Methylation-mediated silencing of TMS1/ASC is accompanied by histone hypoacetylation and CpG island-localized changes in chromatin architecture

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Running Title: Methylation and Chromatin Structure in TMS1 Silencing

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Summary

Aberrant methylation of CpG dense islands in the promoter regions of genes is an acquired epigenetic alteration associated with the silencing of tumor suppressor genes in human cancers. In a screen for endogenous targets of methylation-mediated gene silencing, we identified a novel CpG island-associated gene, TMS1, that is aberrantly methylated and silenced in response to the ectopic expression of DNA methyltransferase-1. In this study, we characterized the methylation pattern and chromatin architecture of the TMS1 locus in normal fibroblasts and determined the changes associated with its progressive methylation. In normal fibroblasts expressing TMS1, the CpG island is defined by an unmethylated domain that is separated from densely methylated flanking DNA by distinct 5' and 3' boundaries. Analysis of the nucleoprotein architecture of the locus in intact nuclei revealed three DNAse I hypersensitive sites that map to within the CpG island. Strikingly, two of these sites coincided with the 5' and 3' methylation boundaries. Methylation of the TMS1 CpG island was accompanied by loss of hypersensitive site formation, hypoacetylation of histones H3 and H4, and gene silencing. This altered chromatin structure was confined to the CpG island, and occurred without significant changes in methylation, histone acetylation or hypersensitive site formation at a fourth DNAse I hypersensitive site 2 kb downstream of the TMS1 CpG island. The data indicate that there are sites of protein binding and/or structural transitions that define the boundaries of the unmethylated CpG island in normal cells, and that aberrant methylation overcomes these boundaries to direct a local change in chromatin structure, resulting in gene silencing.
Cytosine methylation is a post-replicative modification of DNA that plays an important role in epigenetic inheritance. In vertebrates, methylation occurs primarily at cytosines within the dinucleotide CpG. Both the distribution of CpG sites and their methylation status is non-random in the human genome, generating a pattern of DNA methylation that varies with respect to density across the chromosome. CpG sites occur relatively infrequently throughout much of the human genome, except in discreet regions of CpG-dense DNA known as CpG 'islands'. These islands are ~200-1000 bp in length and often coincide with the 5' ends of genes. Accordingly, the identification of such regions in the human genome sequencing projects has been useful in gene prediction (1). Current estimates suggest that there are ~29,000 CpG islands in the human genome (2). Whereas non-CpG island DNA is generally methylated, most CpG islands remain methylation-free in both expressing and non-expressing tissues (3,4). Specialized physiologic cases in which CpG islands are methylated include genes subject to parental imprinting and genes on the inactive X chromosome (5,6). Here methylation functions as part of an epigenetic mechanism ensuring the stable propagation of allele-specific gene repression.

In human cancers, CpG island methylation occurs aberrantly, and is associated with the inappropriate silencing of tumor suppressor genes and other genes that function in the suppression of the malignant phenotype. Methylation-mediated silencing contributes to malignant progression at several levels, and has been implicated in the inactivation of genes involved in tumor suppression (e.g. Rb, VHL and CDKN2A), the regulation of DNA repair and genome integrity (e.g. MLH1, MGMT, BRCA1), and the suppression of metastases (e.g. CDH1 and TIMP3) (reviewed in (7-9)). Recent evidence suggests that aberrant methylation also contributes to human carcinogenesis by conferring resistance to cell death signals through the silencing of genes that promote apoptosis (10-13). In each of these cases, aberrant methylation of
promoter region CpG islands is associated with loss of gene expression in tumor-derived cell lines and primary tumors relative to normal somatic cells. That methylation plays a primary role in the silencing of these genes is supported by the fact that treatment with a demethylating agent such as 5-aza-2'-deoxycytidine is sufficient to restore expression and, in many cases, gene function.

At present, it is not known how or why particular CpG islands succumb to aberrant methylation in cancer cells, and the precise mechanism in which an unmethylated, actively transcribed gene progresses to a methylated and inactive state is unclear. One established consequence of CpG island methylation is transcriptional repression. Methylation of cytosine can affect gene expression directly, by interfering with the binding of transcription factors like E2F and AP-2 (14,15), or indirectly, through the recruitment of proteins that bind preferentially to methyl CpG through a methyl CpG binding domain (MBD)1 (reviewed in (16,17)). The MBD proteins are found within complexes containing histone deacetylases (HDAC) and chromatin remodeling factors (18-21). The accidental methylation of a CpG island during carcinogenesis could serve to re-direct the MBDs and chromatin remodeling machinery to newly methylated DNA, resulting in aberrant gene silencing. The recent observation that the DNA methyltransferases (DNMTs), DNMT1, DNMT3a and DNMT3b possess intrinsic transcriptional repressor activity and interact directly with HDACs (22-26) also raises the intriguing possibility that methylation-dependent gene silencing may be directed by the DNA methyltransferases themselves through the recruitment HDACs to their methylation targets.

