Expression of Prokaryotic HhaI DNA Methyltransferase Is Transforming and Lethal to NIH 3T3 Cells

Jianjun Wu, James G. Herman, Geoffrey Wilson, Ree Y. Lee, Ray-Whay Chiu Yen, Mack Mabry, Andrée de Bustros, Barry D. Nelkin, and Stephen B. Baylin2


ABSTRACT

In neoplastic cells, levels of DNA methyltransferase activity are often increased, and evidence is accruing to suggest an important role for this event in tumorigenesis. To evaluate this possibility further, and to investigate the contribution of increasing de novo, as opposed to maintenance, DNA methylation in mammalian cells, we expressed the bacterial HhaI methyltransferase in cultured murine fibroblasts. This enzyme is a pure de novo DNA methyltransferase that methylates the internal C in the sequence GCGC. We found that both constitutive and induced expression of the wild-type HhaI results, primarily, in lethality to the cells. However, surviving cell clones that express low levels of M. HhaI demonstrate increased tumorigenicity as assessed by soft agar cloning efficiency (8.6% for sense HhaI-transduced PA 317 cells versus 0.4% for antisense controls; 1.7% for sense HhaI-transfected NIH 3T3 cells versus 0% for a mutant HhaI control) and tumorigenicity in nude mouse heterotransplants (75% for M. HhaI control) and tumorigenicity in nude mouse heterotransplants (75% for sense HhaI-transduced PA 317 cells versus 18.5% for antisense control). DNA isolated from the clonogenic sense HhaI clones, versus clones expressing the mutant HhaI gene, has no increase in overall CpG methylation but an average of 27% (range, 16.7–38.9) increase in methylation content at GCGC sites. These findings suggest that eukaryotic cells tolerate a narrow window of increased de novo DNA methylating capacity, above which cell death occurs and within which cell transformation results. Our results further emphasize the potential role of increased DNA methyltransferase activity in the evolution of cancer.

INTRODUCTION

Increased expression of the DNA-MTase3 gene (1, 2) and regional de novo methylation of cellular DNA (for review, see Refs. 3–4) are consistent features of neoplastic cells. We have recently shown that the overexpression of a mammalian DNA-MTase gene, with predominantly maintenance DNA methylation function, can induce hypermethylation of cellular DNA and tumorigenic transformation of cultured murine fibroblasts (5). Furthermore, Laird et al. (6) have shown that a modest decrease (50%) in DNA-MTase gene expression can markedly reduce intestinal polyp formation in a murine model of inherited polyposis. Increased expression of the DNA-MTase gene may then play a critical role in tumor progression. The mechanisms remain to be elucidated, but hypermethylation of DNA, especially if it occurs in normally unmethylated “CpG islands” associated with gene promoter regions, has the capacity to repress gene expression (7–9) and to alter chromatin structures (10–12). Recently, our group and others have shown that such CpG island methylation and associated gene repression can act as a means to inactivate tumor suppressor genes (13–16). Furthermore, increased DNA-MTase activity has the potential to produce increased DNA mutagenicity (17).

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2 To whom requests for reprints should be addressed, at The Johns Hopkins Oncology Center, 424 North Bond Street, Baltimore, MD 21231.
3 The abbreviations used are: DNA-MTase, DNA methyltransferase; M. HhaI, MTase from Haemophilus haemolyticus; LTR, long terminal repeat; CMV, cytomegalovirus.

The increase in DNA-MTase in neoplastic cells, and in our overexpression system for murine DNA-MTase (5), involves an enzyme that is known to have predominantly a maintenance function in adult cells. That is, the enzyme preserves established patterns of DNA methylation rather than establishing new patterns of DNA methylation. This function is well served by the fact that the intact mammalian enzyme prefers hemimethylated DNA as substrate (18, 19). However, the mammalian enzyme does have some activity against nonmethylated DNA. Also, the COOH terminus of the mammalian DNA-MTase possesses high de novo methylating function in vitro when cleaved from its NH2-terminal regulatory domain (18). This COOH-terminal domain is structurally and functionally similar to prokaryotic enzymes that are purely de novo DNA-MTases (19–21). It is then possible that the increases in DNA-MTase gene expression observed during tumor progression (1, 2), and in our experimental model (5), may increase de novo as well as maintenance DNA-methylating capacity.

To learn more about the consequences of increased de novo DNA methylation for mammalian cells, we expressed the cytosine MTase isolated from Haemophilus haemolyticus (M. HhaI) in cultured murine fibroblasts. This enzyme, which is a pure de novo DNA-MTase, methylates the internal C in the sequence GCGC in both strands of the DNA molecule (22). Thus, it can methylate, de novo, a subset of the CpG dinucleotides in the eukaryotic genome that is a natural target for the mammalian DNA-MTase. HhaI contains all the structural motifs common to prokaryotic cytosine MTases and to the terminal portions of both the human and murine DNA MTases (19–21). Our approach has created an experimental system that suggests that mammalian cells can tolerate only a narrow range of DNA methylation, and that slight increases can result in cellular transformation.

