Hybrid mouse–prokaryotic DNA (cytosine-5) methyltransferases retain the specificity of the parental C-terminal domain

Sriharsa Pradhan and Richard J. Roberts¹

New England Biolabs, 32 Tozer Road, Beverly, MA 01915, USA

¹Corresponding author
e-mail: roberts@neb.com

The mouse (cytosine-5) DNA methyltransferase (Dnmt1) consists of a regulatory N-terminal and a catalytic C-terminal domain, which are fused by a stretch of Gly-Lys dipeptide repeats. The C-terminal region contains all of the conserved motifs found in other cytosine-5 DNA methyltransferases including the relative position of the catalytic Pro-Cys dipeptide. In prokaryotes, the methyltransferases are simpler and lack the regulatory N-terminal domain. We constructed three hybrid methyltransferases, containing the intact N-terminus of the murine Dnmt1 and most of the coding sequences from *M.HhaI* (GCGC), *M.HpaII* (CCGG) or *M.SssI* (CG). These hybrids are biologically active when expressed in a baculovirus system and show the specificity of the parental C-terminal domain. Expression of these recombinant constructs leads to *de novo* methylation of both host and viral genomes in a sequence-specific manner. Steady-state kinetic analyses were performed on the murine Dnmt1–*HhaI* hybrid using poly(dG–dC)·poly(dG–dC), unmethylated and hemimethylated oligonucleotides as substrates. The enzyme has a slow catalytic turnover number of 4.38 h^{–1} for poly(dG–dC)·poly(dG–dC), and exhibits 3-fold higher catalytic efficiency for hemimethylated substrates.

Keywords: baculovirus/*de novo* methylation/5-fluoro-2'-deoxycytidine/hybrid DNA (cytosine-5) methyltransferase

Introduction

The DNA cytosine-5 methyltransferases (MTases) are a class of enzymes that transfer a methyl moiety from S-adenosyl-L-methionine (AdoMet) to carbon 5 of the pyrimidine ring of cytosine (Bestor and Verdine, 1994; Kumar *et al.*, 1994) and form 5-methylcytosine (m⁵C) in DNA. In mammals, the predominant methylated sequence is the dinucleotide CG, whereas in prokaryotes methylation usually involves short sequences (4–8 bp) that are recognized by restriction modification systems. In the mammalian genome ~60% of the CG dinucleotides are methylated, accounting for ~3 × 10⁷ 5-methylcytosines. Such methylation appears to be involved in several biological processes such as gene expression (Graessmann and Graessmann, 1993), development (Li *et al.*, 1992), imprinting (Barlow, 1995), X chromosome inactivation (Singer-Sam and Riggs, 1993), genetic diseases (Imbert *et al.*, 1998), mutagenesis (Yang *et al.*, 1996)

and cancer (Schmutte and Jones, 1998). In prokaryotes, methylation is an essential component of host restriction modification systems (Redaschi and Bickle, 1996), DNA replication (Stambuk and Radman, 1998) and mismatch repair (Modrich, 1989).

Both murine and human methyltransferase cDNAs have been cloned (Bestor *et al.*, 1988; Yen *et al.*, 1992). Both enzymes are ~1600 amino acids long. The N-terminal two-thirds of the protein is considered the regulatory domain and the C-terminus is the catalytic domain (Bestor *et al.*, 1988; Yen *et al.*, 1992). The two domains are fused by a run of 13 alternating Gly-Lys residues and the two can be separated by proteolysis of the intact enzyme with V8 protease (Bestor, 1992). In the center of the N-terminal domain is a cluster of eight cysteinyl residues (CX₂CX₂CX₄CX₂CX₂), which has been shown to bind zinc ions (Bestor, 1992). Another part of the N-terminal domain has been implicated in co-localizing the murine Dnmt1 with the replication machinery (Leonhardt *et al.*, 1992). Recently, a peptide region (TRQTTITSHFAKG) of human Dnmt1 has been found to bind proliferating cell nuclear antigen (PCNA) (Chuang *et al.*, 1997). It has been shown that the *de novo* and maintenance methylation activities reside in the same protein and that the enzyme displays a preference for hemimethylated sites (Flynn *et al.*, 1996; Kho *et al.*, 1998; Pradhan *et al.*, 1999). This contrasts with the bacterial MTases, which show little discrimination between unmethylated and hemimethylated DNA. This discrimination was proposed to be mediated through the N-terminus of the mammalian Dnmt1 (Bestor, 1992). Adams *et al.* (1983) observed an increased rate of *de novo* methylation after treatment of a crude murine Dnmt1 preparation with trypsin, and Bestor (1992) confirmed this result using V8 protease to cleave purified murine Dnmt1 from MEL cells (Bestor, 1992). More recently, another family of murine DNA methyltransferases, Dnmt3a and Dnmt3b, has been identified and are suspected to be *de novo* methyltransferases (Okano *et al.*, 1998).

