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Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain

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Mammalian DNA (cytosine-5) methyltransferase contains a C-terminal domain that is closely related to bacterial cytosine-5 restriction methyltransferases. This methyltransferase domain is linked to a large N-terminal domain. It is shown here that the N-terminal domain contains a Zn binding site and that the N- and C-terminal domains can be separated by cleavage with trypsin or *Staphylococcus aureus* protease V8; the protease V8 cleavage site was determined by Edman degradation to lie 10 residues C-terminal of the run of alternating lysyl and glycyl residues which joins the two domains and six residues N-terminal of the first sequence motif conserved between the mammalian and bacterial cytosine methyltransferases. While the intact enzyme had little activity on unmethylated DNA substrates, cleavage between the domains caused a large stimulation of the initial velocity of methylation of unmethylated DNA without substantial change in the rate of methylation of hemimethylated DNA. These findings indicate that the N-terminal domain of DNA methyltransferase ensures the clonal propagation of methylation patterns through inhibition of the *de novo* activity of the C-terminal domain. Mammalian DNA methyltransferase is likely to have arisen via fusion of a prokaryotic-like restriction methyltransferase and an unrelated DNA binding protein. Stimulation of the *de novo* activity of DNA methyltransferase by proteolytic cleavage *in vivo* may contribute to the process of ectopic methylation observed in the DNA of aging animals, tumors and in lines of cultured cells.

Key words: DNA methyltransferase/DNA methylation

Introduction

The modified nucleoside 5-methylcytosine (m^5C) occurs primarily at CpG dinucleotides in vertebrate DNA and is found in tissue-specific patterns that are transmitted by clonal inheritance during DNA replication (Wigler *et al.*, 1981; Stein *et al.*, 1982). Vertebrate methylation patterns control access of diffusible factors to DNA and may be involved in the regulation of gene expression during development (Antequera *et al.*, 1989; Bestor, 1990). Methylation patterns appear to be established and maintained by a single species of DNA (cytosine-5) methyltransferase (DNA MTase), as a single DNA MTase gene has been detected in the mammalian genome and murine cell types with different methylation patterns contain forms of DNA MTase that are identical by several criteria (Bestor *et al.*, 1988). The *de novo* activity of DNA MTase has little sequence specificity beyond the CpG dinucleotide (Bestor and Ingram, 1983, 1985a) and it is not known how tissue-specific methylation patterns are established.

Mammalian DNA MTase is strongly stimulated by hemimethylated DNA substrates, the product of semiconservative DNA replication (Gruenbaum *et al.*, 1982; Bestor and Ingram, 1983), whereas the bacterial cytosine-specific methyltransferases, which are part of a simple immune system, do not discriminate between unmethylated and hemimethylated DNA (Kelleher *et al.*,, 1991). The preference of mammalian DNA MTase for hemimethylated DNA ensures the clonal propagation of tissue-specific methylation patterns while the *de novo* activity of the bacterial enzymes ensures that the bacterial DNA will be resistant to cleavage by endogenous restriction endonucleases.

Mammalian DNA MTase is a large (1501 amino acid) protein whose 500 C-terminal amino acids have strong sequence similarities to all of the ~30 known bacterial DNA cytosine-5 methyltransferases (Bestor *et al.*, 1988; Lauster *et al.*, 1989; Posfai *et al.*, 1989; Bestor, 1990). Data presented here show that the N-terminal domain, which has a cysteine-rich region suggestive of a metal binding site (Berg, 1990), does bind Zn ions, and that proteolytic cleavage between the N- and C-terminal domains stimulates *de novo* methylation without major change in the rate of methylation of hemimethylated DNA. These data indicate that the N-terminal domain acts to inhibit *de novo* methylation. The role of the N-terminal domain in discrimination of unmethylated and hemimethylated sites is surprising on structural grounds, as three regions within the C-terminal domain are known to be close to the target cytosine and might be expected to provide this function. The findings also suggest that ectopic methylation, as observed in certain tumors (Baylin *et al.*, 1987; deBustros *et al.*, 1988; Nelkin *et al.*, 1991), aging animals (Uehara *et al.*, 1989) and in established lines of cultured cells (Antequera *et al.*, 1990), could result from the activation of the *de novo* activity of DNA MTase by proteolysis *in vivo*.