MATERIALS AND METHODS

Cell Culture. NIH 3T3 cells were grown in DMEM medium (Paragon Biotech, Baltimore, MD) containing 4.5 g/liter glucose, 0.11 g/liter sodium pyruvate, 10% bovine calf serum (Hyclone), 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO).

Expression Constructs. The retroviral vector pZipNeo SV (X), which contains the Moloney murine leukemia virus LTR and a neomycin resistance gene (23), was used to introduce the M. HhaI prokaryotic cytosome DNA methyltransferase gene into mouse fibroblast NIH 3T3 cells. A previously described 1.5-kb HindIII fragment containing the bacterial M. HhaI methyltransferase gene (22), was ligated with BamHI linkers and introduced by standard ligation procedures into a BamHI site, in both sense and antisense orientations, downstream from the 5' LTR in pZipNeo SV(X) (23). The orientation of the M. HhaI methyltransferase gene insert was verified by restriction enzyme analysis. In the cotransfection experiments, the eukaryotic expression vector pBabe-Hygro (24), which contains a hygromycin resistance gene under the control of the SV40 early promoter, was also used. pCMV-Neo (25, 26) was also used for the expression of M. HhaI methyltransferase. In this vector, the inserted gene is under the control of the CMV promoter, and the vector also contains the neomycin phosphotransferase gene under the control of a herpes simplex virus type I thymidine kinase gene promoter. Finally, in some experiments, we utilized a zinc-inducible construct with the HhaI gene.

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placed behind the sheep metallothionein Mase promoter (27, 28) in a plasmid containing the neomycin resistance gene.

**PCR Mutagenesis of the M. HhaI Methytransferase Gene.** As a control for the wild-type M. HhaI methyltransferase construct, a mutant gene was prepared by using a PCR approach (29). The cysteine residue at the proline-cysteine dipeptide, which is absolutely required for enzyme function (30, 31), was targeted for the mutagenesis. For this procedure, a 5′ sense primer (5′-AAAGCTTGCATAAATATGCC-3′) located at the utmost 5′ region of the cloned M. HhaI methyltransferase fragment, and a 3′ antisense primer (5′-TATCGACGGAGTTCGAA-3′), corresponding to the last 19 bp of the cloned fragment, were prepared with a BamHI restriction site sequence added to the 5′ ends of each primer. Primers corresponding to nucleotide positions 668–680, where the conserved proline-cysteine is located, were synthesized in both orientations with a single G to A nucleotide substitution at position 678, changing the codon for cysteine (TGC) to the codon for tyrosine (TAC).

**Transfection of Cultured Cells.** The plasmids used for transfection were purified by either cesium chloride centrifugation or Qiagen plasmid purification kits (Qiagen, Chatsworth, CA). Cells (1 × 10^6) that had been plated in 60-mm cell culture dishes 24 h earlier were transfected by the calcium phosphate procedure (32, 33) or lipofection (34), using 40 μg of lipofectin (GIBCO-BRL) and procedures suggested by the manufacturer. Twenty-four h after transfection, cells were trypsinized, plated onto three 100-mm plates, and selected in 500 μg/ml (active strength) geneticin (GIBCO-BRL, Gaithersburg, MD) or 200 μg/ml of hygromycin B (Boehringer Mannheim, Indianapolis, IN) to derive stably transfected cells. Cell colonies were picked at 14–20 days after transfection by using cell cloning cylinders (Bellco Glass, Vineland, NJ) and trypsinization.

**DNA Methylation Level Determination.** A modification of the methyl-acceptance assay (35) was used to determine the methylation status of HhaI recognition sequences in the DNA isolated from the transfected cells. Twenty μg of genomic DNA, isolated as described above, were sheared 5 times with a 25-gauge needle to assure accurate pipetting and further incubated with 5 μg/ml DNase-free RNase A (Boehringer Mannheim, Indianapolis, IN) at 37°C for 2 h, neutralized, precipitated, and resuspended as described above. The purified DNA was quantified by UV absorbance at 260 nm wavelength and diluted to 50 ng/μl, and the concentration was checked again by UV absorbance to assure accurate quantitation. The DNA prepared in this way had no detectable RNA contamination by ethidium staining and was uniform in a size above 20 kb as judged by electrophoresis mobility in 1% agarose gels.