To study the factors involved in aberrant methylation targeting and its consequences in somatic cells, we have utilized a human fibroblast model system in which de novo methylation is induced by the ectopic expression of DNA methyltransferase-1 (DNMT1). In previous studies, we showed that the overexpression of DNMT1 results in the progressive de novo methylation of
endogenous CpG island sequences (27,28). Using this model, we recently identified a novel CpG island-associated gene, TMS1 (Target of Methylation-induced Silencing), that is aberrantly methylated and silenced in response to DNMT1-driven methylation (13). Further studies indicated that TMS1 is silenced in association with aberrant methylation in human breast cancer cell lines and primary tumors, implicating TMS1 as a putative tumor-suppressor in breast cancer (13). TMS1 encodes a caspase recruitment domain protein and functions as a positive mediator of apoptosis (29). TMS1 was independently identified (and named ASC) by Masumoto et al. (30) who showed that decreased expression of TMS1 results in reduced sensitivity to anticancer agents. Methylation-mediated silencing of TMS1 thus may contribute to breast carcinogenesis by allowing cells to bypass apoptosis, and may confer resistance to cancer chemotherapeutic agents or other genotoxic stress.

In this study, we used bisulfite genomic sequencing, DNAse I hypersensitive site mapping and chromatin immunoprecipitation to characterize the methylation pattern and the chromatin architecture of the TMS1 locus in normal human fibroblasts, and to determine the changes associated with its progressive methylation in immortalized and DNMT1 overexpressing derivatives. We find that in normal fibroblasts, the TMS1 CpG island is comprised of an unmethylated domain with distinct 5’ and 3’ methylation boundaries. Three DNAse I hypersensitive (HS) sites mapped to within the CpG island, with two coinciding almost precisely with the positions of the methylation boundaries. De novo methylation of the CpG island in DNMT1 overexpressing cells was accompanied by loss of CpG island-specific HS formation, localized hypoacetylation of histones H3 and H4, and gene silencing. We propose that there are protein binding sites that demarcate the boundaries of TMS1 CpG island in normal cells, and that aberrant methylation overcomes these boundaries to direct a localized change in chromatin architecture, resulting in gene silencing.
Experimental Procedures

Cell Culture and Drug Treatments

Normal diploid human fibroblasts (IMR90) and SV40 immortalized IMR90 cells (referred to as ‘90SV’) were obtained from the National Institute on Aging Cell Repository (AG02804C) and were maintained in EMEM with 2mM glutamine and 10% FCS. The generation of a 90SV derivative cell line stably overexpressing the human cytosine 5-DNA methyltransferase-1 (HMT.1E1) has been reported (27). HMT.1E1 cells were maintained in EMEM plus 2mM glutamine, 10% FCS and 400µg/ml G418. HMT.1E1 cells were seeded in 75cm² flasks overnight and treated the next day with 100ng/ml Trichostatin A (TSA) (Sigma) or 500nM 5-aza-2'-deoxy-cytidine. For the combined treatments cells were treated with 500nM 5-aza-2'-deoxycytidine for 72 hours and 100ng/ml TSA was added for the last 24 hours.

Bisulfite Sequencing and Methylation Specific PCR

Genomic DNA was treated with sodium bisulfite as previously described (31). For methylation-specific PCR, approximately 50ng of bisulfite-modified DNA was amplified by PCR under the following reaction conditions: 67mM Tris-HCl (pH 8.8), 16.6 mM NH₄SO₄, 6.7 µM EDTA, 10mM β-mercaptoethanol, 6.7 mM MgCl₂ and 1µM each primer in a 25µl reaction. A hot start was preformed (5min 95⁰C) followed by the addition of 0.5u Taq polymerase (Life Technologies) and 35 cycles of PCR (95⁰C, 30s; 58⁰C, 30s; 72⁰C, 30s) with a final extension of 4 min at 72⁰C. Primers were designed from the interpolated sequence after bisulfite conversion assuming DNA was either methylated or unmethylated at three CpG sites. Primers used for methylated reactions were 5’-TTG TAG CGG GGT GAG CGG C-3’ and 5’-AAC GTC CAT AAA CAA CAA CGC G-3’. Primers used for the unmethylated reactions were 5’-GGT TGT AGT GGG GTG AGT GGT –3’ and 5’-CAA AAC ATC CAT AAA CAA CAC A-3’. Reaction
products were separated by electrophoresis on a 6% polyacrylamide/Tris-borate-EDTA gel, stained with ethidium bromide and photographed.

For genomic sequencing, 50 ng bisulfite-modified DNA was amplified by PCR using the same reaction conditions described above except that annealing temperature was 55°C and the final extension was increased to 10 minutes. Primers were designed to avoid potential methylation sites (e.g. CpG) such that both methylated and unmethylated DNA would be amplified equally. The resulting amplification pools were cloned into the pCR2 vector using the TOPO TA Cloning kit (Invitrogen). Eight to twelve individual subclones per PCR reaction were isolated and sequenced. Primer pairs used for bisulfite genomic sequencing were: A: 5’-GAA ATT GAA GTT TAG AGA GAT TTG-3’ and 5’-CCC TTA ATC ATG CCC CAC C-3’, B: 5’-TTG GTG TAG AGA TAA GT-3’ and 5’-ACC ATC GCC TCC CAC AAA CCC ATA-3’, C: 5’-AAG TTG GTT AGT TTT TAT TTG GAG-3’ and 5’-CTT CAA AAA TTC CTC TAC AAA CC-3’, D: 5’-TTT TAT TGT AGA GTT TAG TTA TG-3’ and 5’-CTC TAC ACA TAC CAA CAA ACT AAA-3’, E: 5’-GCT ATG TGT AGA GTT TAG TTA TG-3’ and 5’-CAC TAC ACT CCA TAC TAA ATA AC-3’.

