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De Novo Methylation of CpG Island Sequences in Human Fibroblasts Overexpressing DNA (Cytosine-5-)-Methyltransferase

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Recent studies showing a correlation between the levels of DNA (cytosine-5-)-methyltransferase (DNA MTase) enzyme activity and tumorigenicity have implicated this enzyme in the carcinogenic process. Moreover, hypermethylation of CpG island-containing promoters is associated with the inactivation of genes important to tumor initiation and progression. One proposed role for DNA MTase in tumorigenesis is therefore a direct role in the de novo methylation of these otherwise unmethylated CpG islands. In this study, we sought to determine whether increased levels of DNA MTase could directly affect CpG island methylation. A full-length cDNA for human DNA MTase driven by the cytomegalovirus promoter was constitutively expressed in human fibroblasts. Individual clones derived from cells transfected with DNA MTase (HMT) expressed 1- to 50-fold the level of DNA MTase protein and enzyme activity of the parental cell line or clones transfected with the control vector alone (Neo). To determine the effects of DNA MTase overexpression on CpG island methylation, we examined 12 endogenous CpG island loci in the HMT clones. HMT clones expressing ≥9-fold the parental levels of DNA MTase activity were significantly hypermethylated relative to at least 11 Neo clones at five CpG island loci. In the HMT clones, methylation reached nearly 100% at susceptible CpG island loci with time in culture. In contrast, there was little change in the methylation status in the Neo clones over the same time frame. Taken together, the data indicate that overexpression of DNA MTase can drive the de novo methylation of susceptible CpG island loci, thus providing support for the idea that DNA MTase can contribute to tumor progression through CpG island methylation-mediated gene inactivation.

DNA methylation in mammalian cells occurs on the C5 position of cytosines in the CpG dinucleotide. Across the majority of the genome, these CpG sites are underrepresented and heavily methylated (7, 11, 21). In contrast, there are regions of the genome called CpG islands where CpG sites are found at or near their expected frequency. These islands are usually associated with gene promoters and remain unmethylated in normal adult tissue (7, 11). Exceptions include CpG islands associated with genes on the inactive X chromosome in females and some imprinted genes (3, 25, 35). In these cases, CpG island methylation is associated with transcriptional silencing, suggesting that the maintenance of CpG islands in a unmethylated state is important for gene activity.

The normal patterns of DNA methylation are altered during neoplastic transformation. Both loss of methylation from the heavily methylated fraction of the genome and gain of methylation in CpG islands have been described (4, 21). Since CpG island methylation is incompatible with gene transcription (7, 11) and DNA methylation patterns are propagated with cell division, aberrant methylation of CpG islands during tumorigenesis could result in the stable repression of gene expression. Such a change could confer a selective growth advantage on a cell clone if it occurred in a gene with growth suppressor activity. Indeed CpG island methylation and a corresponding lack of expression have been shown for several tumor suppressor genes, including the *VHL* gene in renal cell carcinomas (15), the *Rb* gene in retinoblastomas (32, 36), and the cyclin-dependent kinase inhibitor *p16* in many epithelial cancers (16, 30). CpG island methylation can also contribute to tumor progression by interfering with the expression of genes thought to be suppressors of invasion and metastasis, as shown for the E-cadherin gene in breast and prostate carcinomas (13), or by affecting genes involved in the detoxification of carcinogens, as shown for the *GSTP* gene in prostate cancers (22). Studies with primary tumor tissue comparing patterns of allelic loss, mutational analyses, and methylation status indicate that CpG island methylation is an alternative to mutation or deletion as a mechanism leading to tumor suppressor gene loss (15, 30, 36).

Although it is now clear that CpG island methylation-associated gene inactivation can affect a number of genes in many tumor types, little is known about the mechanism underlying this aberrant event. The loss during tumorigenesis of a mechanism that normally protects CpG islands from becoming methylated, such as *cis*-acting signals that function in this capacity during embryogenesis (8, 28), could allow de novo methylation of CpG islands in cancer cells. Alternatively, one could invoke a more direct role for the enzyme responsible for DNA methylation in mammalian cells, DNA (cytosine-5-)-methyltransferase (DNA MTase). During normal DNA replication, methylation patterns are copied by the maintenance activity of the DNA MTase which acts on the hemimethylated CpG dinucleotide generated by semiconservative DNA replication and transfers a methyl group from *S*-adenosylmethionine to the cytosine of the CpG site in the newly synthesized strand, restoring the parental methylation pattern (23, 37). De novo methylation, or the addition of new methylation to previously unmethylated CpG sites, is thought to be catalyzed in the cell by the same enzyme, although in vitro this reaction proceeds at a significantly reduced rate relative to the maintenance reaction (reviewed in reference 23).

Several recent studies have implicated DNA MTase in the tumorigenic process. DNA MTase levels tend to be higher in tumorigenic than in nontumorigenic cell lines, and a progres-

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sive increase in the expression of this enzyme accompanies colon tumorigenesis (12, 18, 19). The forced overexpression of the murine DNA MTase is sufficient to cause neoplastic transformation of NIH 3T3 cells, while decreasing DNA MTase levels by expression of an antisense construct decreases the tumorigenicity of adrenocortical tumor cells (27, 42). Recently, the use of a combination of genetic and pharmacologic means of reducing the levels of DNA MTase was shown to drastically reduce polyp formation in a mouse model of colon carcinogenesis (20). This latter observation has generated much debate regarding how DNA MTase might facilitate tumor formation. Two proposed mechanisms include the possibilities that DNA MTase activity levels affect the rate of C-to-T transition mutations or the occurrence of CpG island methylation-mediated gene inactivation events (20).

The aim of the present study was to test the hypothesis that cellular DNA MTase levels can directly affect the de novo methylation of CpG islands. We have generated human cell lines overexpressing human DNA MTase and determined the effect of increased cellular DNA MTase activity on the methylation of endogenous CpG island loci. The data indicate that increasing the levels of enzyme activity by at least ninefold can induce the time-dependent de novo methylation of CpG island sequences and therefore support a direct role for DNA MTase in the aberrant CpG island methylation that occurs during tumor progression.

MATERIALS AND METHODS

Production of human cells overexpressing DNA MTase. Overlapping cDNA clones representing the protein coding sequence for human DNA MTase (HMT) were used to construct a full-length cDNA (43). An *Xba*I-*Dra*I fragment representing the open reading frame (nucleotides 316 to 5055 of the cDNA) was subcloned into the *Bam*HI site of the pCMVneoBam vector (2). This construct, pCMV-HMT, puts the DNA MTase cDNA under the control of the cytomegalovirus (CMV) promoter-enhancer and contains the neomycin resistance gene under the control of a separate promoter (2). IMR90/SV40 (simian virus 40) cells were obtained from the National Institute on Aging Mutant Cell Repository (AG02804C) and were maintained in Eagle's minimal essential medium supplemented with 9% fetal calf serum and 2 mM glutamine (complete medium). For transfections, IMR90/SV40 cells at population doubling 136 to 140 were seeded at 1×10^6 to 2×10^6 cells per 100-mm-diameter dish, and 24 to 48 h later, each plate was transfected with 10 μg of either the pCMV-HMT or pCMVneoBam plasmid with 30 μl of Lipofectin reagent (GIBCO/BRL) for 6 h according to the recommended procedure. After 48 h of recovery in complete medium, cells were harvested and reseeded at low density in complete medium containing 400 μg of G418 (Geneticin; GIBCO/BRL) per ml (selection medium). Two to 3 weeks later, individual G418-resistant ($G418^r$) colonies were harvested with cloning cylinders. After the initial expansion to fill a T75 flask (passage 4), clonal cell populations were passaged at a split ratio of 1:8 every 5 days. Cells were maintained in selection medium at 37°C in 95% air–5% CO_2. All cultures tested negative for mycoplasma contamination.

mRNA expression analyses. Total cellular RNA was isolated from cells by the method of Chomczynski and Sacchi (10). Reverse transcriptase PCR was performed as described previously (43), except that 33 ng of random hexamers was substituted for the specific antisense primer in the reverse transcriptase reaction mixture. For the PCR mixture, 5′-CTATGGAAGGCTCGAGTG-3′ was used as the sense primer and 5′-GCCACCACCTTCTGATAG-3′ was used as the antisense primer. The former represents internal DNA MTase cDNA sequences, and the latter hybridizes to the rabbit β-globin poly(A) signal sequences in the pCMVneoBam vector, downstream of the DNA MTase cDNA. These primers allow specific amplification of mRNA synthesized from the pCMV-HMT construct. In some cases, mRNA expression was confirmed by RNase protection analysis as previously described (29), with a 364-bp *Hin*dIII-*Ssp*I fragment in the 3′ end of the human DNA MTase cDNA as the probe. In these analyses, the endogenous DNA MTase mRNA generates a 364-bp protected fragment and the mRNA synthesized from the pCMV-HMT construct generates a 280-bp protected fragment.