Results

Zinc binding by the N-terminal domain

The N-terminal domain of DNA MTase contains a cluster of eight cysteinyl residues of the form $CX_2CX_2CX_4CX_2$-$CX_2CX_{15}CX_4C$ between amino acids 537 and 575. The possibility that this Cys cluster represents a metal binding site (Berg, 1990) was tested by means of the ^{65}Zn blotting technique introduced by Schiff *et al.* (1988) and modified by Treich *et al.* (1991). As shown in Figures 1 and 2, pATH52, a TrpE fusion protein which contains amino acids 137–635 of DNA MTase, binds ^{65}Zn, while pATH32, a

TrpE fusion protein which contains the entire C-terminal domain, shows no detectable binding. Deletion of the Cys-rich region from pATH52 abolished Zn binding activity (Figure 2). Because of uncertainty regarding the efficiency of protein transfer and the degree of recovery of a native Zn binding conformation in the blotted protein the stoichiometry of Zn binding could not be determined.

Selective cleavage between the N- and C-terminal domains of DNA MTase

Domain-specific polyclonal antibodies were prepared against TrpE–DNA MTase fusion proteins produced in *Escherichia coli* (Figure 1). Immunoblot analysis of whole cell lysates of several murine cell types showed that both antibodies bound to a protein species of M_r 190 000, the known size of DNA MTase in human and murine cells (Bestor and Ingram, 1985b; Pfeifer and Drahovsky, 1986). Independently folded protein domains can often be separated by proteolytic cleavage and the domain-specific antibodies were used to identify conditions under which the link between the N- and C-terminal domains of DNA MTase could be selectively cleaved. As shown in Figure 3, the connection between the N- and C-terminal domains of DNA MTase is subject to cleavage by both trypsin (which cleaves at the amino acids Arg and Lys) and *Staphylococcus aureus* protease V8 (which cleaves primarily at Glu, with activity under some conditions towards Asp). The finding that the link between the domains could be cleaved with proteases of different specificities supports the domain structure deduced from sequence analysis. The precise site of cleavage by protease V8 was identified by vapor-phase Edman degradation of the purified C-terminal protease V8 fragment. As shown in Figure 3, cleavage occurs at a glutamyl residue only 10 residues downstream of the alternating lysyl and glycyl residues that mark the junction between the N- and C-terminal domains, and six amino acids upstream of the first sequence feature conserved among bacterial and mammalian DNA methyltransferases (Bestor *et al.*, 1988; Lauster *et al.*, 1989; Posfai *et al.*, 1989). Both trypsin and protease V8 also cleave sequences rich in charged residues at the extreme N-terminus; this region had been previously reported to be sensitive to proteolysis both *in vitro* and *in vivo* (Bestor and Ingram, 1985b). Cleavage of these sequences does not affect enzymatic activity or preference for hemimethylated sites (Bestor and Ingram, 1983, 1985).

Enhancement of de novo methylation by cleavage between domains

The effect of cleavage between the N- and C-terminal domains on the relative preference of DNA MTase for unmethylated and hemimethylated DNA was examined. Samples of purified DNA MTase were digested with a series of increasing protease V8 concentrations, the reactions stopped by addition of protease inhibitors, and the proteolyzed DNA MTase tested for relative preference for hemimethylated and unmethylated DNA substrates. The extent and position of cleavage was analyzed by immunoblotting with the antibody specific for the C-terminal domain. Hemimethylated DNA in which hemimethylated sites occurred only at CpG dinucleotides was prepared by hybridization of *E.coli* DNA methylated with the CpG-specific enzyme M.*Sss*I with an equal amount of unmethylated *E.coli* DNA. As shown in Figure 4, cleavage

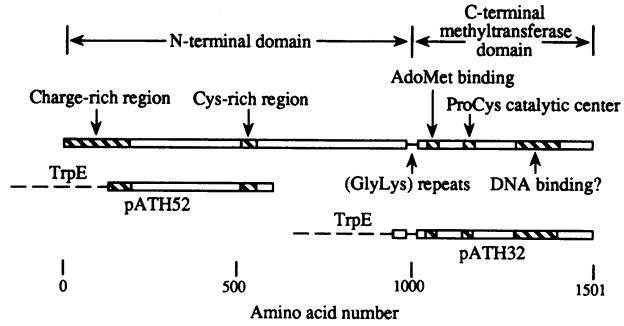

Fig. 1. Sequence features in mammalian DNA MTase. The C-terminal methyltransferase domain is ~500 amino acids in length and shows strong similarities with those bacterial restriction methyltransferases which produce m^5C. Sequence motifs proposed to be involved in *S*-adenosyl L-methionine (AdoMet) binding, DNA binding and catalysis (ProCys) are indicated, as are the cysteine-rich region and a region rich in charged residues at the extreme N-terminus. The N- and C-terminal domains are separated by a run of 13 alternating glycyl and lysyl residues (Bestor *et al.*, 1988). Shown below are regions of the DNA MTase cDNA used in production of the pATH52 and pATH32 fusion proteins. Purified TrpE–DNA MTase fusion proteins were produced in *E.coli* and injected into rabbits to elicit domain-specific polyclonal antibodies.