**DNase I Hypersensitivity**

Cells (1x10^7) were resuspended in Nuclei Isolation Buffer (RBS)(10 mM Tris, pH 7.4, 5 mM MgCl_2, 0.5 mM DTT, 0.3 mM sucrose, 0.4 mM PMSF and 0.1% NP-40) and swollen on ice for 10 min. This was followed by dounce homogenization and nuclei were collected by centrifugation at 1,500g. Nuclei were resuspended in RBS without NP-40 and immediately added to prepared aliquots of DNase I (GibcoBRL) and 0.1mM CaCl_2 for 10min at room temperature. As controls, nuclei were incubated in the presence of CaCl_2 without DNAse I (endogenous nuclease control) or in the absence of DNAse I (nuclease free reference). Reactions were stopped by the addition of an equal volume of 20mM EDTA, 1% SDS, 10μg/ml RNase A.
and incubated at 37°C for 30 min. DNA was then isolated by Proteinase K digestion, phenol:chloroform extraction and ethanol precipitation. Ten µg of DNA was digested with HindIII and electrophoresed on a 25 cm, 1.0 % agarose gel. The gel was transferred to a nylon membrane and hybridized to a random-prime labelled 423bp HindIII-XbaI fragment anchored to the 3’ HindIII site of the TMS1 locus. Blots were washed to a final stringency of 0.1XSSC, 0.1% SDS at 65°C and exposed to X-ray film (Biomax-MS, Kodak).

**Chromatin Immunoprecipitation (ChIP)**

Chromatin immunoprecipitation was performed using antibodies specific for the acetylated isoforms of histone H4 and H3 in accordance with the protocol provided by the supplier (Anti-acetyl Histone H4, Chip Grade and Anti-acetylated Histone H3, Upstate Biotechnology, Lake Placid, NY). DNA fragments immunoprecipitated with anti-acetylated H3 or anti-acetylated H4 were analyzed by multiplex PCR using primers spanning HS1, HS2, HS3 or HS4 of the TMS1 locus in combination with primers specific for the β-actin promoter region. Multiplex PCR was carried out under the following reaction conditions: 67 mM Tris-HCl (pH 8.8), 16.6 mM NH₄SO₄, 6.7 µM EDTA, 10 mM β-mercaptoethanol, 6.7 mM MgCl₂ and 1 µM each primer in a 25 µl reaction. A hot start was performed (5 min, 95°C) followed by the addition of 0.5 µl Taq polymerase (Life Technologies) and 35 cycles of PCR (95°C, 30 s; 55°C, 30 s; 72°C, 30 s) with a final extension of 72°C for 4 min. PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and photographed. The ratio of intensities of the TMS1 product to β-actin product in each reaction was determined from digital images using Scion Image software (Scion Corporation). The PCR conditions used were within the linear range of amplification for input DNA. Primer pairs derived from the TMS1 genomic sequences surrounding the DNase I hypersensitive sites (HS1: 5’-CCA GCT GGA GGG CGC GA-3’ and 5’-GCC GCC GAC CAG GAG GA-3’, HS2: 5’-ACC TGG AGA CCT ACG GCG and 5’-GGC
CAA GCG TGG GGA GC-3’, HS3: 5’-ACC CTC ATC CAA CTG CAT G-3’ and 5’-ATC CAG CAG CCA CTC AAC G-3’, HS4: 5’-GGT CAT AGG TTG CAC TTT GC-3’ and 5’-AGC TTG AAC CTG GGA GGT G-3’) were each paired with primers from the promoter region of the human β-actin gene (5’-CCT CAA TCT CGC TCT CGC T-3’ and 5’-CGC GCC TGC GAA CTG GC-3’).

**Reverse Transcriptase-PCR**

Total RNA was isolated from log-phase cells by the Chomynski and Sacchi method (32). Six µg of total RNA was pretreated with amplification grade DNase I (Life Technologies) and reverse transcribed using random hexamer primers and MMLV-reverse transcriptase (Life Technologies). One-thirtieth of the reverse transcriptase reaction (200ng of starting RNA) was used in a PCR reaction. The PCR conditions were: 67mM Tris-HCl (pH 8.8), 16.6mM NH₄SO₄, 6.7µM EDTA, 10mM β-mercaptoethanol, 4.7mM MgCl₂, 10% DMSO, and 400nM each primer in a 25µl reaction. A hot start was performed (5 min, 95°C), followed by 35 cycles of 95°C, 30s; 55°C, 30s; 72°C, 30s. TMS1 primers were 5’- TGG GCC TGC AGG AGA TG-3’ and 5’-ATT TGG TGG GAT TGC CAG-3’. β-actin primers were 5’-CCT TCC TGG GCA TGG AGT CCT G-3’ and 5’-GGA GCA ATG ATC TTG ATC TTC-3’. For multi-cycle experiments, samples were withdrawn from a 100µl PCR reaction after 20, 30 and 40 cycles for TMS1 or after 15, 25, 35 cycles for β-actin. Samples were run on a 2% agarose gel, transferred to a nylon membrane and hybridized to random prime labeled TMS1 or β-actin cDNA probe. Blots were washed to a final stringency of 0.1XSSC, 0.1% SDS at 65°C and exposed to X-ray film (Biomax-MS, Kodak).
Results

We identified TMS1 in a PCR-based subtractive cDNA screen to isolate transcripts that were downregulated in human fibroblasts stably overexpressing DNMT1 (13). We have shown previously that TMS1 is expressed in normal human diploid fibroblasts (IMR90) and their SV40-immortalized counterparts (90SV cells), but is silent in the 90SV derivative cell line expressing 50-fold increased levels of DNMT1 (HMT.1E1 cells) (13). These cell lines represent a useful model for studying the genesis and consequences of aberrant CpG island methylation in that they are derived from a common parent (IMR90) yet differ in their expression and methylation status at the TMS1 locus.