Generation of anti-Human DNA MTase antibodies. The rabbit polyclonal human DNA MTase antisera HMT 1147 and HMT 1509 were raised against partial human DNA MTase–glutathione-*S*-transferase (GST) fusion proteins. For the HMT 1147 antiserum, a human DNA MTase cDNA representing nucleotides 744 to 2056 (encoding amino acids 129 to 566) of the published sequence (43) was cloned into the *Eco*RI site of the pGEX-2T vector (Promega). For the HMT 1509 antiserum, a human DNA MTase cDNA representing nucleotides 2170 to 4708 (encoding amino acids 604 to 1449) (43) was cloned into the *Eco*RI site of the pGEX-3T vector (Promega). The resulting plasmids were used to transform TOPP *Escherichia coli* cells (Stratagene). GST-DNA MTase fusion proteins were produced by growth of *E. coli* at 30°C to an A_{600} of 0.8 followed by induction with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and growth for an additional 2 to 4 h. Cells were harvested by centrifugation and disrupted by sonication in phosphate-buffered saline (PBS). Triton X-100 was added to a final concentration of 1%, and insoluble material was removed by centrifugation at 10,000 × *g*. GST-DNA MTase fusion protein in the soluble fraction was partially purified by isolation on glutathione-Sepharose (Pharmacia) as recommended by the manufacturer. Fusion protein was isolated on sodium dodecyl sulfate (SDS)–8% polyacrylamide gels and gel slices containing the GST-DNA MTase fusion proteins were excised and used for antibody production. Rabbit polyclonal antisera were generated by HRP Inc. (Denver, Pa.).

Western immunoblot analysis. Log-phase cells were washed twice in PBS, pelleted, and lysed in 50 mM Tris (pH 7.5)–1 mM EDTA–150 mM NaCl–1% Nonidet P-40–0.1% SDS–1 μg of aprotinin per ml–1 μg of leupeptin per ml–1 mM phenylmethylsulfonyl fluoride. Insoluble material was removed by centrifugation at 10,000 × *g*. Protein in the soluble fraction was measured by the Bradford method (Bio-Rad), and 150 μg of cellular protein was separated on an SDS–6.5% polyacrylamide gel and electroblotted to nitrocellulose in 24 mM Tris-base–192 mM glycine–10% methanol. Filters were blocked in 20 mM Tris (pH 7.5)–137 mM NaCl–0.1% Tween 20 (TBS-T) plus 10% nonfat dry milk and then incubated with a 1:1,000 dilution of the rabbit polyclonal antiserum HMT 1147 in TBS-T plus 5% nonfat dry milk. After several washes in TBS-T, immune complexes were detected with a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody and enhanced chemiluminescence detection according to the manufacturer's instructions (Amersham).

Immunohistochemistry. Cells were grown on chamber slides for 48 to 96 h. After the growth chambers had been removed, the slides were fixed in buffered formalin and permeabilized in 0.05% saponin in PBS. The slides were then preblocked in 5% normal goat serum in PBS and incubated with a 1:1,000 dilution of the rabbit polyclonal antiserum HMT 1509 in PBS at 5°C overnight. Slides were washed in PBS and incubated for 1 h with a biotinylated goat anti-rabbit secondary antibody. Immune complexes were detected by peroxidase staining with a Vectastain Elite kit (Vector Laboratories).

DNA MTase activity. Log-phase cells were harvested by trypsinization, washed in PBS, and lysed by successive freezing and thawing in lysis buffer containing 50 mM Tris (pH 7.8), 1 mM EDTA, 1 mM dithiothreitol, 0.01% sodium azide, 10% glycerol, 1% Tween 80, and 60 μg of phenylmethylsulfonyl fluoride per ml. The protein concentration in the lysate was determined by the Bradford method (Bio-Rad). Final reactions were carried out for 2 h at 37°C as described previously (17), and the mixtures contained 1 to 5 μg of cellular protein in 15 μl of lysis buffer, which contained 3 μCi of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (~80 Ci/mmol [Amersham]) and 0.5 μg of poly(dI-dC) · (dI-dC) or poly(dG-dC) · (dG-dC) (Pharmacia) in a total volume of 20 μl. Results are expressed as the mean disintegrations per minute per microgram of protein ± standard deviation of triplicate cell extracts, each of which was assayed in duplicate.

Genomic probes. All probes were of human origin. The estrogen receptor probe was a 300-bp exon 1 fragment generated by *Eco*RI-*Pvu*II digestion of the pOR3 plasmid (American Type Culture Collection). The 1.7-kb genomic *Bam*HI fragment recognizing the pYNZ22 (D17S5) polymorphic marker used to analyze the *HIC-1* locus was a gift from Bert Vogelstein. The α-globin locus was analyzed with a cDNA probe spanning most of exons 1 and 2 that was generated from the pJW101 plasmid (American Type Culture Collection). The E-cadherin probe was synthesized by PCR from human genomic DNA as described previously (13) and represents a 270-bp PstI fragment covering the 5′ flanking sequences of the gene. The probe for the somatostatin gene was a 1.3-kb *Pst*I-*Eco*RI genomic fragment generated from the plasmid pHS7-2.7 (American Type Culture Collection). Probes were labeled with ^{32}P by the random hexamer priming method (GIBCO/BRL).

CpG island methylation analyses. DNA methylation was assessed by digestion of 5 to 10 μg of genomic DNA with a cytosine methylation-sensitive restriction enzyme (*Eag*I, *Sac*II, or *Not*I) at 20 U of enzyme per μg of DNA for 16 h at 37°C and then with an appropriate cytosine methylation-insensitive enzyme (*Hin*dIII, *Eco*RI, or *Pst*I) at 15 U of enzyme per μg of DNA for an additional 16 h at 37°C. Digested samples were separated by electrophoresis on 1% agarose gels, transferred to nylon membranes (Zetaprobe; Bio-Rad), and hybridized overnight at 60 to 65°C with 1×10^7 to 5×10^7 cpm of ^{32}P-labeled DNA probe according to standard procedures (9). Membranes were washed to a final stringency of 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 65 to 70°C and exposed to storage phosphor screens (Molecular Dynamics). Blots were stripped between hybridizations by incubation for 20 min at 95°C in 0.1× SSC–0.1% SDS. Southern blots were subjected to phosphorimage analysis with a Molecular Dynamics PhosphorImager, and band intensities were quantified with ImageQuant software (Molecular Dynamics). For each sample, the percent methylation was calculated as the intensity of the band(s) representing DNA that is not digested by the methylation-sensitive enzyme (and therefore methylated) relative to the combined intensity of all bands in the lane.