DNA (150 ng) was incubated with 5 units of HhaI methylase (New England Biolabs; 1 unit is the amount of enzyme required to protect 1 μg λ phage DNA, during 1-h incubation at 37°C, from cleavage by HhaI restriction endonuclease). The 20-μl reaction mixture also contained 1.25 μM S-adenosyl-L-[methyl-3H]methionine (80–85 Ci/mmolly, Amersham, TRK 581), 50 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 5 mM 2-mercaptoethanol. The reaction was incubated at 37°C for 4 h and stopped by adding 5 μl of 2.5 mM nonradioactive S-adenosylmethionine (New England Biolabs). The reactions were then spotted on a GF/C 2.4-cm² Whatman filter disc that had been presoaked with 100 μl of 1 mg/ml S-adenosylmethionine (Sigma Chemical Co., St. Louis, MO). The filters were then air dried for 15 min, washed with 10 ml 5% TCA-10 ml 70% ethanol, placed in a scintillation vial containing 10 ml Enconofluor (DuPont New England Nuclear, Boston, MA), and counted in a Beckman liquid scintillation counter. Reactions without either DNA or enzyme added were included as background controls. All samples were done in duplicate or triplicate, and values were obtained as dpm/amount of DNA.

For quantitation of the methylation status at all CpG sites, 150 ng of DNA were mixed with 4 units of M. SstI CpG methylase (New England Biolabs; same unit definition as HhaI methylase) in the presence of 1.5 μM S-adenosyl L-[methyl-3H]methionine (80–85 Ci/mmolly, Amersham, TRK 581) and 1.5 μM nonradioactive S-adenosylmethionine (New England Biolabs). The reaction was incubated at 37°C for 4 h in a buffer containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM MgCl₂, and 1 mM DTT in a 20-μl total volume. The rest of the assay steps are identical to the M. HhaI assay described above.

**Soft Agar Cloning.** SeaPlaque agarose (1.6%; FMC Bioproducts, Rockland, Maine) was mixed with an equal volume of 2X culture medium required by the specific cell type to be studied to make a 0.8% bottom layer of agarose. Cells in monolayer were trypsinized to single cells and mixed with 0.4% agarose in 1X medium. The cell-agarose mixtures were plated onto the bottom 50 mM NaCl, 10 mM MgCl₂, and 1 mM DTT in a 20-μl total volume. The rest of the enzyme added were included as background controls. All samples were done for each cell line tested. The mice were observed weekly, and tumor growth was measured at day 18 after injection.

**RESULTS**

**Functional Status of the M. HhaI Gene Expression Constructs.** To evaluate the functional status of our expression constructs, we transformed the Escherichia coli strain MC1061 with the sense, antisense, and mutant forms of the M. HhaI gene in pZip-Neo SV(X) plasmids. Because the HhaI methyltransferase insert in the expression vector contains its own prokaryotic promoter and terminator, the sense and antisense constructs can be bidirectionally transcribed by E. coli, and functional protein can be generated from each construct independent of the eukaryotic promoter/enhancer sequences in the mammalian expression vector pZipNeo SV. The wild-type M. HhaI gene, from both the sense and antisense vectors, is functional as indicated by the fact that the plasmid DNA isolated from the bacteria containing the M. HhaI constructs was resistant to digestion by the methylation-sensitive HhaI restriction enzyme (Fig. 1, compare Lanes 1, 2, and 3). In contrast, the M. HhaI construct, which is mutated in the catalytic site, was completely inactive as a methyltransferase in the E. coli as indicated by the complete sensitivity of the isolated plasmid to HhaI restriction enzyme digestion (Fig. 1, Lane 4).

**Fig. 1.** Functional assessment of the expression constructs containing the wild-type, antisense, and mutant HhaI gene. One μg of each plasmid isolated from the E. coli strain MC1061 was digested with 10 units of the cognate methylation sensitive restriction enzyme HhaI. Lane 1, plasmid without insert; Lane 2, wild-type M. HhaI in sense orientation; Lane 3, M. HhaI in the antisense orientation; Lane 4, the mutant M. HhaI construct. The Hha restriction sites were fully methylated in the sense and antisense wild-type M. HhaI constructs, as indicated by the higher molecular weight plasmid DNA, which was resistant to Hha restriction digestion (Lanes 2 and 3). DNA from the vector and the mutant M. HhaI plasmid was unmethylated, as indicated by complete digestion by the Hha restriction enzyme (Lanes 1 and 4).
Effects of Expressing the Wild-Type M. HhaI DNA Methyltransferase Gene on G418 Selection of NIH 3T3 Cells. When equal amounts of the expression vector containing the sense, antisense, or mutant M. HhaI inserts were transfected into equal numbers of NIH 3T3 cells by either calcium phosphate precipitation or liposome-mediated gene transfer techniques, both the mutant M. HhaI gene (Fig. 2C) and the wild-type gene in the antisense orientation (Fig. 2B) yielded numerous cell colonies surviving G418 selection. In contrast, transfection of cells with the construct containing the wild-type M. HhaI gene in the sense orientation repeatedly resulted in very few cells surviving in G418 (Fig. 2A). These results, summarized in Table 1, suggested that the expression of the M. HhaI methyltransferase gene might be lethal to the majority of the murine cells.