Many prokaryotic MTases have been cloned and sequenced (Roberts and Macelis, 1999). These enzymes lack the long N-terminal domains of the mammalian enzymes. Both prokaryotic and mammalian enzymes function catalytically in a similar manner (Wu and Santi, 1987; Bestor and Verdine, 1994). All the MTases share a common architectural arrangement of 10 conserved motifs within the catalytic domain (Kumar *et al.*, 1994). The mechanism of DNA methylation has been elucidated in some detail for the prokaryotic enzyme *M.HhaI* (Wu and Santi, 1987). The enzyme first forms a covalent intermediate between a cysteine residue (Cys81) and the 6 position of the target cytosine. Crystallographic evidence has shown an unusual mode of DNA binding in which the target cytosine flips out of the DNA helix and into the

Fig. 1. Construction of mammalian-prokaryotic hybrid MTases. (A) Diagram representing mouse Dnmt1. Regions associated with specific functions are indicated as PCNA (proliferating cell nuclear antigen binding), RF (replication foci directing) and Zn (zinc binding). Conserved motifs are indicated with Roman numerals. (B) Schematic diagram of a prokaryotic MTase. Conserved motifs from I to X. The catalytic motif, IV, is represented as a black box. Other motifs are stippled and the protein is shown in grey. (C) Diagram showing the construction of the mouse Dnmt1-M.HhaI hybrid methyltransferase (pVICMH). Genes representing M.HhaI and Dnmt1 are represented by grey and black lines, respectively. Arrows represent primers used for amplification of parts of the M.HhaI and Dnmt1 genes. The amino acid numbers are indicated for the Dnmt1 amplified segment. The primer sequences are in Table III. Restriction enzyme sites are represented as B (*Bss*HII), S (*Sap*I), E (*Eco*RI) and N (*Nhe*I). (D) Amino acid alignment of motifs I for Dnmt1 and hybrid MTases. The conserved amino acids (FxGxG) are underlined. All amino acids from motif I of the mouse Dnmt1 are in upper case type. The amino acids contributed from the parental prokaryotic donor to make the hybrid MTases are indicated in lower case type. For the hybrid enzymes the amino acids indicated in upper case type are from the parental murine Dnmt1.

active site of the enzyme (Klimasauskas *et al.*, 1994). This greatly facilitates the reaction chemistry and avoids the potential steric hindrance if the base were to remain stacked within the helix. Because of the strong sequence conservation it is likely that the murine MTase will have a similar mechanism.

We have expressed the full-length murine Dnmt1 in a baculovirus-mediated insect cell expression system (Pradhan *et al.*, 1997). Others have expressed truncated versions of the same enzyme in *Escherichia coli* (Tollefsbol and Hutchison, 1995), mammalian COS cells (Czank *et al.*, 1991), or a baculovirus expression system (Glickman and Reich, 1994). The biological activity of the murine Dnmt1 is well conserved in all the expression systems described above. To gauge the structural and functional significance of the two domains of the murine Dnmt1, we have constructed three hybrid MTases in which the catalytic C-terminal domain is replaced by essentially complete prokaryotic enzyme sequences. These hybrid methyltransferases have been expressed in the baculovirus expression system. The target specificity and the involvement of the N-terminus in discrimination of *de novo* and

maintenance methylation are described. Steady-state kinetic studies of one hybrid (murine Dnmt1 fused to M.HhaI) have been carried out and are compared with those for both parental enzymes (Wu and Santi, 1987; Pradhan *et al.*, 1997).

