De novo CpG Island Methylation in Human Cancer Cells

Kam-Wing Jair,[1] Kurtis E. Bachman,[4] Hiromu Suzuki,[1] Angela H. Ting,[3] Ina Rhee,[2] Ray-Whay Chiu Yen,[1] Stephen B. Baylin,[1,2,3] and Kornel E. Schuebel[1]

[1]The Sidney Kimmel Comprehensive Cancer Center, [2]The Program in Human Genetics, and [3]Graduate Program in Cellular and Molecular Medicine, The Johns Hopkins University School of Medicine; and [4]The University of Maryland Greenebaum Cancer Center, The University of Maryland School of Medicine, Baltimore, Maryland

Abstract

A major obstacle toward understanding how patterns of abnormal mammalian cytosine DNA methylation are established is the difficulty in quantitating the *de novo* methylation activities of DNA methyltransferases (DNMT) thought to catalyze these reactions. Here, we describe a novel method, using native human CpG island substrates from genes that frequently become hypermethylated in cancer, which generates robust activity for measuring *de novo* CpG methylation. We then survey colon cancer cells with genetically engineered deficiencies in different DNMTs and find that the major activity against these substrates in extracts of these cells is DNMT1, with minor contribution from DNMT 3b and none from DNMT3a, the only known bona fide *de novo* methyltransferases. The activity of DNMT1 against unmethylated CpG rich DNA was further tested by introducing CpG island substrates and DNMT1 into *Drosophila melanogaster* cells. The exogenous DNMT1 methylates the integrated mammalian CpG islands but not the *Drosophila* DNA. Additionally, in human cancer cells lacking DNMT1 and DNMT3b and having nearly absent genomic methylation, gene-specific *de novo* methylation can be initiated by reintroduction of DNMT1. Our studies provide a new assay for *de novo* activity of DNMTs and data suggesting a potential role for DNMT1 in the initiation of promoter CpG island hypermethylation in human cancer cells. (Cancer Res 2006; 66(2): 682-92)

Introduction

Covalent modification of cytosine nucleotides by methylation is a heritable and reversible epigenetic process important to a diverse range of biological processes in multiple species (1). In mammals, DNA methylation is essential for normal embryonic development (2) and plays important roles in the regulation of gene expression (3, 4), X chromosome inactivation (5), genomic imprinting (6), chromatin modification (7), silencing of endogenous retroviruses (8), mutation accumulation (9, 10), and aberrant silencing of tumor suppressor genes in cancer (11).

The existence of a two-component DNA methylation system consisting of an enzyme activity that methylates unmethylated DNA (*de novo*) and one that methylates hemimethylated sites ("maintenance") has long been proposed (12–14). Genetic disruption of mouse *DNMT1* (15) leads to extensive demethylation of several classes of genomic DNA sequences and embryonic lethality during midgestation (15, 16). Consistent with the role of DNA methyltransferase 1 (DNMT1) as the major maintenance methyltransferase in mouse, biochemical analysis shows that this enzyme has a 5- to 30-fold preference for hemimethylated DNA over unmethylated substrates (17). Furthermore, *DNMT1* introduction into the germ line of *Drosophila* does not lead to methylation of the fly genome (18). Taken together, these experiments suggest that Dnmt1 functions exclusively as a maintenance enzyme, ensuring faithful propagation of DNA methylation patterns from parental to daughter genomes.

In contrast, inactivation of both mouse Dnmt3a and Dnmt3b was found to disrupt *de novo* methylation of proviral DNA in embryonic stem cells and genome-wide *de novo* methylation during early development, in the absence of discernible effects on maintenance of preexisting methylation patterns (19). These studies have led to the general consensus that DNMT3a and DNMT3b proteins represent the only bona fide *de novo* DNMTs.

The two-component system satisfactorily explains the establishment of DNA methylation in normal murine cells. However, altered DNA methylation patterns are also important in the etiology of disease states, such as cancer (11). Human neoplasias exhibit methylation defects, including both global loss of 5-methylcytosine (20) and accumulation of 5-methylcytosine in the CpG-rich regulatory regions of tumor suppressor genes concurrent with gene silencing (21). These observations have stimulated a search to identify the molecular components, including the role of the mammalian DNMTs, underlying these aberrant methylation patterns. In colorectal cancer cells, acute down-regulation of DNMT1 expression mediated by transient RNA interference (RNAi) treatment leaves gene promoter hypermethylation intact (22). Furthermore, in these same cells, chronic loss of DNMT1 via targeted gene disruption (23) or stable RNAi-mediated suppression (22) leaves the vast majority of CpG methylation and gene promoter hypermethylation intact. These results were explained by subsequent studies showing that sequential genetic removal of DNMT1 and DNMT3b abolishes virtually all DNA methylation, including promoter CpG island methylation, in these colon cancer cells (24). Thus, maintenance of CpG methylation in human cancer cells does not depend on DNMT1 or DNMT3b methyltransferase alone but on the combined catalytic activity of both enzymes. However, these studies did not address how CpG methylation is initially established; a question that has important implications for understanding how tumor suppressor gene expression is extinguished in human cancer.

To better clarify the role of methyltransferases in establishing DNA methylation patterns in human cancer cells, we sought to develop a more robust assay for measuring *de novo* methyltransferase activity. Our interest in aberrant methylation of promoter CpG islands and associated gene silencing led us to test these DNA sequences for potential quantitation of *de novo* methylation. We now show that such sequences can provide a robust assay for

Requests for reprints: Kornel E. Schuebel or Stephen B. Baylin, Cancer Biology, The Johns Hopkins University, Sidney Kimmel Comprehensive Cancer Center, Room 530, 1650 Orleans Street, Baltimore, MD 21231. Phone: 410-955-8506; E-mail: kornels@jhmi.edu or sbaylin@jhmi.edu.

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de novo methyltransferase activity. Furthermore, through study of colon cancer cells, in which all of the known major mammalian DNMTs have been genetically disrupted, we show that DNMT1 is responsible for most of this activity. Finally, we show that DNMT1 is a robust *de novo* DNMT for CpG islands in both heterologous cell systems and cancer cell settings.

Materials and Methods

Gene targeting in human cancer cells. HCT116 cells and derivatives (American Type Culture Collection, Manassas, VA) were cultured in McCoy's 5A modified media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. *DNMT1* and *DNMT3b* targeting was done as described (25). To target the *DNMT3a* locus, *DNMT1*$^{(+/-)}$, *DNMT3b*$^{(-/-)}$ cells were transfected with the linearized *DNMT3a*-targeting vectors using LipofectAMINE (Invitrogen, Carlsbad, CA) and selected in growth medium supplemented with 0.4 μg/mL geneticin (Invitrogen). General aspects of targeting with bipartite Neo vectors were previously described (25). Each clone had a total of five targeted alleles; the targeting order was *DNMT3b*, allele 1 → *DNMT3b*, allele 2 → *DNMT1*, allele 1 →*DNMT3a*, allele 1 →*DNMT3a*, allele 2. Identification (primers Neo and 3a-KO) and verification of homologous recombinants (Primers HD1 and 2) was done using PCR. Loss of *DNMT3a* mRNA was assessed by reverse transcription-PCR (RT-PCR) using primers 3aRT-up and 3aRT-down and compared with control glyceraldehyde-3-phosphate dehydrogenase levels (26). Primer sequences are Neo, 5′-GTTGTGCCCAGTCATAGCCG-3′; *3a*-KO, 5′-CCCTCGATAGCAACTCTACC-3′, HD1 5′-AAGTGTTGCGTAAGGTTCC-3′; HD2, 5′-GTGATGGAGTCCTCACACAC-3′; 3aRT-up, 5′-TGTGTGAGGACTC-CATCACG-3′; and 3aRT-down, 5′-AACTTTGTGTCGCTAC CTCAG-3′.