To examine the role of DNA methylation in TMS1 silencing, we first characterized the TMS1 locus with regards to structure and methylation status in normal diploid fibroblasts (IMR90). The coding sequence of TMS1 covers approximately 1.5 kb on chromosome 16p11.2. The promoter region of TMS1 lacks a defined TATA box but contains a 600 bp CpG island predicted on the basis of C+G content (69%) and CpG frequency (CpG observed/expected = 0.82) which extends from approximately 200 bp upstream to 400 bp downstream of a single transcription start site (position 1177, Figure 1A and data not shown). Genomic sequencing of bisulfite modified DNA was used to construct a map of the methylation status of 110 CpGs across 2.5 kb of the TMS1 locus. Five overlapping regions covering the TMS1 locus were amplified from bisulfite treated DNA. PCR products were then subcloned and individual clones sequenced. This allows for the assessment of the methylation status of independent alleles derived from the cell population. Figure 1B shows the percent of alleles methylated at each CpG site as determined from the analysis of eight to sixteen individual alleles for each primer set. The TMS1 locus was characterized by an unmethylated domain that extended from ~100 bp upstream to ~1 kb downstream of the transcription start site, and encompassed the entire predicted CpG...
island plus ~500 bp of additional downstream sequence (Figure 1B). CpGs within this region were methylated in no more than 1 allele per 8-16 analyzed with exception of one CpG site in which 3/8 alleles analyzed were methylated. In contrast, sequences upstream of nt 1100 and downstream of 2100 were densely methylated, with distinct 5’ and 3’ boundaries separating the unmethylated CpG island region from heavily methylated flanking DNA (Figure 1B). Primary mammary epithelial cells are also completely devoid of methylation on 8-10 individually sequenced alleles in the region from 1100 to 2000 (data not shown). Thus, the pattern shown here appears representative of normal tissues.

To determine the relationship between methylation of TMS1 and gene silencing, we next analyzed the methylation pattern of 500 bp surrounding the transcription start site (primer set B, see Figure 1) in the IMR90 fibroblasts, their SV40 immortalized derivatives, the 90SV cells, and the DNMT1 overexpressing derivative HMT.1E1 cells. As discussed above, TMS1 was almost completely unmethylated on individual alleles downstream of nt 1100 in IMR90 cells (Figure 2A). Of the few sites that showed methylation, they appeared to be random in that no single CpG site was methylated in more than one allele analyzed (Figure 2A). Relative to IMR90, 90SV cells showed a partially methylated pattern. There did not appear to be any methylation hot-spots in these cells, although there was somewhat more methylation in general in the 5’ end than the 3’ end of the region analyzed. Rather, the analysis of individual subclones was suggestive of two populations of alleles; those that were predominantly methylated and those that were predominantly unmethylated (Figure 2A). This pattern is consistent with a mixed cell population in which some cells are methylated for TMS1 and others are not, or with allelic heterogeneity within each cell. The partially methylated pattern of the 90SV cells correlated with a decreased expression of TMS1 relative to IMR90 cells (Figure 2B). In stark contrast, HMT.1E1 cells showed no detectable TMS1 expression and were methylated at nearly every CpG site on all
alleles analyzed (Figure 2A and 2B).

Next we used DNase I to probe the nucleoprotein architecture of the TMS1 locus in intact nuclei. Four DNase I hypersensitive sites, designated HS1-HS4, were identified in the IMR90 fibroblasts (Figure 3). Interestingly, mapping of the DNase I hypersensitive sites relative to the DNA methylation map from the same cells (Figure 1) indicated that HS1, HS2, and HS3 lay within the unmethylated domain, with HS1 and HS3 corresponding almost precisely to the location of the 5’ and 3’ methylation boundaries (compare Figure 3A with Figure 1B). The fourth site (HS4) mapped to about 1 kb downstream of the end of the TMS1 coding region (Figure 3A). Similar experiments using a probe anchored at the 5’ HindIII site confirmed the position of the DNase I hypersensitive sites (data not shown). To determine the relationship between CpG island methylation, gene expression, and chromatin structure, DNase I hypersensitivity assays were also performed on the 90SV and HMT.1E1 cells. All four DNase I hypersensitive sites found in the IMR90 cells (HS1-HS4) were also observed in the 90SV cells, which express TMS1 and contain a mixed population of unmethylated and methylated alleles (Figure 3B). In contrast, complete methylation of the TMS1 CpG island in the HMT.1E1 cells resulted in loss of the CpG island-associated hypersensitive sites HS1, HS2, and HS3, but had no effect on the formation of HS4 (Figure 3B). HS4 is thus 'constitutive' in that its formation is independent of TMS1 expression and methylation status at the CpG island, whereas the CpG island-associated hypersensitive sites (HS1, HS2 and HS3) form only when the CpG island is at least partially unmethylated and TMS1 is expressed. Analysis of the methylation status of the 500 bp surrounding HS4 (primer set "E", see Figure 1) in the 90SV and HMT.1E1 cells indicated a similar pattern to that observed in IMR90 cells (data not shown). These data show that, in normal cells, the chromatin architecture adopted by the unmethylated CpG island is bounded by
DNAse I HS, and that methylation-mediated silencing of TMS1 is accompanied by localized changes in the conformation of this domain.