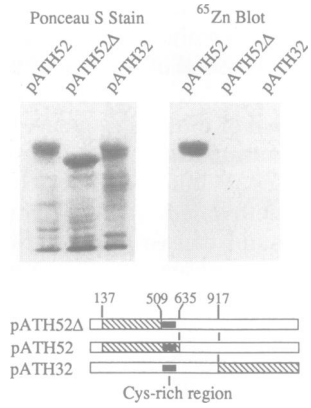

Fig. 2. Binding of ^{65}Zn by the Cys-rich region of the N-terminal domain. TrpE fusion proteins which contained the entire C-terminal domain (pATH32), or amino acids 137–635 (pATH52), or 137–509 (pATH52Δ) of the N-terminal domain were electroblotted and probed with ^{65}Zn(II). Deletion of the Cys-rich region can be seen to eliminate Zn binding. Cross-hatched regions at bottom show the portions of DNA MTase fused to TrpE; numbers are amino acid residues.

between the N- and C-terminal domains caused a large increase in initial velocity of methylation of the unmethylated substrate, without a comparable change in the rate of methylation of hemimethylated substrates or the synthetic substrate poly d(I·C)–poly d(I·C) (Figure 5). Trypsin gave a very similar result in that *de novo* activity was proportional to the accumulation of the M_r 57 000 fragment (unpublished data); however, the C-terminal domain contains trypsin-sensitive sites and activity was lost at higher trypsin concentrations (cleavage within the C-terminal domain is apparent in lanes 10–12 in Figure 3A). In all cases *de novo* activity was proportional to the amount of the M_r 57 000 C-terminal fragment. Trypsin treatment had been reported to stimulate the *de novo* activity of a partially purified preparation of DNA MTase (Adams *et al.*, 1983), but cleavage sites were not mapped and it could not be concluded from that study whether it was cleavage of DNA MTase or another component of the mixture that was responsible for the effect.