Results

Construction and expression of murine-prokaryotic hybrid cytosine-5 DNA methyltransferases

We have constructed three murine-prokaryotic hybrid MTases. These hybrids are between murine Dnmt1 and three different prokaryotic MTases: M.HhaI (GCGC), M.HpaII (CCGG) and M.SssI (CG). For the construction of each hybrid enzyme, the N-terminal domain of the murine Dnmt1 was fused to the catalytic domain of each prokaryotic methyltransferase (Figure 1A, B and C). The site of fusion was just upstream of conserved motif I, which contains the amino acid sequence FxGxG (Kumar *et al.*, 1994) and forms part of the AdoMet binding site. The alignment of the conserved motifs I of M.HhaI,

Fig. 2. Expression and purification of hybrid MTases. (A) Western blot analysis of High Five cell extracts expressing the hybrid methyltransferases using a mouse Dnmt1 specific Ab 334. The relative positions of biotinylated mol. wt markers (165, 105, 76) are in kDa. His-MMT3 is a His₆-tagged full-length mouse Dnmt1 (Pradhan *et al.*, 1997). An uninfected High Five cell extract was used as a control (C). The MTase bands are indicated by the arrow. The hybrid proteins are indicated above each lane. (B) Two purified hybrid MTases, Dnmt1-HpaII and Dnmt1-HhaI resolved on SDS-PAGE and stained with Coomassie Blue. The broad range molecular weight markers are indicated on the left in kDa. The hybrid MTases are indicated by the arrow.

M.HpaII, *M.SssI* and murine Dnmt1 is shown in Figure 1D, and the fusion sites are indicated. To enhance the stability of the mRNAs for the hybrid MTases a phosphoglycerate kinase polyadenylation signal sequence was placed downstream of the stop codon of each individual gene. The hybrid genes were under the transcriptional control of the baculovirus polyhedrin promoter.

Co-transfection of the hybrid constructs with linear *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA resulted in homologous recombination of the hybrid genes. The viral DNA was isolated and checked for the integration and orientation of the hybrid gene by PCR (data not shown). All the recombinant viral DNAs produced the expected length of the PCR products. These supernatants were used for plaque purification to isolate single recombinant viruses. From each transfection, six recombinant plaques were purified and amplified at least twice to reach a titer $>2 \times 10^8$ p.f.u./ml in SF9 cells. However, subsequent expression used High Five insect cell lines (derived from *Trichoplusia ni*) for high level protein production (Davis *et al.*, 1992).

Two days post-infection the cell extracts were tested for recombinant protein expression. This was done either by assaying crude cell extracts for the transfer of [³H]methyl groups onto the substrate DNA or by a Western blot analysis of the cellular proteins separated by SDS-PAGE, using antibody Ab 334 (Pradhan *et al.*, 1997). Poly(dG-dC)-poly(dG-dC) was used as substrate for cell extracts containing both Dnmt1-HhaI and Dnmt1-SssI. λ DNA cut with *HindIII* was used as a substrate for Dnmt1-HpaII. Both these substrates have large numbers of potential sites for DNA methylation. Enzyme activity was detected in all three constructs. However, the activity of the Dnmt1-SssI construct was less than one-tenth of the other two constructs. This is consistent with the amount of hybrid

proteins detected by Western blot assay (Figure 2A). Very little Dnmt1-SssI hybrid protein was detected throughout infection. Both Dnmt1-HhaI and Dnmt1-HpaII hybrid enzymes were purified to homogeneity and had a molecular weight of ~170 kDa (Figure 2B). The yields of homogeneous protein obtained from 1×10^9 cells were between 0.4 and 0.8 mg. The hybrid enzymes were stable at -20°C for several weeks.

Hybrid cytosine-5 DNA methyltransferases retain the sequence specificity of the parental C-terminal domain

The hybrids, Dnmt1-HhaI, Dnmt1-HpaII and Dnmt1-SssI contain the intact murine N-terminus, which constitutes ~75% of the whole protein. Addition of such a large N-terminal region may alter the substrate specificity of the hybrid enzymes. To investigate the influence of the N-terminal domain on sequence specificity, we used a standard methylation protection assay to characterize the hybrid enzymes, Dnmt1-HhaI and Dnmt1-HpaII. Because of the very poor expression of the Dnmt1-SssI hybrid, sufficient enzyme could not be obtained to characterize it properly *in vitro*. Plasmid DNAs were methylated *in vitro* in the presence or absence of AdoMet. If methylation occurs then the substrate will become resistant to the action of the corresponding restriction enzyme. We found 100% protection for the test plasmids when methylated individually by either of the hybrid enzymes (data not shown).