Recent evidence suggests that methylation-associated gene repression involves the recruitment of histone deacetylases (HDACs) and other chromatin modifying factors (22-26). To determine whether methylation-mediated silencing of TMS1 is accompanied by changes in histone acetylation, we analyzed the relative levels of acetylated histone H3 and H4 at the regions corresponding to HS1, HS2, HS3 and HS4 in IMR90, 90SV and HMT.1E1 cells. Formaldehyde-crosslinked chromatin was immunoprecipitated with antibodies against the acetylated isoforms of histone H3 or histone H4. Immunoprecipitated DNA was analyzed by multiplex PCR using a primer pair specific to each of the hypersensitive site regions (HS1-4) in conjunction with primers to the β-actin promoter under conditions of linear amplification of input DNA. β-actin was used as a positive control as it is expressed at similar levels in all three cell lines (Figure 2B). The ratio of intensities of the TMS1 product and β-actin product was determined and normalized to that of the IMR90 cells to allow for comparison between the three cell lines.

Relative to the IMR90 cells, both 90SV and HMT.1E1 cells showed decreased levels of acetylated histones H3 and H4 at all four regions of the TMS1 locus analyzed (Figure 4). At the CpG island-localized DNAse I hypersensitive sites (HS1, HS2, and HS3), where the density of CpG is highest, the relative degree of histone hypoacetylation in the three cell lines correlated well with the degree of methylation. As the level of methylation in the CpG island increased from <10% in the IMR90 cells to ~50% in the 90SV to nearly 100% in HMT.1E1, the relative levels of histone H4 acetylation decreased by ~50-60% in the 90SV cells and >80% in the HMT.1E1 cells. The relative levels of acetylated histone H3 at HS1, HS2 and HS3 were also decreased in the 90SV and HMT.1E1 cells, although to a somewhat lesser extent than acetylated
histone H4 (Figure 4B). In contrast, there was little correlation between methylation and histone acetylation outside of the CpG island, in the region of HS4. Although the levels of acetylated histones associated with HS4 in the 90SV and HMT.1E1 cells was decreased relative to IMR90 cells, there was little difference between the TMS1 expressing 90SV cells and the TMS1 silent HMT.1E1 cells (Figure 4). Together with the finding that the formation of HS4 is independent of the CpG island methylation and gene expression, these data suggest that the effect of aberrant methylation at the TMS1 CpG island on chromatin structure is confined to the immediate vicinity of the CpG island.

To further examine the relative contributions of methylation and histone deacetylation in TMS1 silencing, we determined the effect of treatment with the demethylating agent 5’-aza-2’deoxycytidine (5azadC) or the histone deacetylase inhibitor Trichostatin A (TSA) on the expression and methylation of TMS1 in the HMT.1E1 cells. Treatment of HMT.1E1 cells with 5azadC resulted in a time-dependent re-activation of TMS1 (Figure 5A). This reactivation was accompanied by partial demethylation of the TMS1 CpG island (Figure 5B). In contrast, treatment of HMT.1E1 cells with TSA had no effect on TMS1 expression or methylation of the CpG island, and did not significantly alter the degree of re-expression when combined with 5azadC. The data indicate that the inhibition of histone deacetylation alone is insufficient to re-activate TMS1. As has been found for other CpG island associated genes silenced in human cancers (33,34) these data suggest that methylation plays a primary role in TMS1 silencing.
Discussion

TMS1/ASC is an apoptotic signaling protein whose expression is frequently lost in human breast cancers via aberrant methylation. TMS1 is associated with a 5' CpG island that is unmethylated in normal fibroblasts and mammary epithelial cells, but becomes aberrantly methylated in response to overexpression of DNMT1 and in human breast cancer cell lines and primary tumors. In this study, we showed that the CpG island of the TMS1 gene is unmethylated in normal somatic cells and is separated from densely methylated flanking DNA by distinct boundaries at both the 5' and 3' ends. The methylation boundaries coincided with DNase I HS sites that form only when the CpG island is unmethylated. The *de novo* methylation of the TMS1 CpG island was accompanied by local changes in CpG island conformation and activity, including the hypoacetylation of histones H3 and H4, remodeling of CpG island-specific DNAse I HS sites, and gene silencing. An understanding of the consequences of aberrant methylation of TMS1 on chromatin structure and gene expression is important not only because of its role in breast cancer but because TMS1 may be representative of other CpG island-associated tumor suppressor genes that are silenced in cancer.