Cell Lethality Effects of the M. HhaI Construct Are Directly Due to the Methyltransferase Activity of the Inserted Gene. We first explored whether the wild-type M. HhaI gene was actually responsible for the cell lethality, or whether the sense M. HhaI construct might merely not be functional for another reason. Because the sense M. HhaI plasmid DNA, as extracted from E. coli, was fully methylated at the GCGC sites, the possibility that methylation might cause lethality due to nonexpression of the neomycin resistance gene was first considered. However, the antisense HhaI plasmid, which was also completely methylated at the HhaI sites (Fig. 1, Lane 3), was able to express the neomycin resistance gene and yield colony numbers equivalent to the mutant plasmids that are not methylated (Fig. 2, B and C). This fact strongly suggested that the initial methylation status of the transfected plasmid did not play a significant role in the cell lethality conveyed by the sense orientation M. HhaI construct.

To test further for nonfunction of the G418 resistance gene, we considered that if the observed lethality of the sense M. HhaI construct were caused by this problem, the lethal phenotype should be abolished if neomycin were not used for selection. However, if the M. HhaI enzyme is actively toxic to the cells, the lethality should be dominant. To evaluate these possibilities, we performed cotransfection experiments using a second selectable marker. NIH 3T3 cells were cotransfected with the pBabe-hygro plasmid, which confers hygromycin resistance (24), and either the sense, antisense, or the mutant M. HhaI plasmid. In the pBabe-hygro vector, the hygromycin resistance gene is under control of the SV40 early promoter, which has been shown to be insensitive to HhaI methylation (36). Thus, even if the pBabe-hygro plasmid is methylated when cotransfected into 3T3 cells with the wild-type pZip-Neo-HhaI gene construct, the hygromycin gene should be active and capable of rescuing the cells when selected in hygromycin. In these studies, lethality was still seen in those cells transfected with the sense wild-type M. HhaI as shown in Table 1. In experiments 1 and 2, using a 1:10 ratio of pBabe-hygro to M. HhaI constructs, a marked decrease in cell survival to hygromycin was observed with the sense M. HhaI construct but not with the mutant or antisense M. HhaI constructs. In experiment 3, using a 1:5 ratio of the same plasmids, some increased survival was observed for the sense M. HhaI vector, but the lethality was still evident (Fig. 2, D, E, and F). In addition, we investigated whether one other expression vector would rescue the cells when cotransfected into the NIH 3T3 cells with either the wild-type M. HhaI expression vector or a control plasmid (pBluescript). As shown in Table 1, a pCMV-Neo vector (22, 23) is also unable to rescue cells cotransfected with the M. HhaI gene because these cells have less than 20% of the survival for cells transfected with pBluescript plasmid.

As one final test for the direct toxicity of the M. HhaI gene in the 3T3 cells, we also placed the M. HhaI gene directly under the control of a CMV promoter (37). We again chose the CMV promoter because it contains no HhaI recognition sites (37). The same lethality was seen for the wild-type but not the mutant gene. In an experiment with duplicate transfection plates, the mutant HhaI construct yielded 132 ± 4 colonies, whereas the wild-type HhaI construct yielded only 4.5 ± 0.5 colonies. Thus, direct methylation of the promoter did not seem to explain our results, and these studies with the CMV promoter provided further evidence for a lethal effect of the sense M. HhaI construct.

These data indicate that expression of the wild-type M. HhaI gene is directly toxic to NIH 3T3 cells. That this effect is directly due to the enzymatic activity of the protein is shown by the results with the mutant M. HhaI gene, in which a single bp change in the codon for the cysteine residue at amino acid position 81 completely abolished both the enzyme activity, as assayed by the methylation status of the

Table 1 Transfection and co-transfection studies showing the lethality of wild-type HhaI methylase

<table>
<thead>
<tr>
<th>Transfection method</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cointransfection plasmid</td>
<td>Calcium phosphate</td>
<td>Calcium phosphate</td>
<td>Lipofection</td>
<td>Lipofection</td>
</tr>
<tr>
<td>Selection agent</td>
<td>None</td>
<td>pBabe-hygro (1 μg)</td>
<td>None</td>
<td>pBabe-hygro (1 μg)</td>
</tr>
<tr>
<td>Sense HhaI (10 μg)</td>
<td>G418</td>
<td>0.3 ± 0.3</td>
<td>G418</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>Antisense HhaI (10 μg)</td>
<td>42.7 ± 6.7</td>
<td>41</td>
<td>51</td>
<td>70 ± 6.4</td>
</tr>
<tr>
<td>Mutant HhaI (10 μg)</td>
<td>65.7 ± 4.7</td>
<td>49</td>
<td>38</td>
<td>80 ± 0.8</td>
</tr>
<tr>
<td>pBluescript (10 μg)</td>
<td>319</td>
<td>618</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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HhaI METHYLTRANSFERASE INDUCES CELL LETHALITY AND TRANSFORMATION