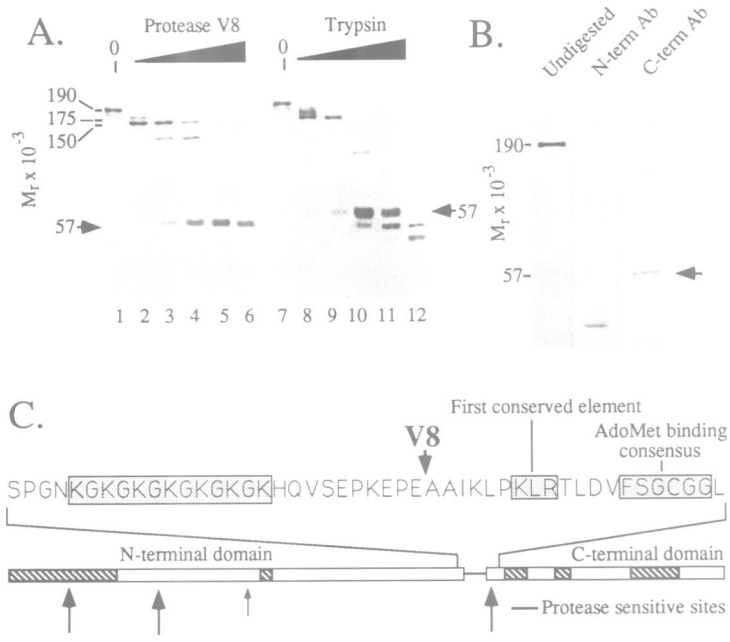

Fig. 3. Proteolytic cleavage between the N- and C-terminal domains of DNA MTase. (**A**) Fragmentation of DNA MTase by trypsin and *S.aureus* protease V8. The immunoblots were probed with antibody against the C-terminal domain. The M_r 57 000 fragment represents the C-terminal methyltransferase domain. The M_r 150 000 and 175 000 fragments are the result of cleavage within charge-rich sequences at the N-terminus; cleavage of these sequences does not affect the *in vitro* enzymatic activity of DNA MTase (Bestor and Ingram, 1985). (**B**) Antibodies to the N- and C-terminal domains recognize different proteolytic fragments of DNA MTase. Cleavage conditions for the material analyzed in the second and third lanes were similar to those of lane 6 in (A). Antibodies to both the N- and C-terminal domains stained the same M_r 190 000 protein prior to proteolysis; the arrow indicates the M_r 57 000 proteolytic fragment which is recognized only by the antibody to the C-terminal domain. (**C**) Site of cleavage between the N- and C-terminal domains. DNA MTase was purified from 50 l of cultured MEL cells (4×10^{10} cells) and cleaved with protease V8 under the conditions of lane 6 in Figure 2. The purified C-terminal fragment was subjected to vapor phase Edman degradation and the V8 cleavage site identified as a glutamyl residue 10 amino acids C-terminal of the alternating glycyl and lysyl (GK) residues which link the domains. The amino acid sequence lysylleusylarginyl (KLR) is common to M.*Sss*I (Renbaum, 1990), the multispecific methyltransferases of *Bacillus subtilis* phages (Lauster *et al.*, 1989) and mammalian DNA MTase. Hatched regions in the diagram at bottom correspond to the sequence features in Figure 1.

Discussion

Cleavage between the domains of DNA MTase

The link between the N and C-terminal domains of DNA MTase is likely to be extended and exposed to solvent, since proteases with different specificities cleave at or near the junction of the two domains. It is interesting in this regard that the bacterial restriction methyltransferase M.*Eco*RII has an N-terminal extension of ~100 amino acids that is either not present or unrelated with respect to sequence in other cytosine-specific restriction methyltransferases. Trypsin rapidly cleaves the M.*Eco*RII N-terminal extension 19 amino acids upstream of the *S*-adenosyl L-methionine binding site without eliminating enzyme activity (Friedman *et al.*, 1991), while protease V8 cleaves mammalian DNA MTase 13 amino acids upstream of this feature. The conservation of cleavage sites suggests strong conservation of both sequence and structure between bacterial and mammalian DNA cytosine methyltransferases.

Discrimination of unmethylated and hemimethylated CpG sites by DNA MTase

The C5 positions of adjacent cytosine residues at CpG dinucleotides are close together in the major groove of the B-form helix (Figure 6). While three regions within the C-terminal domain of DNA MTase [the prolylcysteinyl motif at the catalytic center (Wu and Santi, 1987; Chen *et al.*, 1991), the *S*-adenosyl L-methionine binding site near the glycyllysyl repeats (Ingrosso *et al.*, 1989) and a putative DNA binding region near the C-terminus (Klimasauskas *et al.*, 1991)] are likely to be very near the CpG site during methyl transfer, the results of this study indicate that discrimination of unmethylated and hemimethylated CpG sites is carried out not by contacts with these regions but by a separate large protein domain. Unmethylated and hemimethylated sites can in principle be distinguished by direct contacts in the major groove; amino acid side chains make direct contacts with major groove methyl groups in some specific protein–DNA complexes, as in the case of the glucocorticoid receptor where a valyl side chain makes a van der Waals contact with a thymine methyl (Luisi *et al.*, 1991). However, three lines of evidence suggest that discrimination of unmethylated and hemimethylated sites is not the result of direct contacts with major groove methyl groups. First, proteolysis does not stimulate the initial velocity of methylation of poly d(I·C)–poly d(I·C) (Figure 5). This behavior is more similar to that of hemimethylated DNA than of unmethylated DNA, despite the fact that the major groove of poly d(I·C)–poly d(I·C) lacks methyl groups and resembles unmethylated DNA more than hemimethylated DNA. Second, as mentioned above three regions within the C-terminal domain of DNA MTase must be very close to the CpG site during methyl transfer and it is unlikely on steric grounds that a region of the N-terminal domain could be accommodated in the confines of the major groove (Figure 6). Third, the *de novo* activity of DNA MTase is sensitive to sequence-dependent alternative secondary structures induced in DNA by negative