Recently, 5' methylation boundaries have been mapped for the CpG islands of the GSTP1 and the BRCA1 genes (35,36). This demarcation was absent in cancer cells in which the CpG island is aberrantly methylated and silenced. In the case of GSTP1, the methylation boundary overlapped a small A+T-rich region with a repeated sequence of ATAAA (35). Although the 5' methylation boundary of TMS1 contains one copy of an ATAAA sequence, neither the 5' nor 3' methylation boundary is particularly A+T-rich. We note here that one thing the TMS1 boundaries have in common with those identified at the GSTP1 and BRCA1 genes is small gaps of 50-100 bp that lack CpGs altogether (note the gaps in CpG density in Figure 1A). The 5' and 3' boundaries of the TMS1 CpG island contain a stretch of 60 and 134 base pairs, respectively,
that although C+G rich, are devoid of CpGs. These regions of non-CpG DNA abutting the CpG island DNA may act as a buffer zone ensuring that methylation does not spread into the CpG island.

It is currently unknown what, if anything, constitutes a methylation boundary in normal cells. We found that the unmethylated domain of the TMS1 CpG island is flanked by DNAse I hypersensitive sites that form at the boundaries between the unmethylated CpG island and the surrounding methylated DNA. This finding is significant in that it shows that the CpG island boundary is more than just a transition in methylation pattern; it suggests the existence of a physical boundary occurring at the level of chromatin. These sites were lost when the CpG island was methylated. One model that stems from these findings is that the maintenance of a chromatin boundary at HS1 and HS3, which could be mediated by the binding of a specific protein or complex, may play an important role in preventing the methylation of the CpG island in normal cells. Loss of this boundary function could contribute to aberrant methylation. The idea that there are cis-acting elements that protect CpG islands or other regions from methylation or that specific proteins might block de novo methylation is not without precedent. The binding of transcription factors and other DNA binding proteins blocks de novo methylation by bacterial and mammalian DNA methyltransferases in vivo (37-39). A region of the chicken β-globin locus defined by a constitutive DNAse I HS site was shown to block the progressive de novo methylation and silencing of an integrated transgene (40). Subsequent studies have shown that this site is bound by the methylation-sensitive transcription factor CTCF (41). CTCF binding to the imprint control region of the H19/IGF2 locus is important in the maintenance of normal imprinted expression of H19 and IGF2 (42,43) Interestingly, acquired methylation of these sites in human colon cancers and Wilms tumors leads to loss of imprinting and bi-allelic expression of IGF2 (44,45).
Previous studies have suggested that a cis-element in the 5' end of the mouse or hamster APRT gene containing Sp1-like sites is important in protecting the CpG island from \textit{de novo} methylation (46-49). Mutation or deletion of the Sp1 binding sites leads to \textit{de novo} methylation of the CpG island when propagated in transgenic mice or transfected into embryonic cells. Whether it is Sp1 binding that mediates the protective effect is not entirely clear, however, since neither the Sp1 consensus sites alone nor those from a heterologous locus protected the APRT gene from \textit{de novo} methylation in transgenic embryos (50). Moreover, targeted disruption of Sp1 does not lead to widespread methylation of CpG islands (51). Nevertheless, it is noteworthy that all three CpG island-associated HS sites (not HS4) are in the vicinity of a canonical Sp1 binding site.

If the boundary were occupied by a methylation-sensitive DNA binding protein, the competition between \textit{de novo} methylation of the boundary element and its occupancy by the DNA binding protein during DNA replication might be the first step towards the methylation of the CpG island. In the model system utilized here, that balance might be tipped in favor of aberrant methylation by overexpression of DNMT1. We have shown previously that the hypermethylation of affected CpG island loci was observed only in clones expressing >9-fold increased levels of DNMT1 (27), consistent with a competition model or the existence of a saturable control mechanism. Alternatively, loss of a \textit{trans} factor that mediates the boundary function in the HMT.1E1 cells or other cell types might allow for aberrant methylation. Indeed, we find that in samples exhibiting incomplete methylation of TMS1, such as the 90SV cells studied here or breast cancer cell lines and primary tumors (J. Levine and P. Vertino, manuscript in preparation), there are no "hot-spots" of methylation, rather individual alleles are either predominantly methylated or predominantly unmethylated, suggesting that loss of a boundary function may be a limiting factor.
As with other CpG islands that become aberrantly methylated in disease states, such as the FMR1 locus in fragile X syndrome patients or the BRCA1 locus in human breast cancer cell lines (36,52), methylation of the TMS1 CpG island was associated with hypoacetylation of histones H3 and H4. We found that within the CpG island, there was a good correlation between the levels of methylation, histone deacetylation and expression. Given the methylation pattern in the 90SV cells, the intermediate levels of expression and histone acetylation observed most likely reflect a mixed population of cells/alleles, with some alleles being unmethylated, associated with hyperacetylated histones and expressing TMS1, and others methylated, associated with hypoacetylated histones and silent. These data are consistent with a role for HDACs in TMS1 silencing. The aberrant methylation of TMS1 may drive gene silencing through the binding of MBDs. The MBDs are found in complexes containing HDACs and chromatin remodeling factors and could serve to recruit these complexes to methylated DNA (18-21). This model is supported by recent evidence showing an \textit{in vivo} association of MBD2 with the aberrantly methylated promoter of the p16/INK4A gene in human cancer cells (34). An alternative mechanism would be one in which the aberrant methylation and deacetylation of histones occurs in a coupled process orchestrated by the DNA methyltransferases. DNMT1, DNMT3a, and DNMT3b have been shown to interact directly with HDACs and to repress transcription independently of their methyltransferase activities (22-26). This may occur through direct interaction with sequence-specific transcription factors (22,24). The DNMTs may thus direct HDAC activity to their methylation targets. This latter possibility may be particularly relevant in the case of TMS1 since it was identified as a gene subject to DNMT1-driven gene silencing. At this point, we cannot distinguish between MBD-dependent and -independent silencing mechanisms. However, once established, methylation appears to be the predominant mediator of TMS1 silencing since reactivation of TMS1 required demethylation of the CpG
island induced by 5-aza-2’-deoxycytidine and was not affected by the histone deacetylase inhibitor TSA. This is consistent with other CpG island-associated genes silenced in cancer where methylation plays the dominant role in the maintenance of the repressed state (33).