High performance liquid chromatography. Approximately 40 μg of RNA-free genomic DNA, prepared using the Blood and Cell Culture DNA Midi kit (Qiagen, Chatsworth, CA), was quantitatively digested with nuclease P1 (Roche Molecular Biochemicals, Indianapolis, IN) and calf intestinal alkaline phosphatase (Sigma, St. Louis, MO) as described (27). Samples were separated on a reversed-phase column (Supelcosil LC-18 DB, Sigma) at room temperature, monitoring absorbances at 275 and 285 nm. Peak assignments were confirmed using deoxyribonucleoside standards (Sigma). 5-Methylcytosine content was expressed as a percentage of the total cytosine pool, using peak areas after correction for extinction coefficients.

Plasmid construction. Plasmids p*MLH1*-101/4 and p*TIMP3*-639/73 were generated by amplification of HCT116 genomic DNA with oligonucleotides MLH1.101 and MLH1.104 or TIMP3-2 and TIMP3-3 (see below) followed by insertion into pCR2.1 -TOPO (Invitrogen). An *Xba*I/*Hin*dIII fragment from p*TIMP3*-639/73 was inserted into the *Xba*I and *Hin*dIII sites of pCO-Hygro to generate pCO-Hygro*TIMP3*-639/73. To generate pCO-Hygro*MLH1*-102/4, pMLH1-101/4 was digested with *Eco*RV and inserted into the *Ssp*I site of pCO-Hygro. Plasmid pCO-Hygro*MGMT* was generated by digesting plasmid pKT200 (a gift from Dr. Sankar Mitra) with *Pst*I, blunt ended with T4 DNA polymerase, digested with *Xba*I, and inserted into the *Ssp*I and *Xba*I sites of pCo-Hygro. We inserted a *Spe*I-*Pme*I fragment including full-length hemagglutinin (HA)–tagged version of *DNMT1* from pCDNA3.1-HA-*DNMT1* (28) into pMT/V5-HisA to generate pMT-HA-*DNMT1*. Vector pCDNA3.1-HA-*DNMT1* C1226Y was generated by site directed mutagenesis from pCDNA3.1-HA-*DNMT1* using the Quikchange kit (Stratagene, La Jolla, CA). All plasmids introduced into *Drosophila* Schneider S-2 cells were propagated in *Dam* and *Dcm* methylase-deficient bacteria (SCS110; Stratagene).

PCR primers MLH1.101, 5′-TGCACCTCCAACTCAGGGCC-3′; MLH1.104, 5′-CCACGAACGACATTTTGGCGC-3′; TIMP3-2, 5′-CGGCAGCAGCGGCAAT-GACC-3′; and TIMP3-3 5′-GGTCATTGCCGCTGCTGCCG-3′. Cycling variables and PCR reaction conditions are available upon request.

Cell culture and manipulation. *Drosophila* Schneider S-2 cells (a gift from Dr. Phillip Beachy) were grown in Schneider's *Drosophila* Medium (Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum and 1% penicillin/streptomycin as described. Subconfluent S-2 cells were cotransfected with linearized pCo-Hygro using Effectene (Qiagen). After 48 hours of incubation with nucleic acid complexes, cells were selected with 0.2 μg/mL Hygromycin (Life Technologies) in complete medium. Cell lines harboring stably integrated plasmids were identified by PCR. Transient transfections with pMT-HA-*DNMT1* were done for 48 hours followed by 96 hours of induction as described (29). Cos-7 cells (American Type Culture Collection) were cultured in DMEM supplemented with 10% FCS and 1× PenStrep and were transfected with 5 μg of DNA using LipofectAMINE Plus according to the manufacturers instructions (Life Technologies).

Western blot analysis. Cell extracts from transfected Cos-7, *Drosophila* S2, or double knock-out (DKO) cells were prepared, electrophoresed, and transferred as described (30). Filters were probed with a polyclonal antibody specific for DNMT1 (23), or commercially available antibodies to HA or proliferating cell nuclear antigen (Santa Cruz Biotechnology, Santa Cruz, CA). Visualization was done using the enhanced chemiluminescence method according to the manufacturers instructions (Amersham, Arlington Heights, IL).

Methylation analysis. Bisulfite sequence analysis was done essentially as previously described (23). Briefly, DNA was extracted and treated with sodium bisulfite as described (31). The oligonucleotide primers for amplifying *TIMP3* (TIMP3.BisSeq2F and TIMP3.BisSeq2R), *MLH1* (MLH1.BisSeq.1F and MLH1.BisSeq1R), and *MGMT* (MGMT.BisSeq2F and MGMT.BisSeq4R) are shown below. PCR products were cloned into the vector pCR2.1-TOPO (Invitrogen) according to the manufacturer's instructions. DNA from individual clones was prepared using Promega Wizard (Madison, WI) reagents and analyzed at the JHU Sequencing Facility. For global analysis of 5-methylcytosine content, 5 μg of genomic DNA and 50 units of McrBC (New England Biolabs, Beverly, MA) enzyme was incubated with 1× NEBuffer 2 (50 mmol/L NaCl, 10 mmol/L Tris-HCl, 10 mmol/L $MgCl_2$, 1 mmol/L DTT) containing 100 μg/mL bovine serum albumin and 1 mmol/L γ-GTP for 12 hours at 37°C. One third of each reaction was electrophoresed on 0.8% agarose gels and visualized by UV illumination. Primer sequences are TIMP3.BisSeq2F, 5′-GGTTTGAGGGGGCGGG-TTTTAATAG-3′; TIMP3.BisSeq2R, 5′-CTACTACTCGCCTCTCCAAAAT-TACC-3′; MLH1.BisSeq.1F, 5′-AGTAGTTTTTTTTTTAGGAGTGAAGG-3′; MLH1.BisSeq1R, 5′-TTAACCCTACTCTTATAACCTCCC-3′; MGMT.BisSeq2F, 5′-GAGGATGCGTAGATTGTTTTAGGTT-3′; and MGMT.BisSeq4R, 5′-AAC-TATCCCAACATATCCGAAAC-3′.

Primer sequences used for bisulfite sequencing of the endogenous *Drosophila* X chromosome locus or nucleotides 3276 to 3589 of pCOHygro, encompassing a portion of the Amp resistance gene, are available upon request.

Methyltransferase assay. Templates for measuring methyltransferase activity were generated by amplifying plasmids p*TIMP3*-639/73, p*MLH1*-102/4, or pKT200 with primer sets TIMP3-2 and TIMP3-3, MLH1.101 and MLH1.104 (for sequence, please see Plasmid Contruction above), or MGMT.103 and MGMT.104 (MGMT.103, 5′-AGGAGGGGAGAGACTCGCGC-3′; MGMT.104, 5′-GAGCTCCGCACTCTTCCGGG-3′), respectively. Nucleic acid products were ethanol precipitated, purified with G-25 spin columns (BioMax, Odenton, MD), and quantitated by spectrophotometry. DNMT assays were done essentially as described (23). Briefly, 15 μg of protein lysate or 0.5 to 2 units of recombinant DNMT1 protein (New England Biolabs) was incubated with 3 μCi of *S*-adenosyl-L-[methyl-^{3}H]methionine (Amersham) and 0.5 to 8 μg of the purified DNA template for 120 minutes at 37°C. Reactions were stopped, purified, and the resuspended nucleic acids spotted on GF/C filter discs (Whatman) before analysis by liquid scintillation counting. Reactions without DNA were analyzed in parallel and subtracted from the experimental value. For *in vitro* methylation treatment, 50 μg of DNA were mixed with 1× NEBuffer 2 (50 mmol/L NaCl, 10 mmol/L Tris-HCl, 10 mmol/L $MgCl_2$, 1 mmol/L DTT) containing 320 μmol/L *S*-adenosylmethionine, and 25 units of *Sss*I methylase (New England Biolabs) followed by incubation at 37°C for 4 hours. After addition of 640 μmol/L *S*-adenosylmethionine and 10 units of *Sss*I enzyme, reaction mixtures were further incubated for 24 hours at 37°C. Methylated DNA was extracted with phenol/chloroform, ethanol precipitated, and quantitated by spectrophotometry.