To determine the target site specificity unequivocally we also conducted an assay with a duplex oligonucleotide substrate that has one recognition site in which the target C is substituted by 5-fluorocytosine (FdC). The complementary strand has a methylated cytosine ensuring that FdC is the only acceptor for the methyl group. The reaction

Fig. 3. Target specificity for the hybrid MTases. The results of the FdC assay with the hybrid MTases and parental enzymes are shown. The presence or absence of AdoMet within the reaction is indicated as (+) or (–) on top of the gel. The FdC (F) containing tetranucleotide sequences are shown. Each set of reactions was performed with one enzyme as indicated. The arrow shows the covalent complexes between the radioactive DNAs and the hybrid methyltransferases. Bracket representing two bands indicates the prokaryotic enzyme–DNA complexes. These may reflect alternatively translated proteins or partial degradation (two methyltransferase bands are observed in the purified preparations).

mechanism requires an initial covalent attachment of the MTase to the target cytosine through position 6 (Bestor and Verdine, 1994). Following methyl group transfer to C-5 in a normal oligonucleotide, abstraction of a proton from C-5 allows the enzyme to be released by β elimination. Since fluorine is a poor leaving group for the reaction, the elimination is inhibited and the enzyme remains covalently attached to the DNA. Sets of double-stranded oligonucleotides containing GFGC, CFGG and FG were tested for covalent complex formation in the presence of AdoMet. The recombinant murine Dnmt1 and the respective prokaryotic enzymes were used as controls. The results of the assay are shown in Figure 3. Dnmt1–HhaI formed a covalent complex in the presence of AdoMet with the GFGC oligonucleotide, but not with oligonucleotides containing CFGG or FG. The same pattern was observed with prokaryotic M.HhaI. In the case of Dnmt1–HpaII or M.HpaII, covalent complexes were formed with CFGG oligonucleotides but not with the FG or GFGC oligonucleotides. The murine Dnmt1 formed AdoMet-dependent complexes with all three oligonucleotide duplexes since they all contain at least one FG, the target methylation site. These results confirm that the hybrid methyltransferases retain the specificity of the parental C-terminal domains.

De novo methylation of the viral genomes by hybrid cytosine-5 DNA methyltransferase

We have investigated the methylating capacity of these hybrid enzymes on the baculoviral genome. Total DNA from infected High Five cells was isolated 48 h post-infection. This DNA contains predominantly the recombinant viral DNA. This total DNA was tested for methylation using several methylation sensitive restriction enzymes including *MspI* (CCGG), *HpaII* (CCGG), *AvaI* (CPyCGPuG), *ClaI* (ATCGAT), *BstUI* (CGCG), *HaeIII* (GGCC) and *HhaI* (GCGC). *MspI* and *HpaII* are isoschizomers that differ in their methylation sensitivity (van der Ploeg *et al.*, 1980). *HpaII* digestion is blocked if the internal CG is methylated whereas *MspI* can cut such DNA. *AvaI*, *ClaI*, *BstUI* and *HhaI* are blocked by methylation of the internal CG (Nelson *et al.*, 1993). DNA isolated from Dnmt1, Dnmt1–HhaI, Dnmt1–HpaII and Dnmt1–SssI expressing cells was digested and analyzed by agarose gel electrophoresis. The digestion patterns between individual DNAs were compared (Figure 4A). The digestion patterns of DNA isolated from cells expressing Dnmt1 or the Dnmt1–HhaI hybrid enzyme, were the same with both *MspI* or *HpaII*, indicating that there was neither a gross alteration of the genome nor methylation in the CCGG sequences.

Fig. 4. *De novo* methylation catalyzed by the hybrid MTases. (A) Total DNA from High Five cells expressing Dnmt1–HhaI, Dnmt1–HpaII, Dnmt1–SssI or Dnmt1 was extracted 48 h post-infection. Each lane represents DNA digested with the restriction enzymes indicated (+) above each lane. Lane M contains a *HindIII* digest of λ DNA molecular weight marker. (B) *De novo* methylation of the hybrid MTase genes. Southern blots of total DNA probed with the *BamHI* 2100 bp N-terminal fragment of the mouse *Dnmt1* cDNA. Each lane represents either a single or double restriction digest as indicated (+). The 2100 bp band is indicated by an arrow. (C) *De novo* methylation of viral upstream and polyhedrin promoter sequences. Southern blots of total DNA probed with a 2200 bp *NsiI* fragment consisting of the viral upstream sequences, polyhedrin promoter and a part of the N-terminus of the mouse *Dnmt1*. Each lane represents either a single or double restriction digest as indicated (+). The 2200 bp band is shown by an arrow. (D) Key features of the hybrid MTase and conceptual restriction maps of the *BamHI* (2100 bp) and *NsiI* (2200 bp) fragments. Features of the hybrid MTase are presented schematically. A 3400 bp cDNA containing the mouse *Dnmt1*, indicated by black shading, was fused to the N-terminus of the prokaryotic MTase, as shown in grey. The hatched line represents the upstream baculovirus ORF. pPH marks the polyhedrin promoter. Fragments analyzed by restriction enzyme digests [*BamHI* (~2100 bp) and *NsiI* (~2200 bp)] are indicated by B and N. Restriction enzymes used for methylation analysis are listed on the left. The numbers of target sites and sequences are on the right. Restriction sites are indicated by vertical black lines.