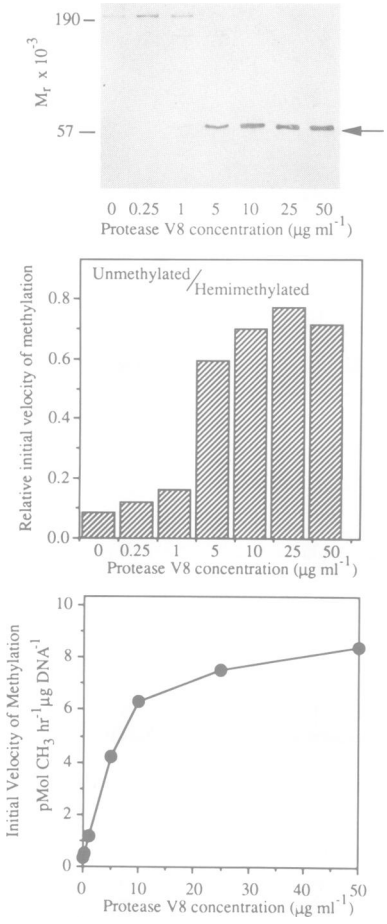

Fig. 4. Effect of proteolysis on initial velocities of methylation of unmethylated and hemimethylated DNA. Purified DNA MTase was treated with a concentration series of protease V8, the reactions stopped by addition of protease inhibitors, and the effect on enzyme activity measured by rate of transfer of ^{3}H-labeled methyl groups from *S*-adenosyl L-methionine to unmethylated or hemimethylated DNA. The top panel shows an immunoblot of samples stained with antibody against the C-terminal domain. Cleavage of the C-terminal M_r 57 000 fragment at higher protease concentrations is apparent. In the middle panel, proteolysis can be seen to stimulate methylation of the unmethylated substrate with respect to hemimethylated DNA; the effect is proportional to the accumulation of the M_r 57 000 C-terminal domain product. The lower plot shows the effect of proteolysis on the initial velocity of *de novo* methylation of purified *E.coli* DNA.

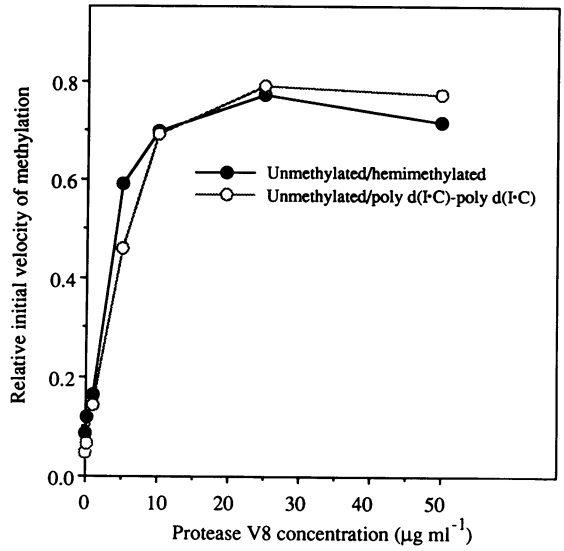

Fig. 5. Stimulation by proteolysis of the initial velocity of methylation of unmethylated DNA with respect to both hemimethylated DNA and the artificial substrate poly d(I·C)–poly d(I·C). Conditions of the reactions and transmethylation assays were identical to those of Figure 4.

supercoiling (Bestor, 1987), as would be expected if features other than nucleotide sequence alone are important in the recognition of target sites by DNA MTase.

As pointed out by Travers (1989), DNA–protein recognition involves both direct interactions with individual bases and the recognition of local and large-scale structural features in DNA. Recognition of static structural features such as bends or cruciforms or dynamic features such as the ability of a sequence to be deformed into a configuration that fits the binding site of a particular protein are examples of this latter type of DNA–protein recognition. Methylation of cytosine residues affects DNA structure by increasing the T_m of G·C base pairs by several degrees (Hausheer *et al.*, 1989) and by favoring the B–Z transition (Felsenfeld *et al.*, 1983). Energy minimization calculations suggest that methylation at the 5 position increases the stacking energy of adjacent bases and changes the sign of electrostatic interactions involving the major groove (Hausheer *et al.*, 1989). The transmethylation reaction itself must involve significant distortion of DNA sequences around the target cytosine due to the non-planar configuration of the 5 and 6 positions during the reaction and the requirement for additional steric changes to allow approach of the cysteinyl thiolate to the 6 position and the methyl group of *S*-adenosyl L-methionine to the 5 position (Chen *et al.*, 1991). The finding that proteolyzed DNA MTase responds to poly d(I·C)–poly d(I·C) as though it were hemimethylated suggests that the structural property that informs DNA MTase of hemimethylated CpG sites is present in poly d(I·C)–poly d(I·C) in a methylation-independent form. A lack of knowledge of the structure of the DNA MTase–DNA complex prevents identification of this structural property but it is likely to be apparent only when DNA is deformed by binding to the enzyme. While it is not known how far the effects of methylation might propagate along the DNA helix or how methylation might affect the ability of particular DNA sequences to adopt alternative conformations upon protein binding, current evidence strongly suggests that it is methylation-dependent conformational information, rather than direct contacts with major groove methyl groups, that is the basis for discrimination of unmethylated and hemimethylated sites by the N-terminal domain of DNA MTase. The finding that the N-terminal domain contains a Zn binding site or sites, which are important DNA binding elements in many proteins (Berg, 1990), is consistent with this proposal.