Recombinant adenovirus generation and infection. High-titer adenovirus expressing DNMT1 or β-galactosidase was generated using the AdEasy system as described (32). Full-length human *DNMT1* was released from pSVK3-5.2 (33) by digestion with *Sal*I and *Kpn*I and inserted into the *Sal*I/*Kpn*I sites of the shuttle vector pADTrack-CMV. After recombination with the vector pAdEasy, high-titer virus was generated in 911 and 293 cells. Viruses were purified on a cesium chloride gradient, and the effective titer was determined by plaque assay of low-passage 293 cells. The efficiency of the infection was normalized to the frequency and intensity of green fluorescent protein (GFP)–positive cells. DKO cells were infected sequentially at 6 days intervals with ~2 plaque-forming units per cell for a total of 24 to 48 days.

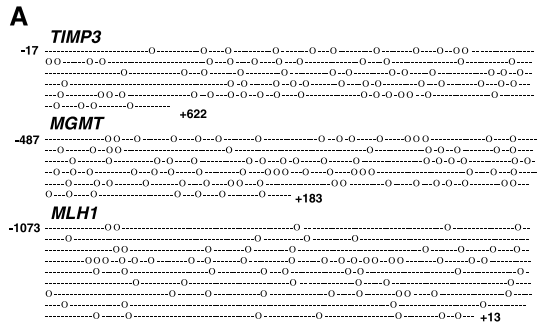
Results

***De novo* methylation of human tumor suppressor gene CpG islands.** To advance the identification of DNMTs responsible for patterns of promoter CpG island hypermethylation in human cancer cells, we wished to develop a biochemical assay, which might quantitate *de novo* methylating activity against such regions. An assay based on the capacity of proteins to incorporate tritiated methyl groups from [methyl-^{3}H]*S*-adenosyl methionine into unmethylated DNA substrates was developed. A similar approach is routinely used to assess the affinity of proteins or extracts for the synthetic substrate poly(deoxyinosinic-deoxycytidylic acid)/poly(deoxyinosinic-deoxycytidylic acid), which mimics hemimethylated DNA and is thought to provide one measure of maintenance DNMT activity *in vitro*. Previous studies have used partially purified (34) or recombinant proteins (35) to measure incorporation into short synthetic oligonucleotides containing a single or few CpG dinucleotides (36, 37), insect DNA (38), or bacterial plasmid DNA (39). In contrast, we wished to analyze *de novo* CpG island methylation in whole-cell extracts, containing intact DNA methylation machinery and potential cofactors.

Initially, when genomic DNA from human and *Drosophila* cells, or short oligonucleotides, were mixed with extracts from human cancer cells, we measured only limited incorporation, equivalent to background levels (data not shown). We then studied CpG islands of three genes (Fig. 1*A*) frequently targeted by DNA methylation in colon cancer, *TIMP3* (40), *O^6-MGMT* (41), and *MLH1* (42). These genes are representative of the diverse categories of genes that are hypermethylated in colorectal cancer and fulfill the criterion of genes containing CpG islands (Fig. 1*B*; ref. 43).

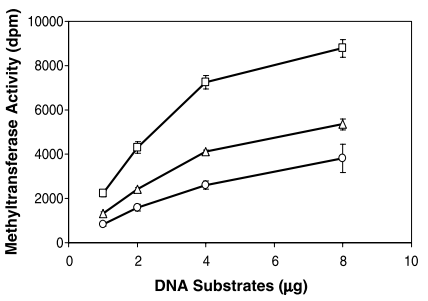
Each of these CpG islands supported robust and reproducible *de novo* methylation in methyltransferase assays using whole-cell protein extracts from HCT116 colon cancer cells (Fig. 1*C*). Levels of incorporation varied over a 3-fold range among the different DNA substrates, independent of their CpG density. To verify that the observed enzyme activity is not unique to this colorectal carcinoma cell line, we tested extracts from small cell lung, breast, hepatocellular carcinoma, and other colorectal cancer cells. Robust incorporation into the *TIMP3* CpG island substrate over a range of 3,178 to 12,610 disintegrations per minute, comparable with those obtained with the HCT116 colorectal cell line was observed (Fig. 1*D*).

DNMT1 accounts for the majority of *de novo* methyltransferase activity in protein extracts from human cancer cells. We next asked whether our enzyme assay could measure *de novo* activity in human cancer cells genetically inactivated for different DNMTs. Incorporation into the CpG island of the human *TIMP3* gene was first assessed using whole-cell extracts from wild-type colorectal cancer cells and compared with



B

	TIMP3	MGMT	MLH1
size (bp)	639	670	1086
CpG (No.)	73	88	74
GC content (%)	75	74	58
CpG/GpC Ratio	0.72	0.91	0.70



D

Tissue of origin	Cancer cell	Activity (dpm)	S.D.
Colon	HCT116	7248	±288
"	RKO	5159	± 491
"	CaCO2	4709	± 646
Lung	H69	8321	±392
"	H249	3178	± 453
Breast	MCF7	6269	± 811
"	MB-MDA-231	5220	± 746
"	MB-MDA-468	12610	± 1758
Liver	HEP3B	8787	± 353

Figure 1. CpG islands as substrates for DNA methyltransferase activity. *A,* structure of the DNA sequences used in this study. *Open circles,* CpG dinucleotides; *dashed lines,* other nucleic acids. *Numbers,* position of nucleotides with respect to the transcription start site. Abbreviated gene names (*italics*). *B,* CpG islands are DNA sequences >200 nucleotides in length, containing >50% GC content and a CpG/GpC ratio of >0.5 (43). Size, CpG content, and GC ratio are shown for the three human CpG islands used as substrates in (*C*). *C,* DNA methyltransferase activity, in disintegrations per minute (*dpm*), of HCT116 cancer cell lysates for the human *TIMP3* (□), *MGMT* (△), and *MLH1* (○) CpG islands. *Points,* mean methyltransferase activity; *bars,* SD. *D,* DNA methyltransferase activity of extracts from different cancer cells for the human *TIMP3* CpG island substrate.

DNMT1$^{(+/-)}$, *DNMT1*$^{(-/-)}$, *DNMT3b*$^{(-/-)}$, or DKO *DNMT1*$^{(-/-)}$ *DNMT3b*$^{(-/-)}$ cells.

As shown in Fig. 2*A*, this experiment revealed that the majority of *de novo* activity was lost after deletion of both alleles of DNMT1, the enzyme predicted to exclusively mediate maintenance methylation. This was reflected by a 40 ± 6% decrease with loss of one DNMT allele [*DNMT1*$^{(+/-)}$], and a 60 ± 5% decrease in the *DNMT1*$^{(-/-)}$ cells. Analysis of cells lacking DNMT3b revealed

a lesser but distinctly measurable loss of *de novo* enzyme activity (17 ± 4%). Experiments done with cells lacking both DNMT1 and DNMT3b produced two interesting results. First, the loss of both of these enzymes reduced methyltransferase activity in an additive manner (60 + 17 = 77%), suggesting that DNMT1 and DNMT3b, as assayed in our cell extract system, catalyze *de novo* methylation independently rather than cooperatively. Second, the DKO cell lines retained ~21 ± 2% of *de novo* activity, suggesting the existence of another methylating activity in these cells.

To further test the observation that *de novo* activity in the cell extracts reflected predominantly the contribution of DNMT1, we studied recombinant human DNMT1 enzyme (34). This preparation catalyzed robust activity against the *TIMP3* CpG island (Fig. 2*B*) as well as the *MGMT* and *MLH1* promoters (data not shown). We also addressed activity generated by DNMT1 expressed exogenously in COS cells from an expression vector containing the full-length human gene (Genbank accession no. NM001379) or a catalytically inactive version in which a single amino acid within the methyltransferase catalytic domain was mutated (Fig. 2*C*). The wild-type construct generated a 10-fold increase in *de novo* methyltransferase activity when compared with lysates from untransfected cells or those transfected with the inactive enzyme (Fig. 2*C*) in experiments where exogenous protein was expressed at comparable levels (Fig. 2*C*).

