from the kit column in 50 μ l elution buffer. The bisulfite reaction was completed by the addition of 5 μ l fresh 3 M NaOH followed by a 5-min incubation at RT. We purified DNA again using a Qiaquick gel extraction kit and eluted it in 30 μ l. For PCR, we amplified 1 μ l of DNA (primers available upon request). We gel-purified all PCR products, cloned them using a TA or TopoII cloning kit (Invitrogen) and then sequenced individual clones.

Expression analysis by RT-PCR

We measured expression of *Rasgrfl* using a quantitative PCR assay. We prepared cDNA using oligo(dT) primers and 0.5 µg total RNA. A fraction of this material (10%) was serially-diluted and used for PCR. A reaction mixture containing four primer pairs was prepared and added to each cDNA dilution. One set of the primers amplified a 378-bp product that spanned a *Rasgrfl* intron–exon boundary, and another set amplified a 128-bp product specific for *Rpl32* which served as an internal control.

PCR primers

Available upon request.

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Fig. 1.

Targeting the *Rasgrf1* repeats. **a**, The wildtype *Rasgrf1* locus, targeting vector and correctly targeted locus are shown. Open rightward-pointing triangles represent the *Rasgrf1* repeat element. The rightward-pointing arrow and black rectangle to the right of it represent the transcriptional start site and first exon of the gene, respectively, which are located approximately 30 kbp 3' of the repeats. Filled rightward-pointing triangles represent *loxP* sites on the targeting vector and targeted allele. **b**, Southern-blot hybridization of *Pst*I-digested DNA from mice using the probe shown in **a** produced band sizes of 8.0 kbp and 3.0 kbp for the wildtype and mutated alleles, respectively. **c**, PCR analysis was carried out on tail DNA from progeny of heterozygous male *Rasgrf1^{tm1Pds}* mice crossed with females containing (+) or lacking (-) the *Zp3cre* transgene. Primers, depicted as arrowheads numbered P1–P5 in **a**, detected the *neo^r* cassette (left panel, P4 and P5), the wildtype *Rasgrf1* allele (WT; right panel, P1 and P2) and the *Rasgrf1^{tm1Pds}* allele (Δ by Cre rightpanel, P2 and P3). Restriction sites are *Pst*I (P), *Bam*HI (B), *Not*I (N), *Hind*III (H) and *Eco*RV (E).

Fig. 2. *NotI* site methylation is lost upon paternal transmission of the *Rasgrf1* repeat mutation. **a**, The wildtype maternal and mutated paternal alleles are shown along with the possible *PstI/NotI* (P/N) restriction fragments arising from methylated or unmethylated *NotI* sites. **b**, Tail DNA from wildtype (+/+) or heterozygous mice with a maternally (-/+) or paternally (+/-) transmitted *Rasgrf1^{tm1Pds}* allele was digested with *PstI* and/or *NotI*, and Southern blots were hybridized with the probe indicated. Mice were on a mixed C57BL/6 and 129S4/SvJae background.

Fig. 3. Loss of paternal allele methylation is due to a failure to establish *Rasgrf1* methylation in the male germ line. **a**, The methylation status of the *NotI* site in DNA isolated from sperm and tail of heterozygous *Rasgrf1^{+/-tm1Pds}* (+/-) mice was analyzed by Southern blot as described in Fig. 2. **b**, Structure of the locus in heterozygotes and location of primers used in PCR analysis of *HhaI* site methylation. **c**, Sperm DNA was amplified by PCR after digestion with *HhaI* (H) or without previous restriction enzyme treatment (U). PCR reactions included primers in **b** specific for the wildtype allele (P1 and P2) or the mutated allele (P3 and P4), or spanned a control sequence devoid of *HhaI* sites, which served as a control for successful PCR amplification (P5 and P6). Mice were on a mixed C57BL/6 and 129S4/SvJae background.

Fig. 4.

Loss of DMD methylation leads to *Rasgrf1* silencing. *Rasgrf1* expression levels in neonatal brain were measured by RT-PCR (left panel). The mice included wildtype progeny of C57BL/6 (+B6/+) or PWK mothers (+PWK/+) as well as animals heterozygous for a paternally transmitted *Rasgrf1*^{tm1Pds} allele, whose mothers were also either C57BL/6 (+B6/−) or PWK (+PWK/−). cDNAs were serially diluted in fivefold increments before PCR amplification. Primer pairs detected *Rasgrf1* (solid triangles) or *Rpl32* (open triangles), which served as an internal control. The methylation status of the *Rasgrf1* DMD in heterozygous animals was assayed by the Southern-blot hybridization method used in Figs 1 and 2 (right panel). In DNA digested with both *Pst*I (P) and *Not*I (N), the 2.8-kbp band is diagnostic for an unmethylated DMD, whereas the 3.0-kb band is diagnostic for a methylated DMD.

Table 1
Results of bisulfite sequencing

CpG	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	
NotI										N																	
HhaI												H						H		H			H			H	
Tail wildtype paternal allele																											
	X	O	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X									
	X	O	X	X	X	X	X	X	X	X	O	X	X	X	X	X	X	X									
	X	X	X	X	X	X	X	O	X	X	X	X	X	X	X	X	X	X									
	X	X	X	X	X	X	X	O	X	X	X	X	X	X	X	X	X	X									
	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X									
	X	X	X	X	O	O	X	O	X	X	X	X	O	X	X	X	X	X									
	O	O	X	X	X	X	X	O	O	O	X	X	X	O	X	X	X	X	O								
	O	O	X	X	X	X	X	O	O	O	X	X	X	X	O	X	X	X	O								
Tail wildtype maternal allele																											
	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O								
	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O	O	O	X	O								
	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O	O								
	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O	O	O								
	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O								
Tail mutant paternal allele																											
	O	O	O	X	O	O	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	X	O	O	O	O
	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O
	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
Sperm wildtype allele																											
	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	O
	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	O
	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	O
	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	X	X	X	X	X	X	X	X	X	X	O	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	O
	X	X	X	X	X	X	X	X	X	X	O	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	O
	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	X	X	X	X	X	X	X	X	X	X	O	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	O
	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Sperm mutated allele																											
	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	X	O	O	O	O	O	O	O	O	O	O	O
	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
	X	O	X	X	X	X	X	X	X	X	X	X	X	X	O	X	X	O	O	X	X	O	X	X	X	X	X
	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O

CpG	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
<i>NotI</i>										<i>N</i>																
<i>HhaI</i>												<i>H</i>						<i>H</i>		<i>H</i>			<i>H</i>			<i>H</i>
	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O

Each line represents the sequence of independent clones of DMD PCR products generated by amplification of bisulfite-treated templates prepared from the indicated tissue and mice. Clones representing the mutated allele were derived from three independent mice for both the tail and sperm DNA samples. Identification of the parental alleles from wildtype tail DNA was based on the methylation state of the *NotI* site (*N*). The relative positions of up to 26 CpGs that were assayed (1–26) are shown. Those contained in the *NotI* (*N*) and *HhaI* (*H*) sites are indicated. O, unmethylated CpG; X, methylated CpG.