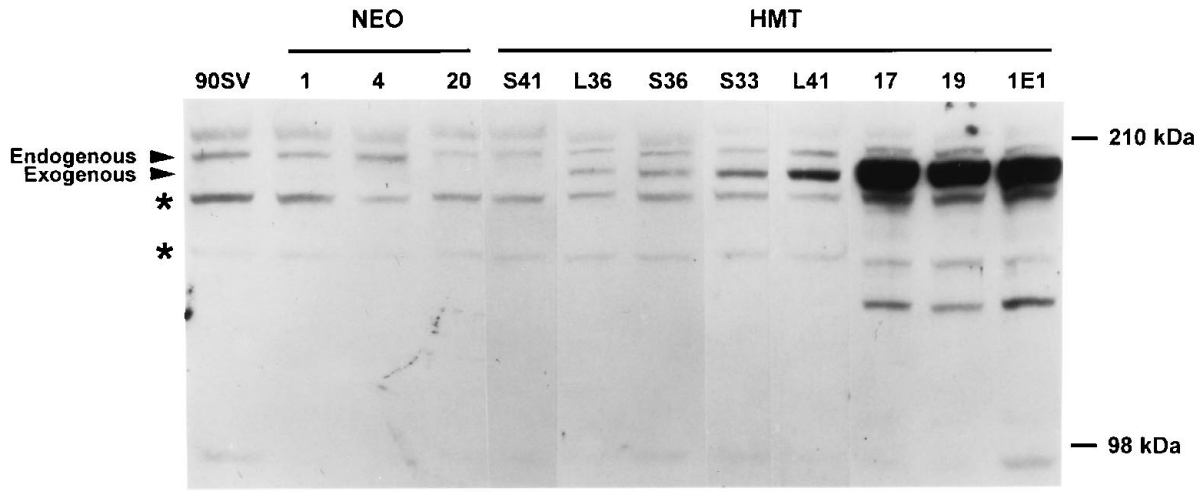

FIG. 1. Western blot analysis of DNA MTase protein in IMR90/SV40 fibroblasts and transfected clones. Total cellular protein (150 μg) isolated from both IMR90/SV40 (90SV) and the indicated Neo and HMT clones (given above each lane) was fractionated on SDS–6.5% polyacrylamide gels, electroblotted to nitrocellulose, and probed with a rabbit polyclonal antiserum to human DNA MTase (HMT 1147). Bands representing the endogenous 200-kDa migrating species and the recombinantly produced (exogenous) 190-kDa migrating species are indicated. The migrations of prestained molecular mass markers are indicated on the right. Nonspecific bands recognized by preimmune serum are starred on the left.

RESULTS

Establishment of cells overexpressing human DNA MTase. Human fetal lung fibroblasts immortalized with SV40 (IMR90/SV40) were chosen for this study for several reasons. An immortalized cell line was used to enable the study of methylation patterns over a long period of time. However, unlike most such permanent cell lines, these cells have a known culture history and were only approximately 140 population doublings from primary culture at the time of transfection. This is an important consideration for the study of CpG island methylation, because, as described by Antequera et al. (1), cell lines that have been in continuous culture for many years are likely to have already accumulated a significant degree of CpG island methylation. In this regard, and as described in more detail below, we had as part of a previous study identified a number of CpG islands that were either unmethylated or had accumulated only low-level methylation in the IMR90/SV40 cells at the time of transfection (40).

IMR90/SV40 cells were transfected with either the pCMV-HMT construct, which contains a full-length cDNA for human DNA MTase (referred to as HMT), or with the vector (pCMVneoBam) alone (referred to as Neo), and a series of G418^r clones were established from three separate transfection experiments. Individual clones were initially characterized for expression of the exogenous DNA MTase at the mRNA level by reverse transcriptase PCR. A sense primer directed at the 3′ end of the HMT cDNA and an antisense primer specific to the vector were used to specifically amplify mRNA generated from the pCMV-HMT construct. In some cases, a RNase protection method capable of distinguishing between the exogenous and endogenous messages was also used (data not shown).

Those clones that were positive for recombinantly produced message were then screened for the production of DNA MTase protein. Western analyses of whole-cell extracts from the various cell lines identified the endogenous 200-kDa DNA MTase protein in the parental cell line and in all transfected clones (Fig. 1). A second DNA MTase-specific species with an apparent molecular mass of 190 kDa was also detected in some of the clones transfected with pCMV-HMT, but not in the parental cells or the pCMVneoBam-transfected clones, indicating that this 190-kDa DNA MTase protein was expressed from the pCMV-HMT construct (Fig. 1). The mobility of the 190-kDa recombinant protein was confirmed by transient transfection of COS-7 cells with an expression vector containing the same DNA MTase cDNA driven by the SV40 early promoter followed by Western analysis for human DNA MTase protein (data not shown).

The underlying reason for the difference in mobility between the endogenous and recombinant DNA MTase proteins is not known but may involve differences in posttranslational modification or the existence of additional mRNA sequences not represented in the cloned cDNA. Despite this difference, the human cDNA used in these studies is equivalent to the murine cDNA (5) used to generate recombinant DNA MTase protein that preferentially methylates hemimethylated DNA substrates (38), localizes to nuclei (24), targets replication foci during the S phase of growth (24), and transforms NIH 3T3 cells (42). As described below, we further demonstrate that the 190-kDa protein generated in the HMT clones has characteristics similar to those of the native human DNA MTase protein.

Analysis of the majority of G418^r clones for expression of the recombinant DNA MTase protein revealed that these were a minority; of the 50 to 60 HMT clones surviving selection, only 8 were found to express the 190-kDa recombinant protein, and of these only 7 maintained expression for at least 20 passages. We also observed that, in general, the clones that were eventually shown to produce the highest levels of DNA MTase (HMT.L41, HMT.17, HMT.19, and HMT.1E1) were among the last clones to be analyzed; i.e., they were the slowest growing and took longer to expand. After the initial expansion, the HMT.17 and HMT.19 clones grew at a rate comparable to that of several Neo clones analyzed (doubling time, 24 to 28 h), while the HMT.L41 and HMT.1E1 clones continued to grow more slowly (doubling times, 31 and 40 h, respectively) for about 20 passages, after which their growth rate gradually increased and approached that of the Neo clones. These combined data suggest that overexpression of DNA MTase may not be well tolerated by cells.

Characterization of DNA MTase overexpressors. The seven HMT clones that stably produced the 190-kDa DNA MTase

TABLE 1. DNA methyltransferase activity in IMR90/SV40 clones

Cell clone	Mean activity (dpm/μg of protein)[a] ± SD		Ratio (maintenance/de novo activity)
	Maintenance[b]	De novo[c]	
IMR90/SV40	1,319 ± 226	66 ± 9.5	20
Neo.1 p21	1,293 ± 177	ND[d]	
Neo.4 p21	1,739 ± 85	59 ± 2.5	29
Neo.20 p21	1,410 ± 233	52 ± 3.7	27
HMT.S41 p21	1,622 ± 274	ND	
HMT.S36 p20	1,867 ± 164	ND	
HMT.L36 p20	2,461 ± 272	ND	
HMT.S33 p21	4,729 ± 459	ND	
HMT.L41 p17	13,441 ± 1,948	ND	
HMT.17 p26	51,963 ± 4,313	1,621 ± 27	32
HMT.19 p28	68,512 ± 9,135	2,610 ± 383	26
HMT.1E1 p20	74,940 ± 6,636	2,260 ± 24	23

[a] Units of activity are for a 2-h assay. Data are expressed as the mean ± standard deviation (SD) for triplicate cell samples assayed in duplicate.
[b] Determined with 0.5 μg of poly(dI-dC) · (dI-dC) as DNA substrate.
[c] Determined with 0.5 μg of poly(dG-dC) · (dG-dC) as DNA substrate.
[d] ND, not determined.

species for at least 20 passages were selected for further study. From the Western analyses, it was apparent that the levels of expression of the exogenously produced 190-kDa protein varied among the various clones. By comparison of serially diluted samples, the amounts of total DNA MTase protein (i.e., endogenous plus recombinantly produced) relative to the levels of endogenous protein were estimated to be >20 in the HMT.19 and HMT.1E1 clones, about 10 to 12 in the HMT.17 clone, about 5 in the HMT.L41 clone, about 3 in the HMT.S33 clone, and 1 to 2 in the HMT.L36 and HMT.S36 clones (Fig. 1 and data not shown). In addition, several of the HMT-overexpressing clones were analyzed by immunohistochemistry to determine whether the recombinant protein was targeting the nucleus. Staining for DNA MTase in the HMT clones (HMT.1E1, HMT.19, and HMT.17) was confined to the nucleus and was markedly more intense than the nuclear staining of two Neo clones studied (data not shown).