The association between hypermethylation of TMS1, the deacetylation of histones and the remodeling of DNAse I hypersensitive sites was specific to the vicinity of the CpG island. At HS4, which maps to only 2 kb downstream of the 3’ end of the TMS1 CpG island, the hypersensitive site formed independently of the changes in methylation and chromatin conformation occurring at the CpG island. The differences in histone H3 acetylation between the cell lines were also less dramatic at HS4, and there was no difference in methylation at this site. Therefore, silencing of TMS1 does not appear to be due to widespread changes in methylation or the structure of the locus, but to the local effects of methylation on the CpG island region. Recently, Shubeler et al. (53) showed that targeted insertion of a methylated transgene resulted in specific changes in transgene conformation, including loss of a promoter DNAse I HS site, hypoacetylation of histones H3 and H4, and transcriptional repression (53). These changes occurred without affecting the methylation or chromatin structure of surrounding DNA. Although the promoter used in that study was not a CpG island, both studies are consistent with the idea that methylation can direct changes in nucleosome modification and chromatin structure within a defined domain, and that these changes are sufficient to drive gene silencing.

We have shown that in normal somatic cells, the TMS1 CpG island is embedded in an unmethylated domain that adopts a chromatin structure characterized by hyperacetylated histones and whose boundaries are defined by DNAse I HS sites. Aberrant methylation of TMS1 results in loss of function at these boundaries, hypoacetylation of histones, and re-organization of CpG island chromatin. One important conclusion of these findings is that the CpG island functions as an independent domain at the level of chromatin, and that the remodeling of this domain
represents a critical step in the silencing associated with aberrant methylation. A similar scenario may be operative at other CpG island-associated tumor suppressor genes silenced by methylation in cancer.
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Footnotes

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2 The abbreviations used are: DNMT, DNA cytosine-5-methyltransferase; HDAC, histone deacetylase; HS, hypersensitive site; MBD, methyl CpG binding domain; PCR, polymerase chain reaction; TSA, Trichostatin A.
Figure Legends

**Figure 1**

**Pattern of Methylation at the TMS1 Locus in Normal Fibroblasts**  
A. Genomic map of the TMS1 locus. The position of 110 CpG dinucleotides (vertical lines) analyzed in this study are indicated above the map. Open boxes, TMS1 exons; numbers indicate nucleotide position relative to an upstream HindIII site (H). Connected arrows represent the locations of the five sets of overlapping primers (A-E) used in bisulfite sequencing.  
B. Methylation pattern of the TMS1 locus in normal fibroblasts. DNA from IMR90 cells was modified with sodium bisulfite which deaminates cytosines to uracil but leaves methyl cytosines unaltered. Bisulfite-modified DNA was amplified by PCR using primer pairs A-E which were designed to avoid potential methylation sites such that methylated and unmethylated DNA is amplified equally. The resulting PCR products were subcloned, and at least eight individual subclones were sequenced for each amplification reaction. Percent methylation was determined from the number of alleles containing a methylated CpG at each position relative to the total number of alleles analyzed.

**Figure 2**

**TMS1 Silencing is Associated with Complete Methylation of the CpG Island.**  
A. Bisulfite sequencing analysis of the methylation status of 53 CpG sites in the TMS1 CpG island. DNA from IMR90, 90SV and HMT.1E1 cells was modified with sodium bisulfite and amplified by PCR using primer set B indicated in Figure 1. The amplification product was subcloned and sequenced. Each row represents the methylation pattern of an individual allele; ◯, unmethylated CpG, ●, methylated CpG. CpG positions are indicated, and are relative to the 5' HindIII site as in Figure 1.  
B. TMS1 expression. Expression of TMS1 was determined by semi-quantitative RT-PCR analysis. RNA from IMR90, 90SV and HMT.1E1 cells was reverse transcribed and amplified with primers specific to TMS1 (top) or β-actin (bottom) for the indicated number of
cycles. TMS1 silencing in HMT.1E1 cells is correlated with the near-complete methylation of all 53 CpG sites in the CpG island.