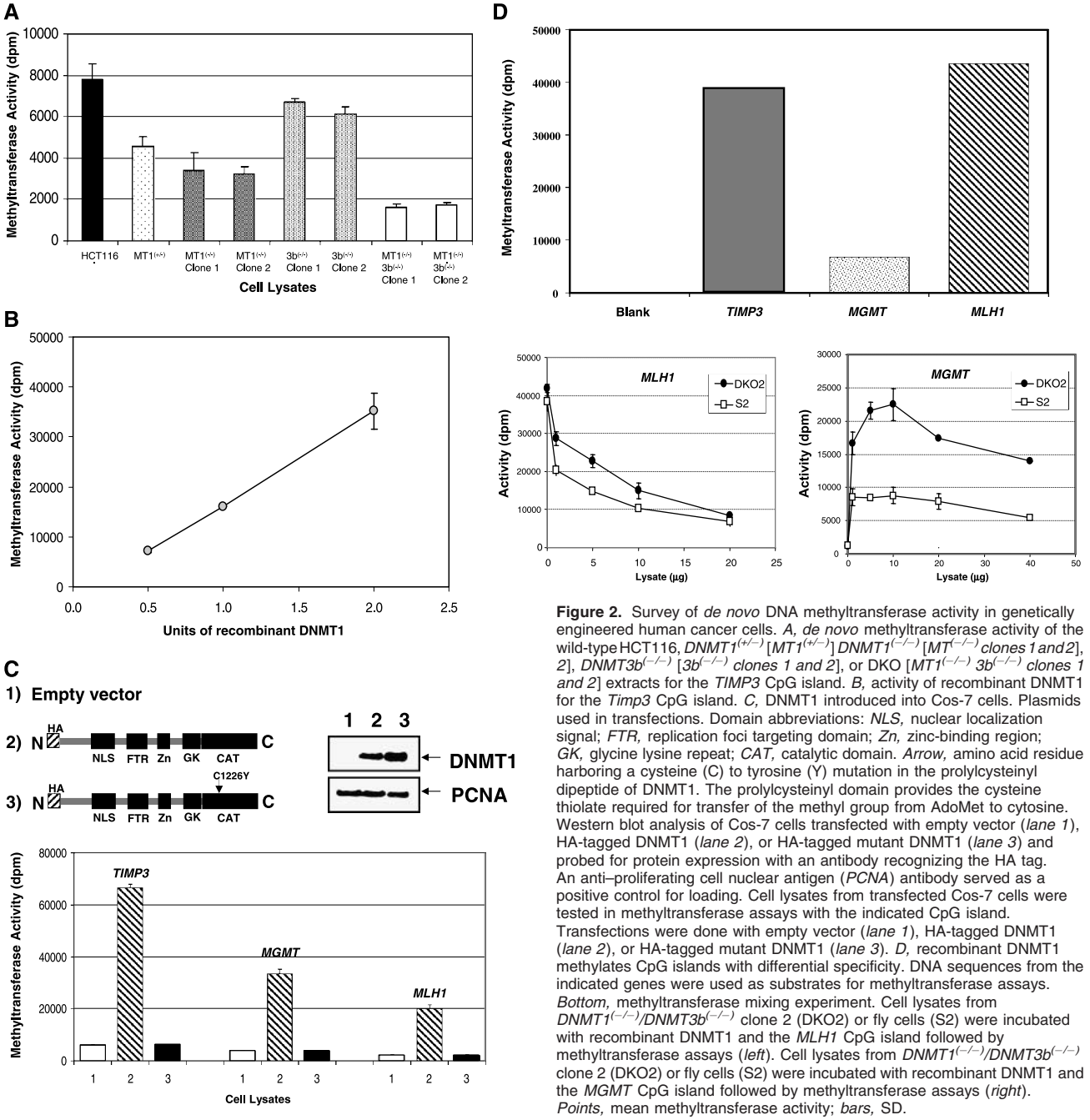
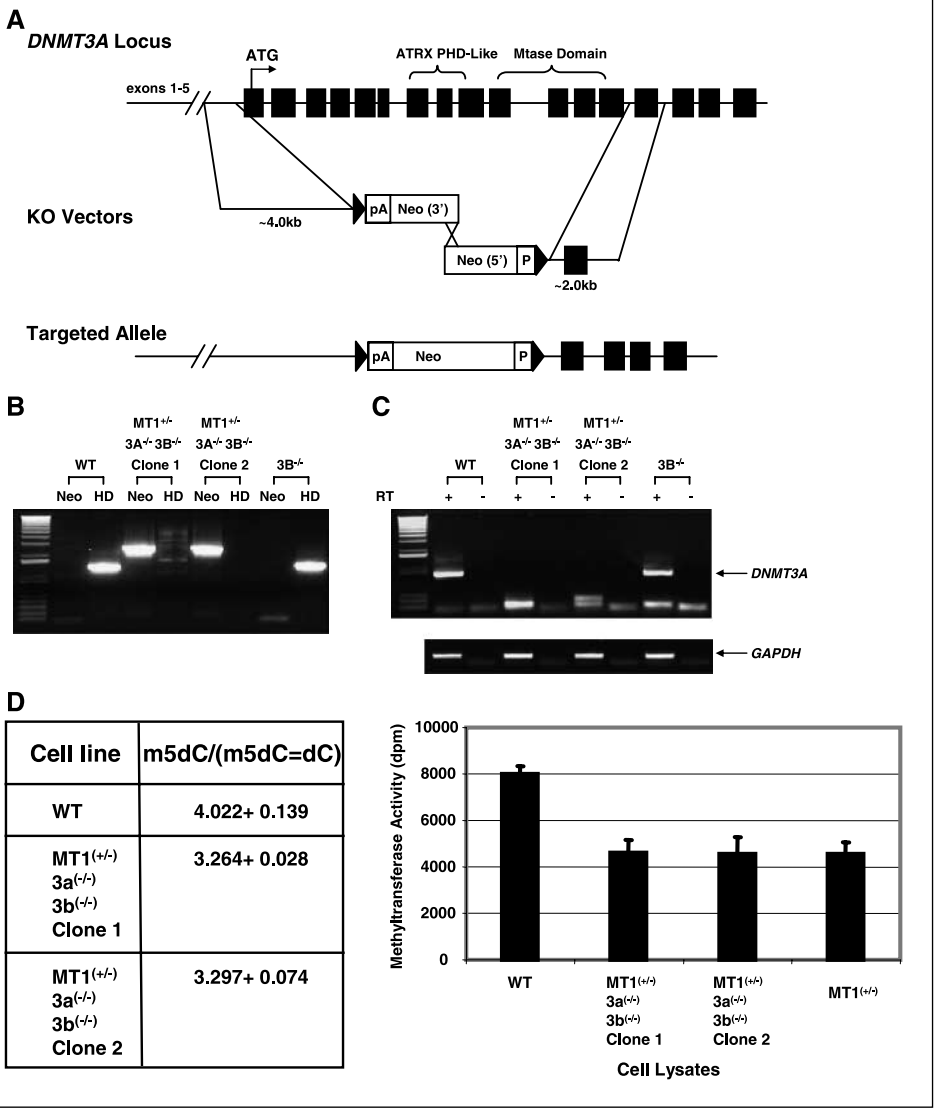