To test whether the recombinant DNA MTase protein in the overexpressing cells was active, DNA MTase enzyme activity was compared in cell extracts from the HMT clones, several Neo clones, and the parental cell line (Table 1). Poly(dI-dC) · (dI-dC) was used as a substrate for these assays because its ability to be methylated by DNA MTases in vitro is comparable to that of hemimethylated DNA substrates (6, 33), thus giving a measure of maintenance DNA MTase activity. The DNA MTase activity of the HMT clones ranged from 1- to 50-fold that of the parental cell line or the Neo clones (Table 1). Cellular DNA MTase activities were relatively consistent with the total amount of DNA MTase protein estimated from Western analyses, indicating that the recombinant 190-kDa protein was active and contributing to total cellular DNA MTase activity.

In addition to maintenance methylase activity, DNA MTases are capable of the de novo methylation of an unmethylated substrate (23). DNA MTase activity was measured with poly

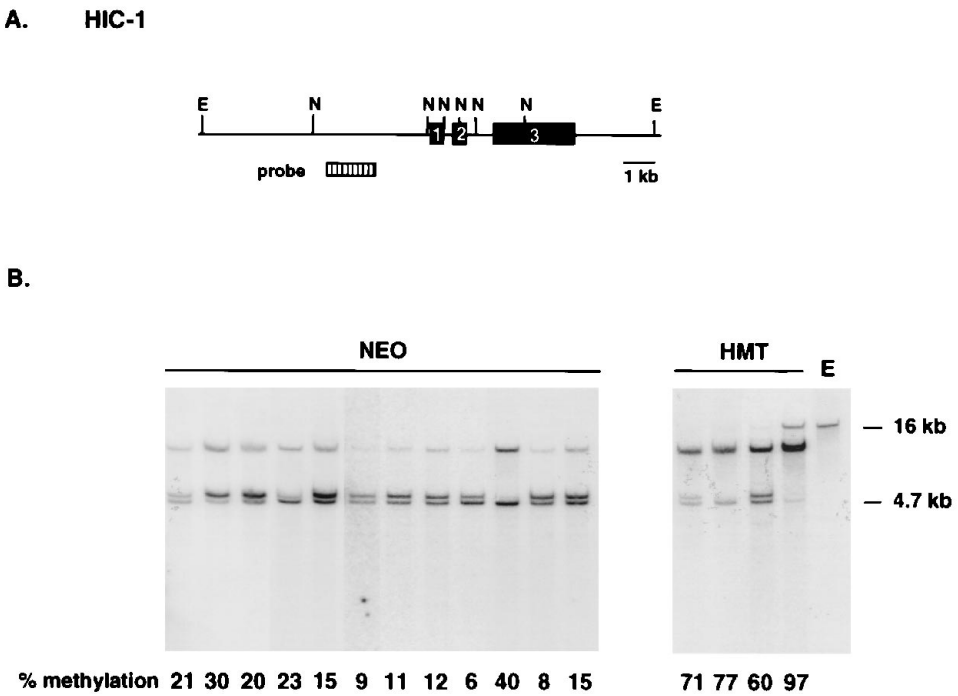

FIG. 2. Methylation of the *HIC-1* CpG island in IMR90/SV40 transfectants. (A) Genomic map of the region surrounding the *HIC-1* gene. *Eco*RI (E) and *Not*I (N) sites are indicated, as are the positions of the *HIC-1* exons (solid boxes) and the probe recognizing the polymorphic marker pYNZ22 used for Southern analysis (striped box). The position of the *Eag*I site mentioned in the text is ~600 bp upstream of exon 1. (B) Methylation at *HIC-1* was examined by digestion of DNA with *Eco*RI alone (E) or *Eco*RI plus *Not*I (all other lanes) followed by electrophoresis on 1% agarose gels, transfer to nylon filters, and hybridization to the probe shown in panel A. Each lane represents DNA from an independent clone transfected with the control vector (Neo) or the DNA MTase-containing construct (HMT). Twelve representative Neo clones and those HMT clones ≥9-fold overexpressing (left to right, HMT.L41, HMT.17, HMT.19, and HMT.1E1) are shown. Absence of methylation at the *Not*I sites in this region yields two polymorphic bands with sizes of ~4.7 kb, and methylation of one or more *Not*I sites results in bands larger than 4.7 kb. Percent methylation (below each lane) was calculated as the intensity of the methylated bands (>4.7 kb) relative to the combined intensities of all bands. Note the presence of a 16-kb band in HMT.19 and HMT.1E1 samples, indicating methylation of all six *Not*I sites.

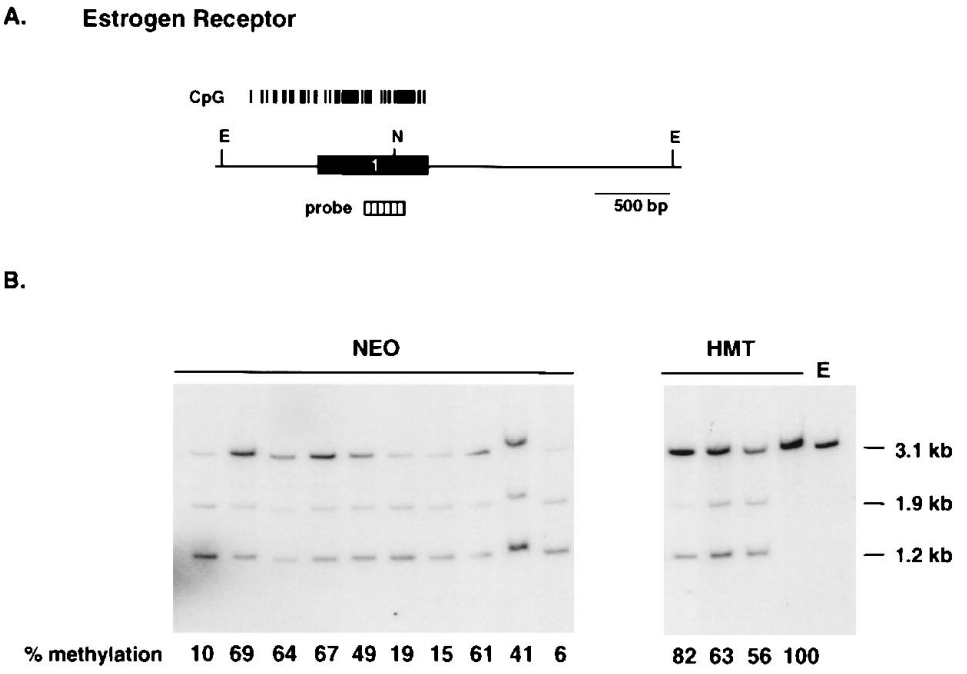

FIG. 3. Methylation of the ER CpG island in IMR90/SV40 transfectants. (A) Genomic map and CpG dinucleotide frequency of the region surrounding exon 1 of the ER gene. *Eco*RI (E) and *Not*I (N) sites are indicated, as are the positions of exon 1 (filled box) and the probe used for Southern analysis (striped box). The position of one of the *Sac*II sites mentioned in the text is 205 bp 5′ of the *Not*I site, and the other is coincident with the *Not*I site. (B) Methylation at the ER locus was examined by digestion of DNA with *Eco*RI alone (E) or *Eco*RI plus *Not*I (all other lanes) followed by electrophoresis on 1% agarose gels, transfer to nylon filters, and hybridization to the probe shown in panel A. Each lane represents DNA from an independent clone transfected with the control vector (Neo) or the DNA MTase-containing construct (HMT). Ten representative Neo clones and those HMT clones ≥9-fold overexpressing (left to right, HMT.L41, HMT.17, HMT.19, and HMT.1E1) are shown. Absence of methylation at the *Not*I site results in two bands with sizes of 1.2 and 1.9 kb, whereas methylation of this site results in a band equal in size to the 3.1-kb *Eco*RI fragment. Percent methylation (below each lane) was calculated as the intensity of the methylated band (3.1 kb) relative to the combined intensities of all bands.