Table 2. Lethality of the Psi-2 cells induced by transfection with the wild-type M. HhaI.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Colony numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p\text{ZipNeoSVX} )</td>
<td>236 ( \pm 8 )</td>
</tr>
<tr>
<td>Antisense M. HhaI</td>
<td>166 ( \pm 9.1 )</td>
</tr>
<tr>
<td>Sense M. HhaI</td>
<td>1.15 ( \pm 0.54 )</td>
</tr>
<tr>
<td>Mutated M. HhaI</td>
<td>174 ( \pm 54 )</td>
</tr>
</tbody>
</table>

a Data are expressed as numbers of colonies surviving G418 selection per \( 1 \times 10^5 \) cells transfected with the M. HhaI constructs.

b Data from a single experiment performed in duplicate. All other data are derived from 2 to 7 separate experiments. Numbers in parentheses, \( \pm 1\) SD.

plasmid in \( E. coli \) (Fig. 1, Lane 4), as well as the lethal effects in the 3T3 cells (Fig. 2C).

Characterization of Cells Surviving Transfection. As shown in Table 1, a few clones transfected with the sense wild-type M. HhaI construct survived G418 selection in each experiment. We analyzed these clones for the integration of the M. HhaI gene insert. In most of the surviving sense M. HhaI-transfected clones, the insert was rearranged. Fig. 3A, Lane 1, shows a typical surviving sense M. HhaI-transfected clone, of many analyzed, in which the M. HhaI gene insert has been rearranged and is detected as two fragments of greater than 1.5 kb, rather than the expected one BamHI 1.5-kb restriction fragment, which should result from removing the intact insert from the vector. In the cotransfection experiments, we also analyzed pools of surviving clones for the integration of M. HhaI. For culture dishes transfected with mutant (Fig. 3, Lanes 2 and 5) or antisense (Fig. 3, Lanes 3 and 6) M. HhaI, regardless of the cotransfection selection agents (Fig. 3: neo for Lanes 2 and 3, hygro for Lanes 5 and 6), an intact 1.5-kb BamHI fragment of the M. HhaI gene was always detected, although some rearranged inserts were also seen (bands above or below 1.5 kb). Intact insert was found in pooled clones, as shown, and in at least 6 individual clones transfected with either the mutant or antisense M. HhaI (data not shown). However, among the few surviving colonies cotransfected with wild-type M. HhaI (Fig. 3, Lanes 4 and 7), no integration of the M. HhaI gene is detected regardless of whether drug resistance plasmid is cotransfected (Fig. 3, Lane 4, pCMV-Neo; Lane 7, pBabe-hygro). To prove that good quality DNA was loaded in those lanes where M. HhaI insert was not detected, the same blot was stripped and rehybridized with a probe for the endogenous DNA-MTase gene. As shown in Fig. 3B, no apparent underloading of DNA was observed. Thus, the obligatory rearrangement in the surviving wild-type M. HhaI-transfected cells, and the absence of detectable M. HhaI sequences in the surviving cells of the cotransfection experiments with the pBabe-Hygro or pCMV-Neo vector, provided further strong evidence that expression of M. HhaI is incompatible with survival of NIH 3T3 cells.

Lethal and Tumorigenic Effects of the M. HhaI Gene in Other Murine Fibroblast Cell Lines. As shown above, the surviving colonies of the wild-type M. HhaI-transfected NIH 3T3 cells were very few in number, and most of them had rearrangements of their inserts regardless of whether the CMV promoter, or the Moloney murine leukemia virus LTR promoter, was used to drive the expression of the M. HhaI gene. Retroviruses are very efficient vehicles for transfer of foreign genes into eukaryotic cells and provide more predictable integration than transfection approaches (38). Because the pZip-Neo construct is a retroviral vector (23), we explored the possibility that infection with this construct, using the Psi-2 helper-free packaging cell line (39), might force survival of sense M. HhaI clones without a rearranged insert. When multiple constructs including the pZip-Neo vector alone, the sense, antisense, or mutant M. HhaI were transfected into the Psi-2 packaging lines, the same lethality is observed as for the NIH 3T3 cells (Table 2). To capture packaged viral particles, we harvested the supernatant of the Psi-2 cells 48 h after transfection with the different constructs mentioned above and used these to infect the PA 317 amphotrophic packaging cell line (40). Five sense clones survived the G418 selection and were compared with five antisense clones derived from the infection of PA 317 cells. Southern and Northern analysis showed integration and expression of the M. HhaI gene for both the sense and antisense constructs (data not shown). One of the sense clones showed morphology changes suggestive of cell transformation. The cells were more spindle shaped with more cells loosely attached (data not shown). This clone had a much higher
control the expression of the M. H/wI gene. When NIH 3T3 cells were
by day 18/no. of animals given injections.