Evolutionary origin of mammalian DNA MTase

Mammalian DNA MTase is likely to have arisen through fusion of genes for a sequence-specific DNA binding protein and a prokaryotic-like DNA cytosine methyltransferase. Some species of the bacterial genus *Spiroplasma* contain a cytosine methyltransferase whose recognition sequence is CpG (Nur *et al.*, 1985; Renbaum *et al.*, 1990). Fusion of a sequence-specific DNA binding protein to such an enzyme might have limited the action of the catalytic moiety to CpG sites in the vicinity of the recognition sequence of the binding

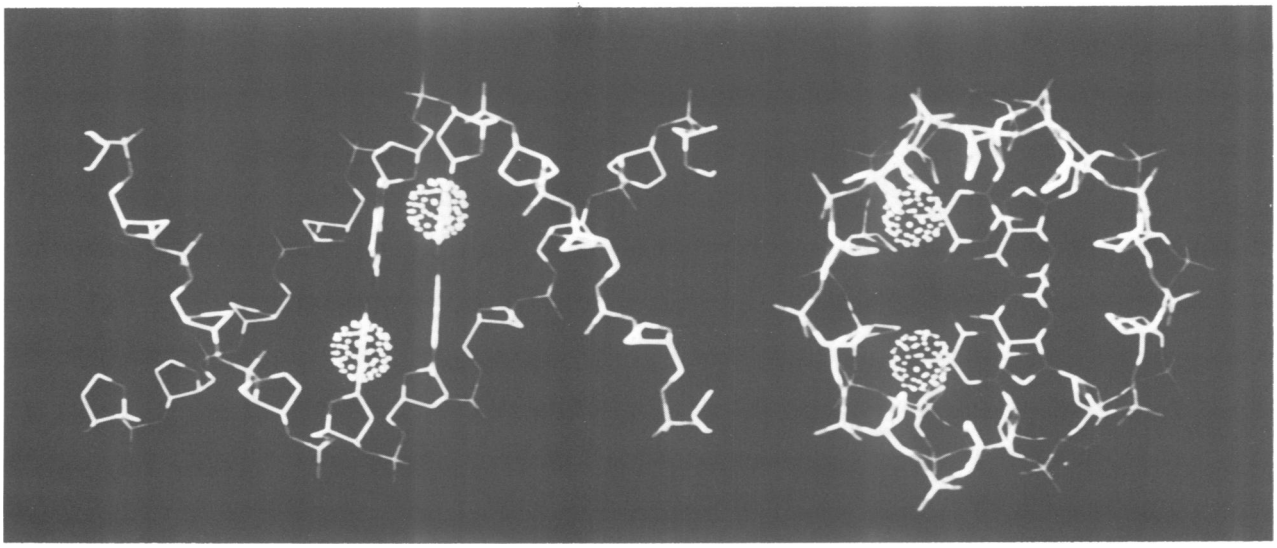

Fig. 6. Molecular graphics image of a symmetrically methylated CpG site in B-form DNA. The van der Waals surfaces of the methyl groups are outlined with white dots and the methyl groups can be seen to lie close together in the major groove. The van der Waals diameter of a methyl group is 5.3 Å and energy minimization studies suggest that methylation does not significantly alter the structure of undistorted B-form DNA (Hausheer *et al.*, 1989). For purposes of clarity only the bases in the CpG dinucleotide pair are shown.

protein; during the evolution of complex metazoa and the expansion of the eukaryotic genome as a result of gene duplication and divergence the regulatory domain could have evolved the capacity to ensure the clonal inheritance of methylation patterns through inhibition of *de novo* methylation (Bestor, 1990). It will be interesting to learn if the N-terminal domain of DNA MTase, when fused to sequence-specific DNA binding proteins, is able to alter or restrict the sequence specificity of such proteins.