(dG-dC) · (dG-dC) as an unmethylated substrate to determine whether there was any difference in the ratio of maintenance to de novo activity in cells producing the recombinant protein compared with that in those expressing only the native DNA MTase protein. The ratios of maintenance to de novo MTase activity were similar in the HMT clones, the Neo clones, and the parental cell line (Table 1). Taken together, these data indicate that the recombinantly produced DNA MTase protein is an active DNA methyltransferase that retains the preference of the endogenous enzyme for the maintenance reaction (23). Furthermore, the HMT clones exhibit up to a 50-fold increased capacity for both maintenance and de novo methylation.

CpG island methylation in DNA MTase overexpressors. Having established several cell clones overexpressing DNA MTase, we next examined the effect of this overexpression on the methylation status of endogenous CpG island sequences. We studied 12 different loci with features characteristic of CpG islands, including a C+G content of >60%, a CpG/GpC ratio of >~0.7, and sites for one or more CpG-containing restriction enzymes that cluster in CpG island DNA (e.g., *Not*I, *Sac*II, *Eag*I, and *Bss*HII) (26). These CpG island loci were found to be either completely unmethylated or to have accumulated only low-level methylation in the IMR90/SV40 cell population prior to transfection (39, 40).

Methylation was assessed by digesting DNA with methylation-sensitive restriction enzymes (*Not*I, *Sac*II, or *Eag*I), followed by Southern blot analysis with CpG island locus-specific probes. The initial analysis compared the methylation status of DNA from the HMT clones expressing the highest levels of DNA MTase (HMT.L41, HMT.17, HMT.19, and HMT.1E1) with those of at least 10 individual Neo clones at representative sites in 12 CpG islands. To minimize differences in methylation due to time in culture, DNAs were isolated from each clone at or near passage 20. Representative Southern blot and percent methylation analyses are shown in Fig. 2 to 6.

At 7 of 12 CpG island loci, there was no consistent difference between 10 Neo clones and the 4 HMT clones expressing the highest levels of DNA MTase. These included the CpG islands associated with the neurofibromatosis I gene, the human *achaete-scute* homolog gene, the collagen(I)α1 gene, the von Hippel-Lindau gene, the cyclin-dependent kinase inhibitor genes *p15* and *p16*, and the manganese superoxide dismutase 2 gene. In fact at most of these loci, there was a complete lack of detectable methylation in all transfected clones tested as well as in the parental cell population (data not shown).

At the remaining five loci, including the recently described *HIC-1* gene (29) on chromosome 17p13.3, the estrogen receptor gene on chromosome 6q25 (ER), the α_1 and α_2 genes of the α-globin locus (HBA), the E-cadherin gene on chromosome 16q22 (E-CAD), and the somatostatin gene on chromosome 3q28 (SST), there was some methylation detected in most of the transfected clones. This was not an unexpected finding, since the parental IMR90/SV40 population, as an immortalized cell line, has accumulated some aberrant CpG island methylation and was determined to have low-level methylation at these loci prior to transfection (approximately 10% at the *HIC-1* locus, 30% at the ER locus, and <10% at the E-CAD locus; the HBA and SST loci were not tested) (39, 40). Any clones derived from this heterogeneous population would therefore be expected to show some methylation, and the av-

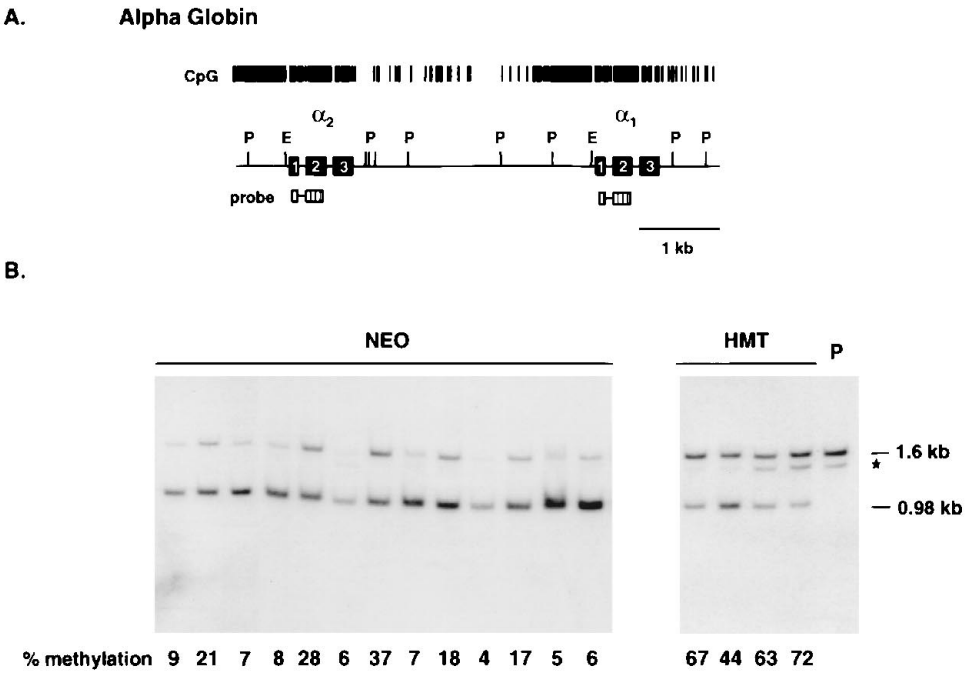

FIG. 4. Methylation of the CpG islands of the α_1 and α_2 genes of the α-globin locus in IMR90/SV40 transfectants. (A) Genomic map and CpG dinucleotide frequency of the region surrounding the α_1- and α_2-globin genes. *Pst*I (P) and *Eag*I (E) sites are indicated, as are the positions of the α_1 and α_2 exons (solid boxes) and the probe used for Southern analysis (striped box). (B) Methylation at the α-globin locus was examined by digestion of DNA with *Pst*I alone (P) or *Pst*I plus *Eag*I (all other lanes) followed by electrophoresis on 1% agarose gels, transfer to nylon filters, and hybridization to the probe shown in panel A. Each lane represents DNA from an independent clone transfected with the control vector (Neo) or the DNA MTase-containing construct (HMT). Thirteen representative Neo clones and those HMT clones ≥9-fold overexpressing (left to right, HMT.L41, HMT.17, HMT.19, and HMT.1E1) are shown. Absence of methylation at the *Eag*I site gives a band with a size of 0.98 kb, whereas methylation of this site results in a band equal in size to the 1.6-kb *Pst*I fragment. Since the α_1 and α_2 genes represent a nearly perfect tandem gene duplication, the relative positions of the *Pst*I and *Eag*I sites are the same for both, and the probe recognizes both genes. Percent methylation (below each lane) was calculated as the intensity of the methylated band (1.6 kb) relative to the combined intensities of all bands. A band of unknown origin also recognized by the probe is starred. Hybridization to this band was disregarded in percent methylation calculations.

erage pattern of the control clones should reflect that of the parent population.