However, total DNA from cells expressing the Dnmt1-*HpaII* and Dnmt1-*SssI* hybrids were resistant to *HpaII* digestion indicating methylation of the internal CG. As expected, total DNA from cells expressing Dnmt1-*HhaI* was resistant to *HhaI*. Similarly, total DNA from Dnmt1-*SssI* expressing cells was resistant to all of the enzymes tested except *HaeIII* (Figure 4A). We also used the McrBC endonuclease to probe the methylation status of the total DNA. This enzyme cleaves DNA containing m^5C on one or both of the strands but will not cleave unmethylated DNA. Sites on DNA recognized by McrBC consist of two half-sites of the form Pu m^5C (Stewart and Raleigh, 1998). All four DNAs were tested for methylation of McrBC sites. DNA extracted from cells expressing Dnmt1 was apparently refractory to McrBC-mediated hydrolysis, suggesting the absence of m^5C in the DNA. This observation reflects the fact that Dnmt1 is a maintenance methyltransferase that only works efficiently when hemimethylated DNA is available as substrate. During a typical baculovirus infection the viral DNA is presumably packaged quickly, preventing the long exposure to MTase that would be needed for *de novo* methylation. When DNA from either a clone expressing functional Dnmt1 or a clone with a catalytically impaired mutant was digested with McrBC, identical patterns were observed. As expected, there was no digestion of Dnmt1-*HpaII* expressing host genomic DNA, since the sequence methylated is Cm^5CGG , which is not a substrate for McrBC. DNA from clones expressing Dnmt1-*HhaI* and Dnmt1-*SssI* contain (A/G) m^5C sites and so were digested by McrBC (Figure 5A). These results demonstrate that the hybrid methyltransferases are capable of global methylation.

The polyhedrin promoter is blocked by CG methylation

The poor expression of the Dnmt1-*SssI* hybrid protein led us to investigate whether methylation of the CG sequences was repressing the expression of the *Dnmt1-SssI* gene. This was investigated by Southern blotting after the genomic DNA digest (Figure 4A) and probing with a 2100 bp *BamHI* cDNA fragment containing the N-terminal region of the murine Dnmt1 (Figure 4D). These sequences are common to all of the hybrid constructs, thus allowing a comparison of the methylation status of the three hybrid genes. The restriction enzymes selected each have at least one site within this region. All four DNAs were digested either with *BamHI* alone or with *BamHI* and the enzymes indicated (Figure 4B). Digestion with *BamHI* will result in a 2100 bp fragment and double digestion will result in fragments smaller than 2100 bp if the genome is not methylated for that particular restriction enzyme (Figure 4B).

Genomic DNA from cells expressing native Dnmt1 showed no detectable methylation of the 2100 bp fragment (Figure 4B). Extracts from cells expressing Dnmt1 showed MTase activity *in vitro*. However, our observations do not address the global ability of Dnmt1 to methylate other parts of the host or viral genomes. When the constructs Dnmt1-*HhaI*, Dnmt1-*HpaII* and Dnmt1-*SssI* were expressed in High Five cells, the *BamHI* fragments were protected in a predictable fashion. There was complete protection against the cognate restriction endonucleases *HhaI* and *HpaII* (Figure 4B), while *BstUI* was partially

blocked by methylation in Dnmt1-*HhaI* expressing cells. This is because the *BstUI* (CGCG) and *HhaI* (GCGC) recognition sites overlap. Most of the CG sites were methylated in the viral DNA extracted from the Dnmt1-*SssI* expressing cell, as shown by the presence of a predominant 2100 bp band in double digests of *BamHI* with *HpaII*, *AvaI*, *ClaI*, *BstUI* and *HhaI*. Similar methylation patterns were also observed for total DNA isolated from Dnmt1-*HhaI* and Dnmt1-*SssI* expressing cells with a 2200 bp *NsiI* fragment probe spanning a part of viral ORF 603, the polyhedrin promoter and the N-terminal sequences of mouse Dnmt1 (Figure 4C and D). Once again viral DNAs from Dnmt1-*SssI* and Dnmt1-*HhaI* showed strong methylation of *HpaII* and *HhaI* sites, respectively, as indicated by the presence of a predominant 2200 bp band. These results show methylation of the hybrid MTase genes in both coding and upstream viral sequences.