Artifactual cleavage of DNA MTase

DNA MTase is very sensitive to endogenous and exogenous proteases, and cleavage within the charge-rich region at the N-terminus and between the N- and C-terminal domains commonly occurs during purification and enzyme assays (unpublished data); cleavage within the charge-rich region at the N-terminus occurs *in vivo* in MEL cells grown to saturating densities (Bestor and Ingram, 1985). Purification procedures which favor proteolysis will give the highest apparent yields, as *de novo* enzyme activity is the usual basis for assay of fractions during purification. Cleavage during purification is likely to be part of the reason for the wide disagreement in published reports of the size (Bestor and Ingram, 1985; Pfeifer and Drahovsky, 1986; Hitt *et al.*, 1988; Smith *et al.*, 1991), preference for hemimethylated versus unmethylated substrates (Sano *et al.*, 1983; Bestor and Ingram, 1983, 1985; Pfeifer *et al.*, 1985) and other properties of DNA MTase. Immunoprecipitation and immunoblot analysis of lysates of a number of tissues and cell types has shown that intact murine and human DNA MTase has an apparent M_r upon SDS–PAGE of 190 000 (Pfeifer and Drahovsky, 1986). Smaller forms are likely to be the product of proteolysis. Much of the published biochemical characterization of DNA MTase is likely to describe partially proteolyzed enzyme preparations, and appreciation of the sensitivity of DNA MTase to proteolysis and its effect on enzyme behavior *in vitro* will be essential for an understanding of the biochemical properties of this enzyme.

Activation of the de novo activity of DNA MTase by proteolysis in vivo

Ectopic *de novo* methylation occurs around certain genes in aging animals (Uehara *et al.*, 1989) and in human tumors (Baylin *et al.*, 1986, 1987; Silverman *et al.*, 1989), and is a frequent cause of gene inactivation in cultured cells (Antequera *et al.*, 1990). The effects of chance *de novo* methylation events are transmitted to daughter cells through the heritable nature of methylation patterns. Gene inactivation by ectopic *de novo* methylation may lead to somatic variation in tissues (if a tumor suppressor locus is affected, for example) and if it occurs in the germ line could contribute to genetic disease as may occur in the case of a gene at the fragile X locus in humans (Pieretti *et al.*, 1991). Results of the present study suggest that activation of DNA MTase by proteolysis would increase the frequency of gene inactivation by inappropriate *de novo* methylation and may contribute to the processes of tumorigenesis and senescence.

Materials and methods

Preparation of domain-specific antibodies

The N-terminal fusion construct (pATH52) encoded amino acids 137–635 and the the C-terminal fusion construct (pATH32) directed expression of amino acids 927–1522 (see Figures 1 and 2). In-frame fusions to the *E.coli TrpE* gene were constructed by means of the pATH series of expression vectors (Dieckmann and Tzagoloff, 1985). Fusion proteins were induced and inclusion bodies purified from *E.coli* strain JM105 as described (Klempnauer and Sippel, 1987). Fusion proteins were further purified by electroelution from SDS–polyacrylamide gels and precipitation with chloroform–methanol (Wessel and Flugge, 1984) prior to injection into rabbits.

Analysis of Zn binding

The fusion proteins encoded by pATH52 and pATH32 were isolated from induced cultures of *E.coli* JM105 as inclusion bodies and dissolved in 2% SDS, 5% β-mercaptoethanol, 0.0625 M Tris–HCl (pH 6.8), 10% sucrose and 0.02% bromphenol blue by heating to 100°C for 10 min. The denatured proteins were subjected to electrophoresis on SDS–8% polyacrylamide gels and transferred to Immobilon P membranes (Millipore Corp) by electroblotting in a Hoefer semidry transfer apparatus with 0.3 M Tris–HCl (pH 10.5), 20% methanol at the anode and 0.025 M Tris, 0.04 M ε-aminocaproic acid, 20% methanol at the cathode. The transfer was carried

out at 4 mA/cm^2 for 30 min. Analysis of ^{65}Zn(II) binding was as described (Schiff *et al.*, 1988; Treich *et al.*, 1991) except that denaturation with guanidine HCl was found not to be necessary in this case and was omitted. ^{65}Zn binding required the presence of 5 mM dithiothreitol (DTT) in the binding and washing steps. $^{65}ZnCl_2$ (7 Ci/g; New England Nuclear) was used at a final concentration of 5 μCi/ml.