As expected, the individual Neo clones varied in their levels of methylation at these five loci, but the average level (Table 2) was relatively low and was similar to that of the uncloned parental population. In contrast, the four HMT clones were, as a group, significantly more methylated than the Neo clones. As summarized in Table 2, expression of ≥9-fold increased DNA MTase levels in almost all cases correlated with increased DNA methylation at the CpG islands associated with the *HIC-1* gene, which is hypermethylated in many tumor types (Fig. 2), the ER gene (Fig. 3), the HBA locus (Fig. 4), the E-CAD gene (Fig. 5), and the SST gene (Fig. 6). Interestingly, one HMT clone in particular (HMT.1E1) showed the greatest degree of methylation at all five loci and was ~100% methylated at the *HIC-1* and ER loci (Fig. 2 to 6, second lane from the right).

To confirm that the observed hypermethylation was not unique to the restriction site examined but rather was representative of the methylation status of that region, we examined additional methylation-sensitive restriction sites in the *HIC-1*, ER, and E-CAD loci. Consistent with the above results, those four clones expressing ≥9-fold increased DNA MTase levels were also distinctly more methylated than at least six independent Neo clones at an *Eag*I site ~600 bp upstream of exon 1 in the *HIC-1* locus (Fig. 2A), two *Sac*II sites in the first exon of the ER gene (Fig. 3A) (17), and three *Bst*UI sites spanning 270 bp of the proximal promoter region of the E-CAD gene (Fig. 5A) (13) (data not shown).

In addition to affecting a greater number of the alleles in the population at each susceptible locus, de novo methylation in the HMT clones appeared capable of spreading across broad genomic regions. For example, the HMT.1E1 clone and, to a lesser extent, the HMT.19 clone showed evidence of methylation at all six *Not*I sites in the *HIC-1* locus, indicating that de novo methylation in a fraction of these cells must have occurred over at least the 8-kb region encompassing these sites (Fig. 2).

To further examine the relationship between the levels of DNA MTase activity and hypermethylation of CpG islands, we analyzed the methylation status of the five sensitive CpG islands in the HMT.S36, HMT.L36, and HMT.S33 clones which have low levels of DNA MTase overexpression (one- to threefold that of the parental cell line). At the *HIC-1*, ER, and HBA loci, the methylation levels in the low-level HMT expressors were, as a group, well within the range obtained for the Neo clones (Table 2). In contrast, the SST and E-CAD loci were somewhat more methylated in the HMT.L36 and HMT.S33 clones (1.3- and 3-fold overexpressing) than in the Neo clones with the highest level of expression. Whereas the HMT.L41 clone (ninefold the endogenous levels of DNA MTase) was more methylated than the most methylated of the Neo clones at all five sensitive CpG island loci, the HMT.S33 clone (threefold overexpressing) fit this criterion at only two loci. This suggests that within the scope of this analysis (i.e., 20 passages), there is a greater potential to consistently affect CpG island methylation when DNA MTase activity is above a certain (in this case, ninefold overexpressed) level. It also appears that once this threshold has been reached, there is little correlation between the levels of DNA MTase activity and CpG island methylation. At four of the five sensitive CpG island loci, HMT.L41 (9-fold overexpressing) was more methylated than

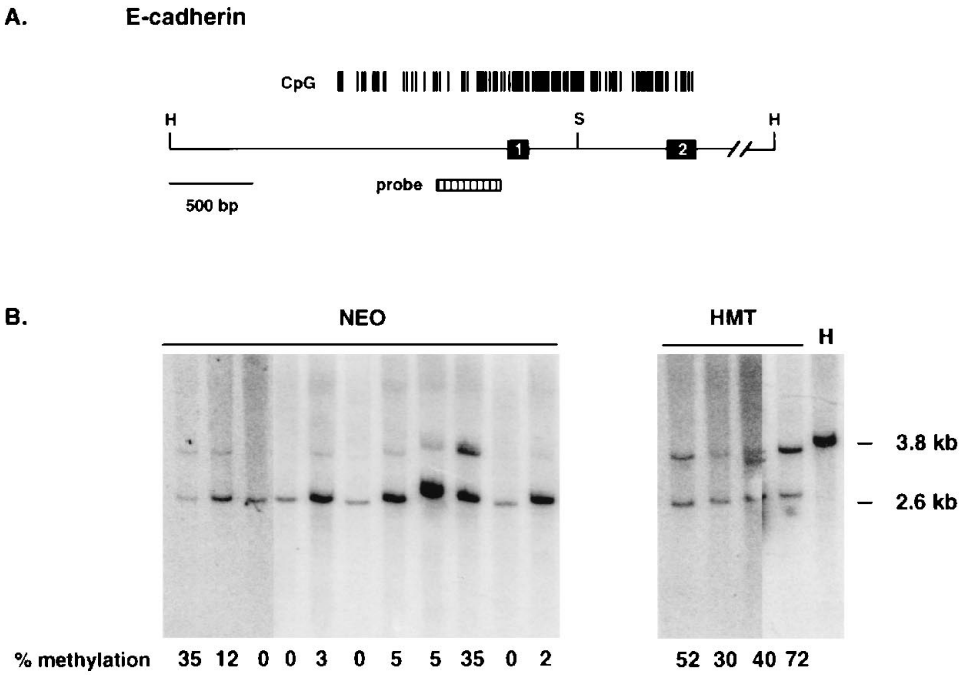

FIG. 5. Methylation of the E-CAD CpG island in IMR90/SV40 transfectants. (A) Genomic map and CpG dinucleotide frequency of the 5′ end of the E-CAD gene. *Hin*dIII (H) and *Sac*II (S) sites are indicated, as are the positions of exons 1 and 2 (solid boxes) and the probe used for Southern analysis (striped box). The three *Bst*UI sites mentioned in the text span the 270 bp covered by the probe. (B) Methylation at the E-CAD locus was examined by digestion of DNA with *Hin*dIII alone (H) or *Hin*dIII plus *Sac*II (all other lanes) followed by electrophoresis on 1% agarose gels, transfer to nylon filters, and hybridization to the probe shown in panel A. Each lane represents DNA from an independent clone transfected with the control vector (Neo) or the DNA MTase-containing construct (HMT). Eleven representative Neo clones and those HMT clones ≥9-fold overexpressing (left to right, HMT.L41, HMT.17, HMT.19, and HMT.1E1) are shown. Absence of methylation at the *Sac*II site gives a band with a size of 2.6 kb, whereas methylation of this site results in a band equal in size to the 3.8-kb *Hin*dIII fragment. Percent methylation (below each lane) was calculated as the intensity of the methylated band (3.8 kb) relative to the combined intensities of all bands.

at least one of the 40- to 50-fold overexpressors, HMT.17 or HMT.19. In addition, the HMT.1E1 clone was the most highly methylated at all five CpG island loci, despite DNA MTase levels that were similar to those of HMT.17 and HMT.19.

Accumulation of CpG island methylation with time. CpG island methylation accumulates with aging (17), during tumor progression (4, 17), and with time in culture for cell lines (1, 40). To determine whether DNA MTase levels affect the rate of accumulation of methylation in CpG islands, we examined methylation of the *HIC-1* and ER CpG islands in four independent Neo clones and the four HMT clones expressing greater than ninefold normal levels of DNA MTase activity over the course of 40 to 50 passages (6 to 7 months) in culture. In these analyses, growth to passage 4 is equivalent to the time it took for a single cell to populate a T75 flask, or about 20 to 23 population doublings. After that, each passage represents about 3 population doublings. At the *HIC-1* locus, individual Neo and HMT clones varied in their levels of methylation, and a passage four, there was little difference in the range of methylation levels between the two groups (Fig. 7A and B). However, while the percent methylation at this locus remained virtually stable in the Neo clones over the course of 40 passages, it increased markedly in the HMT clones (Fig. 7A and B). Likewise, at the ER locus, the levels of methylation remained relatively stable in the Neo clones, while the level of methylation increased substantially in the HMT clones (Fig. 7C and D). After 40 to 50 passages, nearly 100% of the *HIC-1* and ER alleles in the HMT cell populations had become methylated at the sites examined (Fig. 7). Interestingly, even those few Neo clones that were relatively heavily methylated at passage 4 failed to show any increase over the next 30 passages at either locus. The data are consistent, therefore, with the idea that overexpression of DNA MTase can promote the time-dependent accumulation of methylation at susceptible CpG island loci.