**Figure 3**

**Mapping of DNase I Hypersensitive Sites in the TMS1 locus.** A. Map of the 5.5 kb Hind III (H) genomic fragment containing the TMS1 gene. The unmethylated domain identified in IMR90 cells (Figure 1) is indicated. Open boxes, TMS1 exons; numbers indicate nucleotide position relative to the 5' HindIII site. The HindIII-XbaI (X) probe used in indirect end-labeling experiments is indicated. Arrows represent the positions of the DNase I hypersensitive sites, which were mapped to the approximate base pair positions 1094 (HS1), 1515 (HS2), 2054 (HS3), and 3090 (HS4) relative to the 5' HindIII site. B. DNase I hypersensitive site mapping. Nuclei isolated from the indicated cell line were incubated in the absence (lanes 2 and 3 of each gel) or presence of increasing concentrations of DNase I (open triangles). Genomic DNA was then isolated, digested with HindIII and subjected to Southern blot analysis using a random-prime labeled probe indicated in panel A which was anchored to the 3' HindIII site. Genomic DNA digested with HindIII plus XbaI, XhoI or BamHI was included as a marker (M, Lane 1).

**Figure 4**

**Histone acetylation at the TMS1 locus.** The indicated cell lines were treated with formaldehyde to crosslink protein to DNA and acetylated histones were immunoprecipitated using antibodies specific to the acetylated isoforms of histone H3 (A) or histone H4 (B). Immunoprecipitated DNA was analyzed by multiplex PCR using primers specific to the regions surrounding HS1, HS2, HS3, or HS4 of the TMS1 locus coupled with a common primer set specific to the β-actin promoter. Immunoprecipitation reactions lacking antibody (No Ab) or of 1/40 of chromatin input (Input) were used as a negative and positive controls for each of the four primer pairs (only the
reaction with HS3 primers is shown). Amplification products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide and photographed. A representative experiment is shown. C, D. Band intensities were quantified from the digital images using Scion Imager software and the ratio of intensities of the TMS1 product to the β-actin product calculated for each reaction. Ratios obtained for the IMR90 cells at each site were set to one to allow for comparison between the cell lines. The data represent the mean +/- standard deviation of four independent immunoprecipitation experiments using anti-acetylated Histone H3 (C) or anti-acetylated Histone H4 (D).

**Figure 5**

Demethylation and re-expression of TMS1 in response to treatment with the demethylase 5-aza-2’-deoxycytidine but not to treatment with the HDAC inhibitor, Trichostatin A. HMT.1E1 cells were treated for the indicated times with 500nM 5-aza-2’-deoxycytidine (AZA), 100ng/ml trichostatin A (TSA) or a combination of both. A. Analysis of TMS1 expression by RT-PCR. Following treatment, total RNA was isolated, reverse transcribed and amplified by PCR using primers specific to the TMS1 transcript (top) or the β-actin transcript (bottom). Control reactions in which the reverse transcriptase was omitted (-RT) were amplified under the same conditions. B. Methylation of the TMS1 locus analyzed by methylation-specific PCR. DNA isolated from parallel HMT.1E1 cultures treated as described in panel A was modified with sodium bisulfite and amplified using primers in the TMS1 CpG island that overlap 3 potential methylation sites each. Parallel reactions were performed using primers specific to the unmethylated (U) or methylated (M) DNA.
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A.

CpG Island

B.  

Percent Methylation

Position
A. Anti-acetylated Histone H3

|     | HS1   | HS2  | HS3   | HS4   | No Ab | Input |
|-----|-------|------|-------|-------|-------|-------|
| M   | IMR90 | 90SV | HMT.1E1 | IMR90 | 90SV | HMT.1E1 | IMR90 | 90SV | HMT.1E1 | IMR90 | 90SV | HMT.1E1 |

↓β-actin
↓TMS1

B. Anti-acetylated Histone H4

|     | HS1   | HS2  | HS3   | HS4   | No Ab | Input |
|-----|-------|------|-------|-------|-------|-------|
| M   | IMR90 | 90SV | HMT.1E1 | IMR90 | 90SV | HMT.1E1 | IMR90 | 90SV | HMT.1E1 | IMR90 | 90SV | HMT.1E1 |

↓β-actin
↓TMS1

C.

|      | HS1   | HS2  | HS3   | HS4   |
|------|-------|------|-------|-------|
| IMR90 90SV HMT.1E1 | IMR90 90SV HMT.1E1 | IMR90 90SV HMT.1E1 | IMR90 90SV HMT.1E1 |

D.

|      | HS1   | HS2  | HS3   | HS4   |
|------|-------|------|-------|-------|
| IMR90 90SV HMT.1E1 | IMR90 90SV HMT.1E1 | IMR90 90SV HMT.1E1 | IMR90 90SV HMT.1E1 |
### A.

|                | Untreated | 24hr AZA | 48hr AZA | 72hr AZA | 24hr TSA | 48hr TSA | 72hr AZA+ 24hr TSA |
|----------------|-----------|----------|----------|----------|----------|----------|-------------------|
| **RT**         | +         | -        | +        | -        | +        | -        | +                 |
| **TMS1**       |           |          |          |          |          |          |                   |
| **β-actin**    |           |          |          |          |          |          |                   |

### B.

|                | Untreated | 24hr AZA | 48hr AZA | 72hr AZA | 24hr TSA | 48hr TSA | 72hr AZA+ 24hr TSA |
|----------------|-----------|----------|----------|----------|----------|----------|-------------------|
| **M**          | M         | U        | M        | U        | M        | U        | M                 |
| **U**          |           |          |          |          |          |          |                   |
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