Figure 2. Survey of *de novo* DNA methyltransferase activity in genetically engineered human cancer cells. *A, de novo* methyltransferase activity of the wild-type HCT116, *DNMT1*$^{(+/-)}$ [*MT1*$^{(+/-)}$] *DNMT1*$^{(-/-)}$ [*MT*$^{(-/-)}$ *clones 1 and 2*], *2*], *DNMT3b*$^{(-/-)}$ [*3b*$^{(-/-)}$ *clones 1 and 2*], or DKO [*MT1*$^{(-/-)}$ *3b*$^{(-/-)}$ *clones 1 and 2*] extracts for the *TIMP3* CpG island. *B,* activity of recombinant DNMT1 for the *Timp3* CpG island. *C,* DNMT1 introduced into Cos-7 cells. Plasmids used in transfections. Domain abbreviations: *NLS,* nuclear localization signal; *FTR,* replication foci targeting domain; *Zn,* zinc-binding region; *GK,* glycine lysine repeat; *CAT,* catalytic domain. *Arrow,* amino acid residue harboring a cysteine (C) to tyrosine (Y) mutation in the prolylcysteinyl dipeptide of DNMT1. The prolylcysteinyl domain provides the cysteine thiolate required for transfer of the methyl group from AdoMet to cytosine. Western blot analysis of Cos-7 cells transfected with empty vector (*lane 1*), HA-tagged DNMT1 (*lane 2*), or HA-tagged mutant DNMT1 (*lane 3*) and probed for protein expression with an antibody recognizing the HA tag. An anti–proliferating cell nuclear antigen (*PCNA*) antibody served as a positive control for loading. Cell lysates from transfected Cos-7 cells were tested in methyltransferase assays with the indicated CpG island. Transfections were done with empty vector (*lane 1*), HA-tagged DNMT1 (*lane 2*), or HA-tagged mutant DNMT1 (*lane 3*). *D,* recombinant DNMT1 methylates CpG islands with differential specificity. DNA sequences from the indicated genes were used as substrates for methyltransferase assays. *Bottom,* methyltransferase mixing experiment. Cell lysates from *DNMT1*$^{(-/-)}$/*DNMT3b*$^{(-/-)}$ clone 2 (DKO2) or fly cells (S2) were incubated with recombinant DNMT1 and the *MLH1* CpG island followed by methyltransferase assays (*left*). Cell lysates from *DNMT1*$^{(-/-)}$/*DNMT3b*$^{(-/-)}$ clone 2 (DKO2) or fly cells (S2) were incubated with recombinant DNMT1 and the *MGMT* CpG island followed by methyltransferase assays (*right*). *Points,* mean methyltransferase activity; *bars,* SD.

Interestingly, lysates from Cos-7 cells expressing exogenous human DNMT1 showed a consistent and reproducible preference (*TIMP3* > *MGMT* > *MLH1*) for the different substrates (Fig. 2*D*) identical to that observed with HCT116 colon cancer cell lysates (see Fig. 1*C*). To address whether this specificity was inherent to DNMT1, we incubated each of the three human CpG islands with pure DNMT1 enzyme (Fig. 2*D*). Surprisingly, the pure enzyme showed a markedly different specificity (*TIMP3* = *MLH1* >>>>> *MGMT*). We speculated that the specificity of DNMT1 for various CpG islands was not inherent to the enzyme but may be due to associated cellular factors present in the protein extracts. To test this hypothesis, we incubated recombinant human DNMT1 with lysates from human or fly cells lacking DNMT1 and measured methylation of the human CpG islands by incorporation assays (Fig. 2*D*). Both of these lysates caused suppression of DNMT1-mediated methylation of MLH1 from levels similar to those observed using pure enzyme, down to levels using whole-cell extracts from HCT116 colon cancer cells. On the other hand, methylation of the MGMT CpG island was only achieved after incubation of recombinant DNMT1 with the colon cancer cell lysate. Thus, specificity for MGMT methylation may be due to the presence of an accessory factor present in human cells, accounting for the lack of methylation observed using the recombinant protein alone.

Generation and characterization of human cancer cells lacking DNMT3a. Because DNMT3b and DNMT3a are the only known bona fide *de novo* DNMTs in mammals, it would be predicted that the residual activity in the DKO cells, which have lost DNMT1 and DNMT3b, would be derived from DNMT3a. To test this hypothesis, we used strategies for the targeted inactivation of genes in human colorectal cancer cells. The human *DNMT3a* locus was inactivated by targeted gene disruption, using an approach that deleted 13 exons, including the catalytic domain of the enzyme, in HCT116 cells lacking *DNMT3b* and containing only a single allele of *DNMT1* (Fig. 3*A*). Amplification of genomic DNA identified two clones containing successful targeting events within the *DNMT3a* locus (Fig. 3*B*). Loss of expression of the DNMT3a gene product in these cells was confirmed by RT-PCR (Fig. 3*B*).

To analyze the effects of DNMT3a gene deletion on global 5-methylcytosine content, we analyzed the DNMT3a- and



Cell line	m5dC/(m5dC=dC)
WT	4.022+ 0.139
MT1(+/-) 3a(-/-) 3b(-/-) Clone 1	3.264+ 0.028
MT1(+/-) 3a(-/-) 3b(-/-) Clone 2	3.297+ 0.074

Figure 3. Targeted disruption of the human *DNMT3a* locus. *A,* schematic of the strategy for targeted disruption of the *DNMT3a* locus. *Closed rectangles,* exons. *B,* verification of targeting at the *DNMT3a* locus by PCR of genomic DNA from the indicated cell line. Neo primers detect homologous recombinants using one primer from the *Neo* gene and one from outside the targeting vector. Homozygous deletion (*HD*) primers were used to verify the absence of the *DNMT3a* locus. Loss of *DNMT3a* expression in targeted cell lines monitored by RT-PCR. Control reactions (−) are without reverse transcriptase. *C,* reverse-phase HPLC of wild type (*WT HCT116*) and two independent *DNMT1*$^{(+/-)}$*DNMT3a*$^{(-/-)}$*DNMT3b*$^{(-/-)}$ [*MT1*$^{(+/-)}$*3a*$^{(-/-)}$*3b*$^{(-/-)}$] knockout cell lines. *D, de novo* methyltransferase activity in wild-type HCT116, *DNMT1*$^{(+/-)}$*DNMT3a*$^{(-/-)}$ *DNMT3b*$^{(-/-)}$ [*MT1*$^{(+/-)}$*3a*$^{(-/-)}$*3b*$^{(-/-)}$] or *DNMT1*$^{(+/-)}$ [*MT1*$^{(+/-)}$] extracts for the *TIMP3* CpG island.