clones; mutant HhaI, 162 clones). However, in the cells exposed to 50
promoter, and in the presence of zinc, had levels of M. H/ia! tran
all tested surviving clones with M. H/ia! driven by a zinc inducible
promoters, such as CMV, it was impossible to directly test this
associated with the wild-type M. H/iaI gene controlled by constitutive

shown in Table 3, 18 days after injection, the tumorigenicity of the
those that are lethal to the cells. Because of the extensive lethality
few clones surviving infection with the retroviral construct. It seemed
the metallothionein promoter appeared to be transformed, as were the
clones containing the mutant M. Hhal gene behind the metallothionein promoter were
not clonogenic in soft agar (cloning efficiency, 0%) regardless of the
presence or absence of zinc. The wild-type clones with the M. Hhal, 162 clones). However, in the cells exposed to 50
μm zinc, a 71% reduction of colony number (45 clones) was observed
when compared to cells transfected with the wild-type M. Hhal
constructs in the absence of zinc.

The phenotype of the clones surviving the above transfection was
assessed by testing the clonogenicity in soft agar. Clones containing
the mutant M. Hhal gene behind the metallothionein promoter were
not clonogenic in soft agar (cloning efficiency, 0%) regardless of the
presence or absence of zinc. The wild-type clones with the M. Hhal, 162 clones). However, in the cells exposed to 50
μm zinc, a 71% reduction of colony number (45 clones) was observed
when compared to cells transfected with the wild-type M. Hhal
constructed in the absence of zinc.

Thus, the cells transfected with the wild-type M. Hhal gene behind
the metallothionein promoter appeared to be transformed, as were the
few clones surviving infection with the retroviral construct. It seemed
probable that the inducible promoter allowed, for those cells that
survived, the expression of the M. Hhal gene at levels lower than
those that are lethal to the cells. Because of the extensive lethality
associated with the wild-type M. Hhal gene controlled by constitutive
promoters, such as CMV, it was impossible to directly test this
hypothosis using the wild-type gene. Thus, we compared expression
of the mutant M. Hhal gene driven by the CMV promoter to expres
of both the wild-type and mutant genes driven by the inducible
promoter. By Northern analysis, the CMV promoter appeared to be at
least 3 times as efficient as the inducible promoter in expressing the
mutant M. Hhal gene (Fig. 4; compare Lanes 2 and 6). Furthermore,
all tested surviving clones with M. Hhal driven by a zinc inducible
promoter, and in the presence of zinc, had levels of M. Hhal tran
scripts much lower than for the mutant M. Hhal driven by the CMV
promoter (Fig. 4; compare Lane 2 to Lanes 4, 6, and 7–10). Thus, all
of these data suggest that high levels of M. Hhal expression in NIH
3T3 cells produce a lethal effect, whereas low levels are compatible
with survival of cells with a transformed phenotype.

Methylcytosine Content of the Surviving Cells. If transformation of the cells surviving expression of the Hhal from the inducible
metallothionein promoter is directly due to the enzymatic activity of
this gene, then alterations in the methylation status of Hhal sites in

Table 3 Nude mice tumorigeninity of PA 317 cells infected with sense or antisense
M. Hhal constructs

<table>
<thead>
<tr>
<th>Antisense M. Hhal</th>
<th>Sense M. Hhal</th>
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<tbody>
<tr>
<td>Clone A</td>
<td>0/5</td>
</tr>
<tr>
<td>Clone B</td>
<td>1/10</td>
</tr>
<tr>
<td>Clone C</td>
<td>0/5</td>
</tr>
<tr>
<td>Clone D</td>
<td>3/3</td>
</tr>
<tr>
<td>Clone E</td>
<td>1/4</td>
</tr>
<tr>
<td>Total</td>
<td>5/27</td>
</tr>
</tbody>
</table>

a Numbers in column = number of animals with a mass of 10 mm diameter or greater
day 18/no. of animals given injections.

b Statistically significantly different from antisense by Fisher's exact and χ² tests
(P < 0.0001).