DISCUSSION

Several recent studies showing a positive correlation between DNA MTase levels and tumorigenicity have implicated this enzyme in the carcinogenic process (20, 27, 42). Furthermore, the aberrant hypermethylation of promoter region CpG islands appears to be one mechanism leading to the inactivation of tumor-suppressor genes. Increased DNA MTase levels might, therefore, contribute to tumor progression by promoting CpG island hypermethylation. The aim of the current study was to determine whether increased levels of DNA MTase could drive the hypermethylation of CpG islands. The data demonstrate that increasing the levels of DNA MTase activity by greater than ninefold induces the time-dependent accumulation of de novo methylation at susceptible CpG island loci.

That the methylation detected at endogenous CpG islands in the DNA MTase-overexpressing cells represents de novo methylation, or the gain of new methylation, is supported by several findings. Although there was substantial baseline methylation in a few Neo clones at some of the loci, individual Neo clones were random in their methylation levels from locus to locus. Of 11 independent Neo clones that were studied at all five sensitive CpG island loci, none were more methylated than the mean of the Neo clones at more than two loci (for example, compare Fig. 2B, lane 5; 3B, lane 2; 4B, lane 5; 5B, lane 5; and 6B, lane 5). The chances of randomly selecting a cell clone that

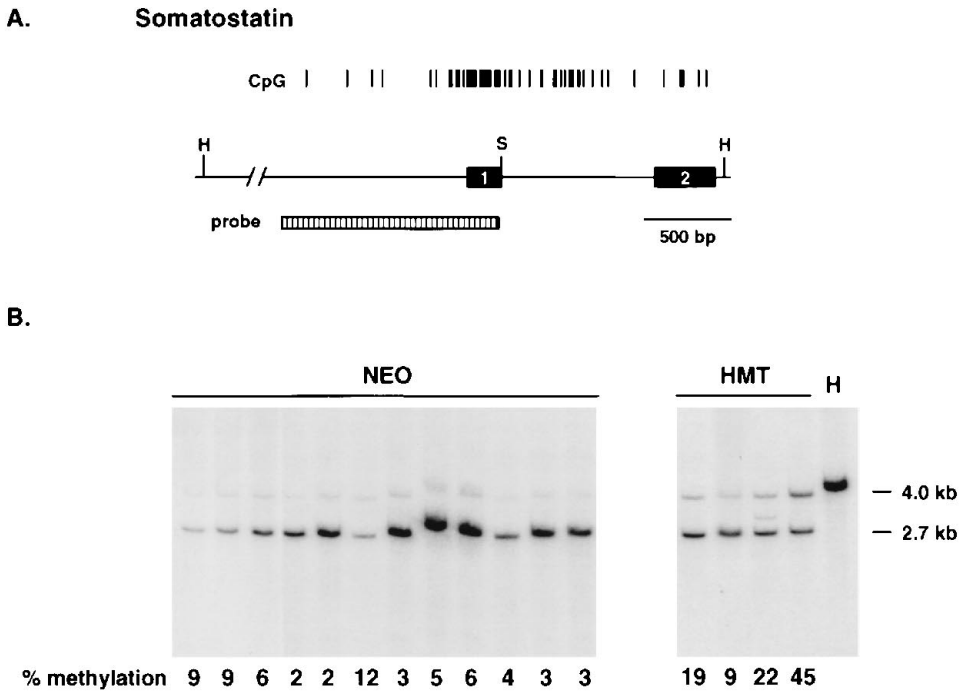

FIG. 6. Methylation of the SST CpG island in IMR90/SV40 transfectants. (A) Genomic map and CpG dinucleotide frequency of the 5′ end of the SST gene. *Hin*dIII (H) and *Sac*II (S) sites are indicated, as are the positions of exons 1 and 2 (solid boxes) and the probe used for Southern analysis (striped box). (B) Methylation at the SST locus was examined by digestion of DNA with *Hin*dIII alone (H) or *Hin*dIII plus *Sac*II (all other lanes) followed by electrophoresis on 1% agarose gels, transfer to nylon filters, and hybridization to the probe shown in panel A. Each lane represents DNA from an independent clone transfected with the control vector (Neo) or the DNA MTase-containing construct (HMT). Twelve representative Neo clones and those HMT clones ≥9-fold overexpressing (left to right, HMT.L41, HMT.17, HMT.19, and HMT.1E1) are shown. Absence of methylation at the *Sac*II site gives a band with a size of 2.7 kb, whereas methylation of this site results in a band equal in size to the 4.0-kb *Hin*dIII fragment. Percent methylation (below each lane) was calculated as the intensity of the methylated band (4.0 kb) relative to the combined intensities of all bands.

is more methylated than the mean at all five loci is therefore very low. Each of the four HMT clones with >9-fold increased DNA MTase levels was more methylated than the mean of the Neo clones at all five loci, indicating that the increased CpG island methylation in these clones was not due to random selection but rather was due to the overexpression of DNA MTase.

Alternatively, one could argue that the CpG island methylation seen in the DNA MTase overexpressors, particularly the apparent increase with time in culture, is the result of the clonal expansion of cells with preexistent methylation of these loci. This implies that the methylation of these loci provides the cell with a selective growth advantage. If this were the case, one would expect a similar time-dependent increase to occur in the Neo clones. Since we found no such change in methylation at the *HIC-1* and ER loci in four Neo clones with time, despite moderate initial methylation levels in some, the kinetics of CpG island hypermethylation in the HMT overexpressors are unlikely to be the result of the selective outgrowth of cells methylated at these loci.

The data are therefore most consistent with the idea that the overexpression of DNA MTase affects the actual number of allelic de novo methylation events, which then accumulate in the cell population over time. Antequera et al. (1) have estimated that most of the CpG islands associated with nonessential genes have become methylated in standard laboratory cell lines that have been in culture for many years. De novo methylation at CpG islands is therefore likely to occur at some low frequency in immortalized cells in culture, and the longer the cells are in culture the more methylation accumulates. In

TABLE 2. Percent methylation at CpG island loci in IMR90/SV40 transfectants

Cell clone	% Methylation at CpG island locus[a]:									
	HIC-1		ER		HBA		SST		E-CAD	
	Mean (range)	*n*	Mean (range)	*n*	Mean (range)	*n*	Mean (range)	*n*	Mean (range)	*n*
Neo	18.8 (7–40)	14	37.3 (6–69)	11	12.2 (0–37)	13	5.3 (2–12)	12	9.6 (0–35)	12
HMT (≥9-fold)[b]	76.3 (60–97)	4	77.0 (56–100)	4	63.0 (44–79)	4	24.0 (9–45)	4	48.5 (30–72)	4
HMT (≤3-fold)[c]	21.0 (21–21)	3	38.0 (19–63)	3	12.3 (0–20)	3	10.3 (4–16)	3	35.3 (12–48)	3

[a] DNAs from cells at passages 20 to 27 were subjected to methylation analyses at the indicated CpG island loci as described in the legends to Fig. 2 to 6. Data are presented as the mean and range of values obtained from *n* individual clones.
[b] HMT clones expressing ≥9-fold the levels of DNA MTase activity of the parental cell line (clones HMT.L41, HMT.17, HMT.19, and HMT.1E1).
[c] HMT clones expressing ≤3-fold the levels of DNA MTase activity of the parental cell line (clones HMT.S36, HMT.L36, and HMT.S33).