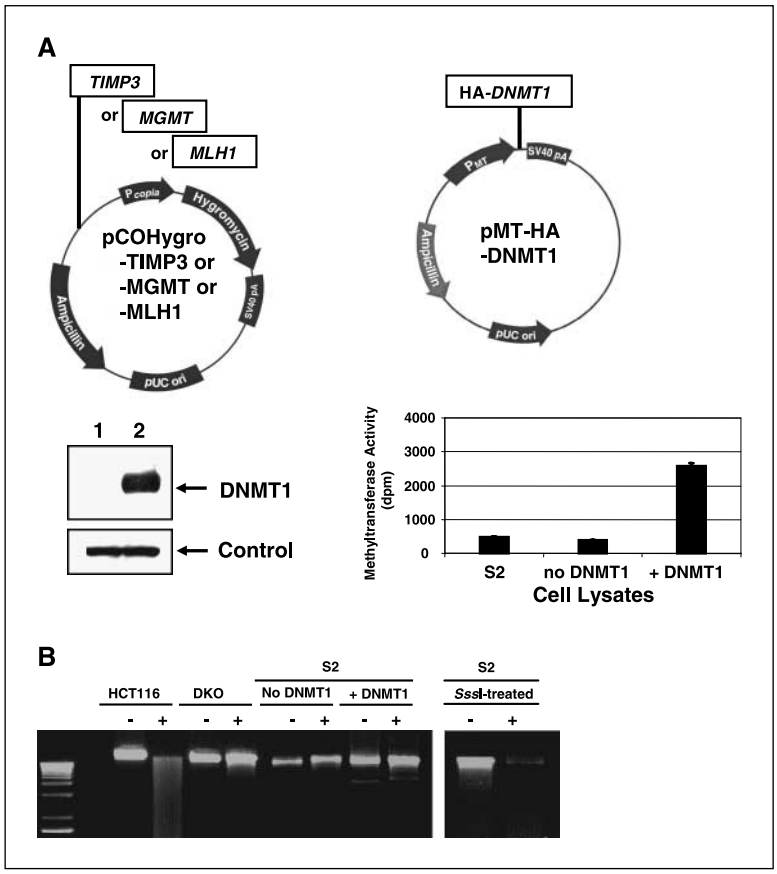


Figure 4. CpG methylation in *Drosophila* cells expressing human DNMT1. *A,* plasmids used in this study. *Left,* vectors containing the hygromycin selection gene driven by the *Drosophila* copia promoter (P_{copia}), with integrated human CpG islands (*rectangles*). *Right,* a schematic depiction of the HA-tagged DNMT1 expression vector under control of the inducible metallothionein promoter (P_{MT}). Representative Western blot analysis of *Drosophila* S2 cells before (*lane 1*) or after (*lane 2*) induction of DNMT1. *Arrows,* position of DNMT1 or a control protein (nonspecific band) after probing with an antibody recognizing HA. Methyltransferase activity of *Drosophila* cells alone (S2) or cells transfected with DNMT1 before (*no DNMT1*) or after induction (+ *DNMT1*). *B,* methylation assessed by McrBC endonuclease digestion. Genomic DNA from HCT116, DKO cancer cells, or *Drosophila* cells (S2) before and after induction of DNMT1. As a positive control, the bacterial *Sss*I methylase was used to methylate fly DNA before digestion with the McrBC endonuclease.

DNMT3b-deficient cells by reverse-phase high-performance liquid chromatography (HPLC). When compared with the wild- type HCT116 cells, the new cell lines registered an ~20% reduction (from 4.022 ± 0.139 to 3.297 ± 0.074) in 5-methylcytosine content (Fig. 3*D*). We showed previously that cells lacking DNMT1 had a minimal loss (~20%) of 5-methylcytosine content (23), whereas cells deleted for DNMT3b had virtually no loss (<3%) of 5-methylcytosine content (24). However, disruption of both DNMT1 and DNMT3b resulted in a reduction of nearly all (>95%) genomic methylation. In light of these data, and from the analysis of our new knockout cell lines, we conclude that DNMT3a contributes minimally to the maintenance of genomic DNA methylation patterns.

Having derived cells with both *de novo* DNMTs, 3a and 3b genetically inactivated we assayed extracts for *de novo* methyltransferase activity against the *TIMP3* CpG island substrate. $DNMT1^{(+/-)}DNMT3a^{(-/-)}DNMT3b^{(-/-)}$ cells revealed no further measurable loss of *de novo* activity against the CpG island substrates (Fig. 3*D*) when compared with the parental $DNMT1^{(+/-)}$ cells. From these experiments, we conclude that a majority of the *de novo* methyltransferase activity in extracts from human cancer cells is provided by DNMT1.

DNMT1 methylates human DNA integrated in the fly genome. Because our cell extract experiments showed high *de novo* DNMT1 activity against human CpG island substrates, we wondered if DNMT1 could preferentially methylate these same sequences in the context of an intact cell. To assess this in a CpG methylation-deficient background, and in the absence of the *de novo* DNMT 3a and 3b proteins, we introduced an inducible human DNMT1 construct into *Drosophila melanogaster* cells into which we had first stably integrated human CpG islands (Fig. 4*A*).

Induction of human DNMT1 expression in the various *Drosophila* cell lines yielded high protein production (Fig. 4*A*) and potent activity against our CpG island substrates in cell lysates (Fig. 4*A*). We then assessed methylation levels in the *Drosophila* genome by cutting the DNA with McrBC endonuclease, which cleaves DNA containing 5-methylcytosine on one or both strands but will not catalyze cleavage of unmethylated DNA (44). When probed with this enzyme neither DNA from uninduced or DNMT1-expressing *Drosophila* cells nor control DNA from the HCT 116 DKO cells, which have little DNA methylation, showed any cleavage. In contrast, genomic DNA from human HCT116 cells or fly genomic DNA artificially methylated with the bacterial *Sss*I enzyme were fully digested (Fig. 4*B*). Our *Drosophila* results are in agreement with previous studies showing that DNMT1 was unable to methylate *Drosophila* genomic DNA *in vivo* as measured by McrBC digestion (18). To verify these results, we sequenced a portion of the fly X chromosome as well as the pCOHygro vector used to introduce the mammalian CpG islands into the fly genome. As shown in Fig. 4*C*, not a single cytosine was methylated in these samples. Finally, we did HPLC analysis of DNA samples before and after DNMT1 expression; again, no 5-methylcytosine was detected (Table 1), consistent with previous observations (45).

We next asked, using bisulfite genomic sequencing, whether the integrated human CpG-rich sequences were methylated in *Drosophila* cells expressing full-length human DNMT1. We first sequenced DNA from fly cells with the human CpG islands for *TIMP3, MLH1*, or *MGMT* integrated but in which DNMT1