Fig. 4. Northern analysis of RNA from NIH 3T3 cells transfected with the M. Hhal
gene controlled by an inducible promoter. Thirty μg of total RNA were loaded in each
cane. Lane 1, RNA isolated from a mix of 9 clones that survived a transfection with
pCMV-Hhal construct (note: no M. Hhal transcript can be detected); Lane 2, RNA
isolated from pooled clones of cells transfected with pCMV-mutant Hhal; Lane 3, RNA
isolated from pooled clones of cells transfected with pMT-Hhal in the absence of zinc;
Lane 4, RNA isolated from pooled clones of cells transfected with pMT-Hhal in the
presence of 50 μM zinc for 10 days; Lane 5, RNA isolated from pooled clones of cells
transfected with pCMV-mutant Hhal in the absence of zinc; Lane 6, RNA isolated from
pooled clones of cells transfected with pMT-mutant Hhal in the presence of 50 μM zinc
for 10 days; Lanes 7–10, 4 individual clonogenic pMT-Hhal-transfected clones isolated
from a soft agar plate and expanded in the usual culture conditions before RNA isolation.
These four clones have been exposed to zinc from the start of soft agar assay to the cell
harvest for RNA preparation for about 30 days. The blot is first probed with Hhal insert
(top). The size difference between the CMV and metallothionein constructs is due to the
different lengths of the untranslated sequences in the vectors. The same blot was reprobed
for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, bottom).

Fig. 5. Increased methylation level at Hhal sites in the clonogenic pMT-Hhal
transfectants. Results for clones transfected with a metallothionein-inducible promoter-driven
wild-type versus the mutant Hhal insert are depicted. The methyl acceptance assay (see
"Materials and Methods") was used to compare 4 wild-type Hhal clones and 3 mutant
Hhal clones. The values represent the changes in labeled methyl groups between a vector-
alone transfected clone (100%) and each individual wild-type and mutant clone as
indicated in the graph. sl–s4, sense clones; m1–m3, mutant clones.
HhaI METHYLTRANSFERASE INDUCES CELL LETHALITY AND TRANSFORMATION

Table 4 Methylation levels at HhaI sites (GCCG) vs. all CpG dinucleotides

<table>
<thead>
<tr>
<th>HhaI Methylase</th>
<th>3T3 Control</th>
<th>3T3 Methylase</th>
<th>Sense</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense</td>
<td>Control</td>
<td>3T3 Control</td>
<td>3T3 Methylase</td>
<td></td>
</tr>
<tr>
<td>3678.9</td>
<td>4950.8</td>
<td>96372.5</td>
<td>100654</td>
<td></td>
</tr>
<tr>
<td>3018.4</td>
<td>4823.2</td>
<td>95068.5</td>
<td>99262</td>
<td></td>
</tr>
<tr>
<td>3848.9</td>
<td>5213.3</td>
<td>97201</td>
<td>95293.5</td>
<td></td>
</tr>
<tr>
<td>4115.7</td>
<td>4942.6</td>
<td>95717.5</td>
<td>97591</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>3665.5</td>
<td>4982.5</td>
<td>96089.9</td>
<td>98208.6</td>
</tr>
<tr>
<td>SE</td>
<td>233.7</td>
<td>82.3</td>
<td>456.1</td>
<td>1156.2</td>
</tr>
<tr>
<td>P value</td>
<td>0.006</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The values in the table represent methyl-acceptance capacity in dpm/150 ng of DNA. The control group consists of one vector alone and 3 mutant M. HhaI-transfected clones. The sense M. HhaI-transfected group includes four independent clones. The P values are derived from t tests between the control and sense clones for each methylase.

3T3 cell DNA would be expected. To test this possibility, we assayed for the methylcytosine content of the surviving cells by a methyl-acceptance assay, in which the DNA isolated from the transfected cells was incubated with HhaI methylase. In this assay, the capacity of the DNA to accept radiolabeled methyl groups is inversely proportional to the degree of endogenous methylation at these sites. Four clonogenic wild-type M. HhaI clones isolated from soft agar plates were compared with 3 mutant M. HhaI, and one vector alone transfected clones. Among the 4 wild-type M. HhaI-transfected clones, there was a 16.7–38.9% (average, 27%) reduction in methyl acceptance, as compared to the vector alone transfected clone. In contrast, methyl acceptance levels for the mutant M. HhaI clones are within 5.5% of those for the vector alone control clone (Fig. 5 and Table 4). Statistically, the difference between the wild-type clones and the control clones is highly significant (P = 0.006). To evaluate whether this methylation increase was truly specific for the HhaI sites, we also used a generalized CpG MTase, M. SssI (41, 42), for the methylcytosine content of the surviving cells by a methyl acceptance assay, in which the DNA isolated from the transfected clone. In contrast, methyl acceptance levels for the mutant M. HhaI clones are within 5.5% of those for the vector alone control clone (Fig. 5 and Table 4). Statistically, the difference between the wild-type clones and the control clones is highly significant (P = 0.006). To evaluate whether this methylation increase was truly specific for the HhaI sites, we also used a generalized CpG MTase, M. SssI (41, 42), for the methylcytosine acceptance assay. This enzyme de novo methylates all CpG dinucleotides. Because HhaI sites mathematically represent a tiny fraction of these sites, differences in overall DNA methylation should not be present if methylation changes have occurred specifically at only HhaI targets. Indeed, we saw no significant divergence in overall CpG methylation between the wild-type and mutant transfected clones (Table 4).