Purification and proteolysis of DNA methyltransferase

The M_r 190 000 form of DNA MTase was purified from log-phase cultures of murine erythroleukemia cells as described (Bestor and Ingram, 1985), except that the DEAE sephacel step was replaced with an HPLC fractionation on a 2.1×15 cm Bio-Rad TSK DEAE 5PW column eluted with a 0–0.4 M NaCl gradient in 20 mM Tris–HCl (pH 7.4), 0.5 mM DTT and 0.2 mM phenylmethylsulfonylfluoride (PMSF). Active enzyme eluted between 0.15 and 0.2 M NaCl. Proteolysis reactions contained ~0.2 μg of DNA MTase and the amounts of protease indicated in the figures in a final vol of 20 μl 10 mM Tris–HCl (pH 7.4), 0.5 mM Na_3EDTA. After 30 min at 25°C, freshly prepared stock solutions of the protease inhibitors PMSF, aprotinin and leupeptin were added to final concentrations of 0.5 mM, 100 μg/ml and 100 μg/ml, respectively. Proteins were denatured by heating to 100°C in the presence of 2% SDS and 100 mM DTT and separated by electrophoresis through SDS–8% polyacrylamide gels. The contents of the gel were transferred to nitrocellulose sheets (BA85, Schleicher and Schuell) by electroblotting as described above. Blots were probed with domain-specific rabbit antibody, followed by alkaline phosphatase conjugated goat anti-rabbit IgG and chromogenic substrates as described (Harlow and Lane, 1988).

N-terminal protein sequencing

Purified DNA MTase (20 μg, ~100 pmol) from 4×10^{10} cultured MEL cells was cleaved with protease V8 at a protein:protease ratio empirically determined to yield quantitative cleavage between the domains (see Figure 3). Protein fragments were precipitated with 10% trichloroacetic acid (TCA) in the presence of 100 μg/ml Na deoxycholate, washed twice with acetone and dissolved in 20 μl of 2% SDS, 100 mM DTT, 10% sucrose, 0.0625 M Tris–HCl (pH 6.8) and 0.02% bromphenol blue by heating to 65°C for 15 min. Proteins were fractionated by SDS–PAGE and transferred to Problott PVDF (Applied Biosystems) membranes as described above for Immobilon P membranes. The band of interest was identified by staining with 0.2% Ponceau S in 3% TCA, excised and washed extensively in water, and subjected to N-terminal sequencing (Chen *et al.*, 1991) in an Applied Biosystems 470A vapor phase sequencer equipped with a model 120A on-line PTH amino acid analyzer at the Harvard Microchemistry Facility.

Assay of DNA MTase on unmethylated and hemimethylated DNA substrates

Hemimethylated DNA was produced by methylating purified *E.coli* DNA to completion at CpG sites with M.*Sss*I (New England Biolabs) in the presence of 20 μM non-radioactive *S*-adenosyl L-methionine, mixing and heat-denaturing equal quantities of methylated and unmethylated DNA, and hybridization for 48 h at 65°C at 1 mg/ml in 2×SSC. Unmethylated DNA was treated in the same way but without prior M.*Sss*I treatment. Renatured DNA was treated with an excess of nuclease S1 to remove any single stranded DNA, purified by phenol extraction and ethanol precipitation, and dissolved in water. Synthesis of hemimethylated DNA was confirmed by demonstration of resistance to *Hpa*II cleavage. Poly d(I·C)–poly d(I·C) was obtained from Boehringer Mannheim or Pharmacia. Proteolysis reactions were as described above; at the end of the reaction each sample received 100 μl of a solution consisting of 20 mM Tris–HCl (pH 7.4), 5 mM Na_3EDTA, 20% glycerol, 5 μg DNA, 4 μCi [^{3}H-methyl]*S*-adenosyl L-methionine (Amersham, 12 Ci/mmol), 200 μg/ml bovine serum albumin and protease inhibitors as described above. The samples were incubated at 37°C for 15 min, at which time one-third of the reaction volume was subjected to gel electrophoresis on SDS–8% polyacrylamide gels. The contents of the gel were transferred by electrophoresis to nitrocellulose sheets and probed with domain-specific antibody. The remainder of the reaction mixture was precipitated with 10% TCA and processed for scintillation counting. Control experiments showed that the transmethylation reactions were done under conditions of DNA excess and were pseudo-first order with respect to DNA MTase. Other controls showed that the protease inhibitors prevented appreciable cleavage during the transmethylation reaction.

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Note added in proof

Another example of indirect recognition of methylated DNA occurs in the case of DNase I, which makes no major groove contacts. [Lahm,A. and Suck,D. (1991) *J. Mol. Biol.*, **221**, 645–667] but shows enhanced cleavage 5′ of the methylated base within the sequence Gm^5CGC [Fox,K.R. (1986) *Biochem J.*, **234**, 213–216].