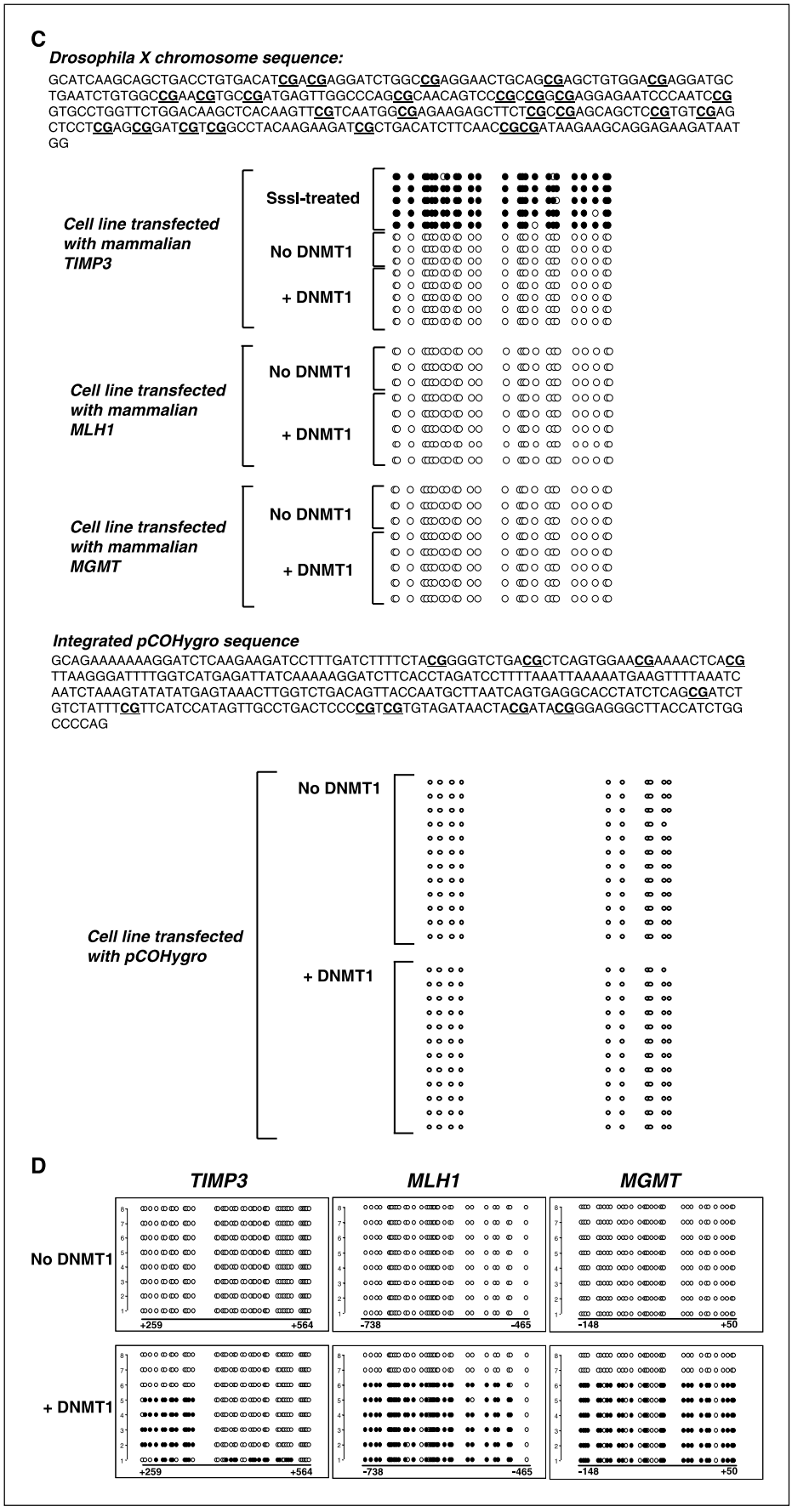


Figure 4 *Continued*. *C,* bisulfite sequencing of fly genomic DNA. *Top,* region of *Drosophila* X chromosome containing 26 CpG dinucleotides (*boldface and underlined*) within a 297-nucleotide span used for sequence analysis. *Bottom,* results of methylation analysis of individual S2 cell clones harboring stably integrated human CpG islands. Samples were induced to express DNMT1 (+ *DNMT1*) or remained uninduced (*No DNMT1*). *Sss*I-treated DNA is included as a positive control. Bisulfite sequencing of vector DNA integrated in the fly genome. The sequence of pCOHygro (nucleotides 3276-3589) spanning a portion of the ampicillin resistance gene (*top*), with CpG dinucleotides (*boldface and underlined*). *Open circles,* unmethylated CpG dinucleotides; *closed circles,* methylated residues. *D,* bisulfite sequencing of mammalian CpG islands integrated in the fly genome. Sequenced alleles from the indicated gene promoter integrated into the *Drosophila* genome. *Open circles,* unmethylated CpG dinucleotides; *closed circles,* methylated residues. *Numbers,* position relative to the transcription start site in the mammalian genome. Sequenced alleles from both uninduced (*No DNMT1*) and induced (+ *DNMT1*) *Drosophila* cells.

Table 1. Global 5-methylcytosine levels in different cell lines

Cell line	Peak area (285 nm)			Response		% MdC/total dC
	dC	MdC	dT	dC	MdC	
HCT116	790,416	36,319	529,567	82.36932	3.503327867	4.080
β-Galactosidase/DKO	8,863,378	22,767	5,291,743	923.6534	2.196103019	0.237
DNMT1/DKO	5,552,113	26,186	4,768,101	578.5862	2.525899489	0.435
S2 no DNMT1	7,501,686	0.00	6,373,960	781.7514	0	0.000
S2 + DNMT1	4,495,968	0.00	4,149,234	468.5252	0	0.000

NOTE: The cell lines used for DNA isolation are indicated in the first column. DKO cells were infected with β-galactosidase or DNMT1 encoding adenovirus, whereas fly S2 cells were uninduced or induced for DNMT1 expression. HPLC was used to quantitate the peak area for the indicated nucleotide in a given DNA sample. Response numbers indicate normalized peak height; cellular 5-methylcytosine ratio is shown in the last column.

expression had not been induced. Of the 864 CpG dinucleotides that were analyzed in these sequences, not a single residue was methylated at CpG or any other cytosine (Fig. 4*D*). In stark contrast, cell lines with high level induction of DNMT1 protein catalyzed vigorous *de novo* methylation of all three integrated human CpG islands (Fig. 4*D*). Interestingly, for the integrated human *MLH1* and *MGMT* sequences, virtually all of the CpG dinucleotides analyzed were methylated in 75% of the alleles analyzed. Alternatively, half of the alleles analyzed in the *TIMP3* promoter showed a clear boundary between nearly complete methylation of CpG dinucleotides and unmethylated residues.

We conclude that expression of the human DNMT1 enzyme is sufficient to specifically recognize and *de novo* methylate human CpG-rich sequences embedded in the fly genome.

DNMT1 can catalyze gene-specific *de novo* methylation of tumor suppressor genes in human cancer cells. We next wished to establish whether DNMT1, when reintroduced into *DNMT1*$^{(-/-)}$, *DNMT3b*$^{(-/-)}$ cancer cells harboring a >95% reduction in 5-methylcytosine content, including loss of DNA methylation in hypermethylated promoters, was capable of reestablishing DNA methylation *de novo*. To address this question, we made replication-defective adenoviruses encoding full-length human *DNMT1* or β-galactosidase and infected the DKO cells. High-level expression was confirmed by microscopy, as these adenoviruses coexpress GFP (Fig. 5*A*). We also detected high DNMT1 protein levels (Fig. 5*B*) and *de novo* methyltransferase activity for the CpG substrates in cell extracts (Fig. 5*B*).

When genomic DNA from infected DKO cells was digested with the McrBC endonuclease, detectable methylation of bulk genomic DNA was observed after infection with DNMT1 but not β-galactosidase encoding virus (Fig. 5*C*). These results are in contrast to those obtained with *Drosophila* cells, which do not methylate genomic sequences after expression of DNMT1. We asked whether this remethylation included repeat sequences, which comprise the major source of 5-methylcytosine in mammalian cells and are completely demethylated in the DKO cells. When Southern blot analysis with probes specific to *Alu* repeat sequences was done, we failed to detect remethylation after infection with DNMT1 (data not shown).

We next analyzed the methylation status of the three endogenous CpG islands in the DKO cells. As a point of reference, we first bisulfite sequenced the *TIMP3, MLH1*, and *MGMT* promoters in the parental HCT116 cells. In this analysis, *TIMP3* showed complete methylation of the promoter region, whereas the *MLH1* and *MGMT* promoters displayed an incomplete and heterogeneous pattern of methylation. In contrast, all three loci showed a complete absence of methylation in the DKO or β-galactosidase-infected cells (Fig. 5*D*). However, in these same cells, we observed robust methylation in six of the eight sequenced *MGMT* alleles but not in any alleles of *TIMP3* or *MLH1* following adenovirally mediated overexpression of DNMT1 (Fig. 5*D*). We confirmed this *MGMT*-specific CpG methylation in several independent experiments. For example, when we infected different DKO cell lines or serially infected these cells with variable amounts of the virus, CpG methylation was detected exclusively in the *MGMT* gene. Interestingly, when we analyzed DNMT1-infected and uninfected DKO cells by HPLC (Table 1), we observed an increase from 0.237 to 0.435 in total 5-methylcytosine content, roughly doubling the amount of endogenous 5-methylcytosine, suggesting the existence of other CpG islands that may be targeted for methylation by DNMT1. These data show that when introduced exogenously, DNMT1 could catalyze promoter hypermethylation in human cancer cells in a nonrandom, gene-specific manner.

Discussion

We describe a novel approach, using natural human CpG island sequences as substrates, to measure *de novo* CpG methylation in human cancer cells. Unlike substrates previously used, these CpG-rich sequences provide a reliable assay to quantitate such activity in cell extracts. Somewhat unexpectedly, when we surveyed genetically modified human cancer cells, including cells deficient for DNMT3a and DNMT3b, we find that DNMT1 is the major *de novo* methyltransferase for three different CpG island substrates in extracts from these cells. This enzyme specifically methylated these substrates imbedded in genomic DNA, in the absence of any other significantly methylated DNA or the presence of DNMT3a and DNMT3b, when inducibly overexpressed in *D. melanogaster* cells. Exogenously expressed DNMT1 could also methylate an endogenous gene CpG island in a virtually methylation deficient human cancer cell genome (colon cancer cells with deleted *DNMT1* and *DNMT3b* genes).

Taken together, our data indicate that DNMT1 might be considered to have more diverse and broad-ranging catalytic activities than previously suspected for a simple maintenance

enzyme. The potential capacity of this protein for catalyzing *de novo* methylation of CpG islands, as indicated in the systems we have tested, makes this enzyme an attractive candidate for a role in such processes as initiating hypermethylation of CpG-rich promoters in human cancer cells, and in other CpG island methylation-associated processes, such as imprinting and X inactivation occurring during human development.