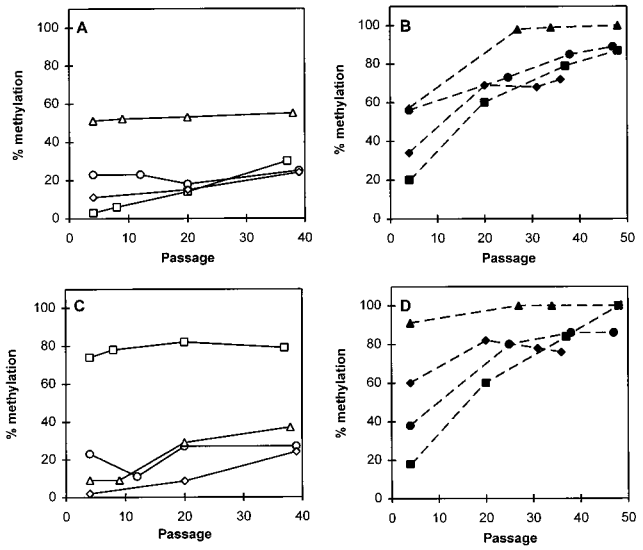

FIG. 7. Time-dependent accumulation of de novo methylation at the *HIC-1* and ER CpG islands. (A and B) Methylation of the *HIC-1* CpG island in the Neo (A) and HMT (B) clones. DNA was isolated from the Neo.1 (○), Neo.4 (□), Neo.20 (△), and Neo.S35 (◇) clones or the HMT.L41 (◆), HMT.17 (●), HMT.19 (■), and HMT.1E1 (▲) clones at the indicated passages and analyzed for percent methylation at the *HIC-1* locus as described in the legend to Fig. 2. (C and D) Methylation of the ER CpG island in the Neo (C) and HMT (D) clones. Data were generated exactly as described for panels A and B, except that the individual clones were analyzed for methylation at the ER locus as described in the legend to Fig. 3.

this regard, we previously determined that the immortalized IMR90/SV40 cell population had stable levels of DNA MTase and did not accumulate more than 10% methylation at the *HIC-1* locus or 30% methylation at the ER locus over the course of 60 to 80 population doublings (39, 40). In the DNA MTase-overexpressing clones, both of these loci became nearly completely methylated within 20 passages, or about 70 population doublings, of the insertion of DNA MTase. Therefore, in the absence of selective pressure, it would appear that the overexpression of DNA MTase can increase the number of de novo methylation events that occur per population doubling, or the de novo methylation rate. A DNA MTase-mediated increase in the de novo methylation rate could well occur during progression of human tumors, since the overexpression of DNA MTase activity necessary to affect CpG island methylation (ninefold) is within the range of relative increase reported for primary colon tumors (18).

Even under the added pressure to de novo methylate, i.e., by DNA MTase overexpression, some endogenous CpG islands did not become methylated in the DNA MTase transfectants within the 20 passages examined. Of 12 loci tested, 7 remained unmethylated. Interestingly, one feature that distinguished the CpG islands that were subject to de novo methylation in the HMT clones is that they initially had low but detectable levels of methylation in the IMR90/SV40 cell population, prior to the insertion of DNA MTase, whereas the CpG islands that were resistant to methylation were devoid of methylation in the parental population. These findings suggest that CpG island loci differ in their inherent susceptibility to de novo methylation and emphasize that the primary effect of DNA MTase overexpression is on the number of de novo methylation events rather than their location.

What are the locus-specific factors that might dictate susceptibility to de novo methylation? This question has only recently been addressed, but these factors may include the presence of Sp1 sites, which seem to function in protecting CpG island sequences from methylation (8, 28), or proximity to methylation centers which can direct methylation to adjacent sequences (14, 31). As a cautionary note, however, these de novo methylation signals have only been defined in cells of embryonic origin, and methylation patterns are known to go through dramatic changes during embryonic development (34). Since methylation patterns are more stable in adult cells, it is unclear whether similar methylation signals will also be operative in differentiated cells undergoing neoplastic transformation.

Alternatively, de novo methylation may be a stochastic process, and the CpG island locus and tumor-type specificity observed may be reflective of the propagation of particular methylation events because they provide a cell with a growth advantage during tumor development. For example, the *VHL* gene is found methylated (or mutated) only in renal cell carcinomas in which its inactivation presumably plays a role in the pathogenesis of the disease (15). Although this might explain the locus specificity seen in established tumors that have survived selective pressure, it is probably not the case in this in vitro system in which cell growth has not been challenged (other than by selection for neomycin resistance). With the exception of the *HIC-1* gene, which is expressed in the parental IMR90/SV40 cells but whose function is unknown, the other four genes are not expressed (ER) (39) or would not be expected to be expressed in fibroblasts (HBA, E-CAD, and SST). It is not immediately clear, therefore, whether methylation of these five loci would lead to a growth advantage. Also, the dynamics of methylation accumulation discussed above suggest that clonal expansion is not involved in this system. There is the possibility, however, that these loci lie within broader areas of hypermethylation or that other unidentified loci are affected. Indeed, methylation in the overexpressors appeared capable of spreading across large (>8-kb) genomic regions. Studies addressing the ability of the HMT clones to produce tumors in nude mice, thereby selecting cells in vivo that have inactivated loci critical to tumor formation, are under way.

What remains to be determined is how excess DNA MTase contributes to de novo CpG island methylation. The primary function of this enzyme is the conversion of newly replicated hemimethylated CpG sites into fully methylated sites so that methylation patterns are maintained during cell division. This activity is thought to be regulated, at least in part, by the recruitment of DNA MTase to replication foci during the S phase of the cell cycle (24). This regulation is presumably mediated through binding of an N-terminal domain of the DNA MTase to other proteins (24). Overriding the normal levels of the enzyme could saturate binding sites on these proteins, allowing the DNA MTase access to the DNA at times other than during DNA replication or at sites other than the replication fork. The finding that there was a difference in the degree of CpG island methylation in HMT clones with 3- versus 9-fold increased DNA MTase activity, but no difference between those with 9- and 50-fold increased activity, suggests the existence of a saturable process. Given that the enzyme has significant activity on unmethylated DNA and that this activity is also increased by up to 50-fold in the HMT cells, uncontrolled access of the enzyme might increase the frequency of an otherwise rare event, i.e., de novo methylation. In this regard, constitutive expression of a bacterial cytosine DNA MTase, which lacks a functional counterpart to the N-terminal S-phase-targeting domain of the mammalian enzyme, is toxic to mouse cells (41). That other cellular factors might modulate DNA MTase-mediated CpG island methylation is further sug-

gested by the fact that one clone (HMT.1E1) was the most highly methylated at every CpG island susceptible to de novo methylation despite similar DNA MTase activity levels among the three highest expressors (HMT.1E1, HMT.17, and HMT.19). The isolation of HMT clones which apparently differ in a de novo methylation-modulating activity may allow the identification of such a factor.

In summary, we have created cell lines expressing up to 50 times the normal levels of DNA MTase, and one consequence of this event is the de novo methylation of endogenous CpG island sequences. Given the growing number of studies supporting a role for CpG island methylation in the inactivation of genes important to tumorigenesis, one explanation for the decreased tumorigenicity seen in animals with genetically or pharmacologically reduced DNA MTase levels (20) might be the concomitant inhibition of aberrant CpG island methylation. As such, the DNA MTase overexpression system presented here should be a useful model not only for determining the cellular factors involved in CpG island methylation but also for the testing of pharmacologic agents designed to block specific de novo methylation events.

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