DISCUSSION

Our present data indicate that expression of the prokaryotic de novo methyltransferase gene, M. HhaI, which catalyzes the methylation of the 5’C in the sequence GCCG, profoundly alters the phenotype of murine fibroblasts in at least two ways. High constitutive expression of this enzyme appears to be predominantly lethal to the cells, whereas lower levels of expression lead to cell transformation. These results then suggest that, within a narrow range, the degree of increase in de novo methylation of cytosines may be critically important for the cell phenotype. This effect is specific for modifying cytosine at the CpG dinucleotides because expression of a bacterial adenine methyltransferase, which resulted in exclusive methylation of adenines in cellular DNA, has no apparent phenotypic affects in eukaryotic cells (43).

The precise mechanism by which increased DNA-MTase activity causes either lethal or tumorigenic changes of cultured cells remains to be determined. However, we favor the likelihood that the HhaI overexpression may cause alterations of DNA methylation similar to those consistently observed in rodent and human immortalized and neoplastic cells (3, 11, 12, 44–49). One such alteration involves hypermethylation in normally unmethylated CpG islands located in the promoter regions of genes. These changes can lead to ablation of the transcription of the genes involved (7, 10, 50). It has been postulated that such changes, when occurring in genes that are vital for basic cell functions, will lead to cell death (12). However, in our surviving cell clones with less HhaI expression, the hypermethylation might selectively involve promoters of tumor suppressor genes for which loss of expression favors unbridled growth, loss of differentiation capacity, or prolongation of cell survival. In fact, such regional hypermethylation has now been associated with at least 3 known tumor suppressor genes [Rb (13, 14), VHL (15), and CDKN2/p16 (16)]. For the latter two genes, the methylation change is associated with loss of transcription, the involved genes can be reactivated by demethylation (15, 16), and the aberrant methylation acts as an alternative mechanism to mutations in the coding regions of the gene for functional inactivation (15, 16).

The overexpression of the HhaI gene might also alter cellular phenotype by causing a high mutation rate of genes receiving new methylcytosines. Methylcytosines are sites of endogenous mutations in multiple genetic diseases such as hypercholesterolemia and cancer (51, 52) and are also hot spots for somatic mutation. For instance, in colon cancer, more than 67% of the mutations in the p53 tumor suppressor gene are C to T transitions (53). Finally, the increased DNA-MTase activity itself, through the catalysis mechanism proposed for the enzymatic process, can theoretically directly cause C to T transitions (17, 54, 55).

Finally, we cannot rule out that the HhaI overexpression might cause cell lethality through less physiological mechanisms. Thus, the increased HhaI site methylation could affect regions in and around the inserted constructs containing the G418 or hygromycin resistance genes. Such methylation, through altered chromatin configuration, might inactivate the resistance gene and account for the sharp decrease in selection efficiency. Given the variable insertion sites observed for each of our constructs during either cell transfection, or cell infection with the retroviral constructs, we consider this mechanism less likely. Also, this process would not explain the transformed state of those cells that survive with a lower overall HhaI expression. Whichever mechanism proves operative, our above observed dual effects of methylation on cell viability and transformation are most compatible with the classic tumorigenesis model suggested by Nowell (56). In his schema, during tumor progression, the majority of tumor cells that undergo DNA changes, such as mutation or altered chromosome structure, are predicted to die, whereas only occasional cells will derive changes that favor growth or increased cell survival. Our data suggest that dynamic changes in DNA methylation could be one of the key factors contributing to this process of cell death and occasional clonal expansion, which is the hallmark of tumor evolution.

We believe our present data can now serve as a useful model for recent observations concerning DNA-MTase activity and tumorigenesis. Potential capacity for DNA methylation appears to increase through all stages of colon tumor progression (1, 2), and our previous studies show that an exogenous murine DNA-MTase gene can transform NIH 3T3 cells (5). These collective data suggest that, during tumor progression, changes induced by moderate DNA-MTase increases are not merely bystander effects, but rather could constitute one of the earliest and fundamental changes of neoplastic development. In support of this concept, recent data of Laird et al. (6) show that a modest reduction of DNA-MTase activity in mice with a genetic form of colon polyps dramatically reduces tumor formation. Our present observations further emphasize the potential importance of DNA methylation balance in mammalian cells. Overexpression of a de novo DNA-MTase, and the resultant hypermethylation of cellular DNA, can be detrimental for cell survival. The more modest increases in DNA-MTase observed in immortalized and tumorigenic cells appears to reflect a key step in the selective advantage gained by cells during neoplastic progression.

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Expression of Prokaryotic \textit{HhaI} DNA Methyltransferase Is Transforming and Lethal to NIH 3T3 Cells

Jianjun Wu, James G. Herman, Geoffrey Wilson, et al.


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