These above possibilities, especially for the role in cancer, correlate with other previous findings. For example, constitutive expression of exogenous DNMT1, accompanied by increased enzyme activity, is sufficient to transform NIH3T3 cells and induce tumors when introduced as xenografts in nude mice (46) and to accelerate the spread of methylation in gene promoter CpG islands (30). Efficient oncogenic transformation by the c-*fos* oncogene requires elevated DNMT1 expression and increased cellular 5-methylcytosine content (47). Furthermore, genetic disruption of *Dnmt1* in mice reduces gastrointestinal tumors in the Min mouse model (48), whereas a hypomorphic *DNMT1* allele induces tumor formation (49). The molecular signaling pathways (50, 51) linking DNMT1 to oncogenesis suggest a capacity to mediate aberrant CpG island methylation. Our current data provide compelling evidence that DNMT1 could participate in neoplastic progression because it is functionally equipped to initiate promoter CpG island hypermethylation associated with

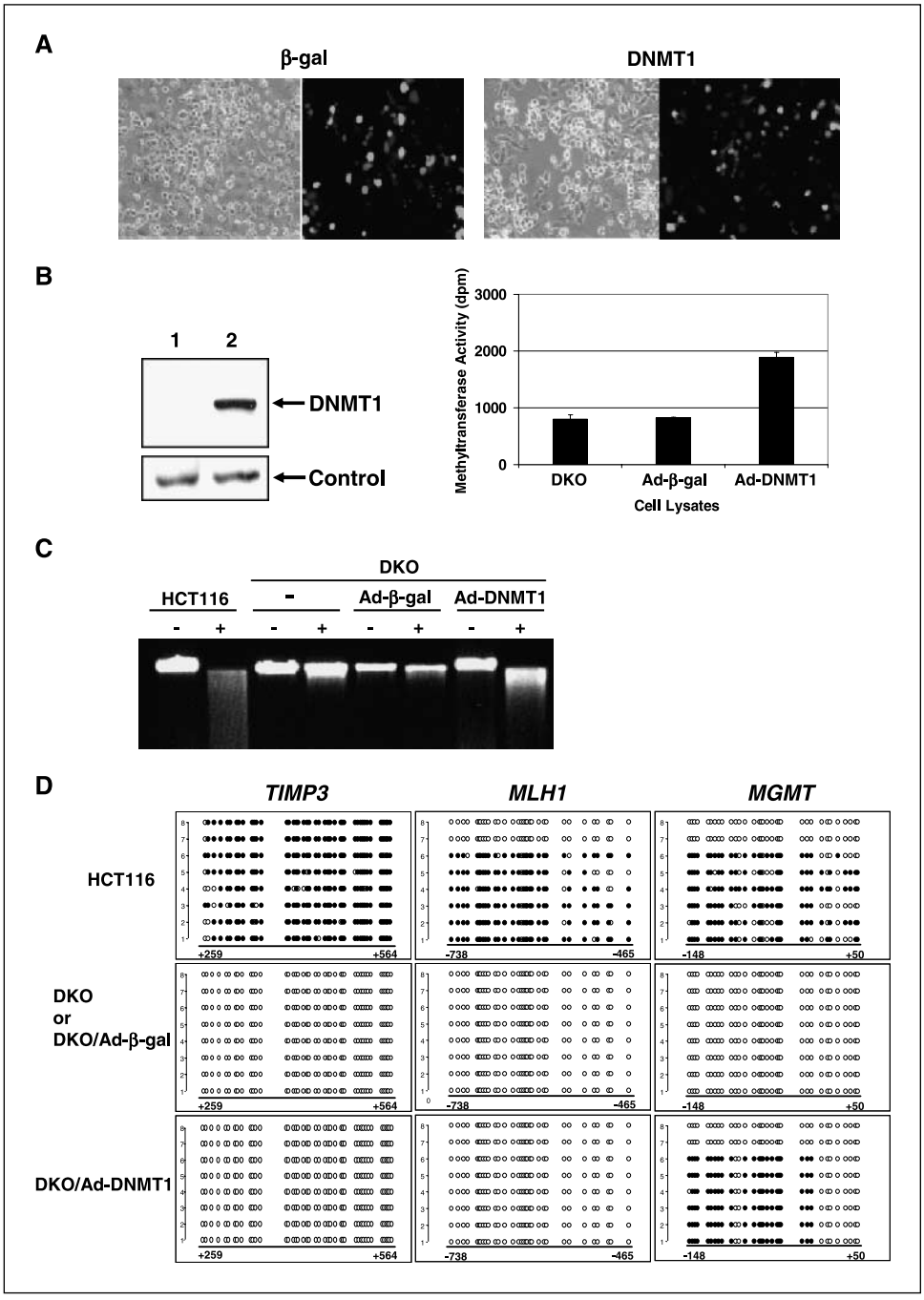


Figure 5. DNMT1-mediated reconstitution of CpG methylation in human cancer cells lacking 5-methylcytosine. *A,* DKO cells infected with adenoviruses encoding β-galactosidase or DNMT1. Cells were monitored for viability by phase-contrast microscopy (*left*) or for viral expression by fluorescence microscopy (*right*). *B,* Western blot analysis of DKO cells after viral infection. A representative filter probed for expression of DNMT1 (*arrow*) after infection with β-galactosidase (*lane 1*) or DNMT1 (*lane 2*). *Bottom,* control protein (nonspecific band). *De novo* methyltransferase activity after adenovirus infection. *C,* McrBC digestion of genomic DNA from HCT116, DKO cells alone (−), or infected with the indicated virus. *D,* bisulfite sequencing of CpG islands in DKO cells. Unmethylated (*open circles*) or methylated (*closed circles*) CpG dinucleotides. Genomic DNA was isolated from HCT116, uninfected DKO cells, or DKO cells infected with either β-galactosidase (*Ad-β-gal*) or DNMT1 (*Ad-DNMT1*) encoding adenoviruses. Eight sequenced alleles from the indicated gene. *Numbers,* position of nucleotides with respect to the transcription start site.

gene silencing. Our data also indicate that during the initiation of such aberrant methylation, the targeting of promoters in a native chromatin setting may be gene specific. This is apparent in our findings showing selective remethylation of O^6-*MGMT* in the DKO cells after reintroduction of DNMT1. Future studies designed to identify other genes targeted for epigenetic inactivation by DNMT1 could lead to important discoveries in our understanding of human cancer. In addition, how protein interactions known to occur with DNMT1 (52) or chromatin structures that normally protect CpG islands from aberrant methylation (4) influence the genesis of DNA methylation patterns in cancer cells, and how they may direct sites for the *de novo* functions of DNMT1 is clearly a rich area for future experimentation.

A final point raised by our data and showing the value of our sensitive *de novo* methylation assay concerns the detection of residual activity in the DKO cells that have genetically disrupted DNMT1 and DNMT3b. Clearly, from characterization of mammalian DNMTs to date, it might have been predicted that this remaining activity would be provided by DNMT3a. Yet, when this was rigorously tested using the *DNMT1*$^{(+/-)}$, *DNMT3a*$^{(-/-)}$, *DNMT3b*$^{(-/-)}$ cells, CpG methylation was maintained in an identical manner to *DNMT1*$^{(+/-)}$ cells. Thus, we detected no loss of *de novo* methyltransferase activity in cells rendered deficient for DNMT3a. All of the activity remaining in these cells, ~50% of wild-type HCT116 cells, can be accounted for by the one allele of *DNMT1*. Other studies have reported increased levels of DNMT3a RNA in cell lines and tumors (53), and our data cannot rule out the possibility that DNMT3a plays an important role in establishing and/or maintaining methylation patterns in other cell types. Most importantly, the possibility that another DNMT, such as DNMT2, which has recently been suggested to have some *de novo* CpG methylating capacity (54), may be responsible.

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