Using the McrBC endonuclease, we examined the methylation status of the *BamHI* fragment from Dnmt1-*HhaI*, Dnmt1-*HpaII* and Dnmt1-*SssI* hybrid DNAs. When only McrBC was used for digestion of either Dnmt1-*HhaI* or Dnmt1-*SssI* hybrids, a smear was observed because of the random distribution of the cleavage sites. However, in double digests with *BamHI*, a predominant 2100 bp band was observed, representing the unmethylated fraction plus the smear, and minor bands, representing the methylated fraction of the genome. Comparing the intensities of the 2100 bp *BamHI* band it was concluded that >75% of the CGs were methylated either in ACG or GCG sites (Figure 5B). These results confirm that *de novo* methylation is taking place in cells expressing the hybrid enzymes. Thus, it is seems likely that methylation of CG sites in the Dnmt1-*SssI* hybrid gene and its surrounding DNA sequences results in suppression of gene expression.

Steady-state kinetic analysis of murine Dnmt1-HhaI hybrid C-5 DNA methyltransferase

Poly(dG-dC)-poly(dG-dC) has been used to determine the kinetic parameters of M.*HhaI* (Wu and Santi, 1987). This polymer provides a large number of potential sites for methylation to occur and was chosen to study the kinetic properties of the Dnmt1-*HhaI* hybrid. The methylation reaction was linear up to 40 min with 1, 5 or 10 μM AdoMet (Figure 6A). During the reaction, each molecule of the enzyme was capable of methylating several CGs on the DNA strand. Linearity of the reaction progression curve was also observed with enzyme concentrations between 10 and 50 nM (Figure 6B). A series of double reciprocal plots were made at six different AdoMet and six different DNA concentrations (as described in Materials and methods). The velocity curves were linear with respect to the variable substrate, i.e. they converged to the left of the y-axis and above the x-axis (data not shown). The slope and y-axis intercept ($1/V_{maxapp}$) for each of the regressions were replotted, and the results also showed linear responses (Figure 7). The intercept and slope values were used for replots to calculate the steady-state kinetic constants. The average of both sets of kinetic constants is shown in Table I along with a comparison of values for the parental enzymes M.*HhaI* and Dnmt1. The k_{cat} for the hybrid enzyme was $4.38 h^{-1}$, which is between 20- and 40-

Fig. 5. McrBC digest of the genomic DNA from Dnmt1 and hybrids. (A) The total DNAs were digested with *Bam*HI or *Bam*HI and McrBC as indicated. C is undigested control DNA. Lane M contains a *Hind*III digest of λ DNA. The source of DNA is also indicated on the top of the gel. (B) Southern blots of the gel probed with the 2100 bp *Bam*HI N-terminal probe of the mouse Dnmt1 cDNA. The 2100 bp band is indicated by an arrow.

Fig. 6. Time course of methylation catalyzed by the hybrid Dnmt1-*Hha*I. (A) Reactions contained 30 nM hybrid Dnmt1-*Hha*I, 50 μ M CG [poly (dG-dC)-poly (dG-dC)] and 1, 5 or 10 μ M AdoMet and were incubated at 37°C. Duplicate 25 μ l samples of each reaction were spotted onto DE81 paper, processed as described in Materials and methods, and 3 H incorporation was measured. The mean values, with standard deviation, for the time points are plotted as filled diamonds (1 μ M AdoMet), open circles (5 μ M AdoMet) or filled circles (10 μ M AdoMet). (B) Linearity of methyltransferase reaction as a function of enzyme concentration. Duplicate reactions in 25 μ l contained 50 μ M CG, 5 μ M AdoMet and various mouse Dnmt1-*Hha*I concentrations (5, 10, 20, 30, 40 and 50 μ M) and were incubated at 37°C for 30 min and processed. The solid circles depict the mean values of the [3 H]methyl group incorporation.

fold less than *M.Hha*I but comparable to that for Dnmt1. The kinetic constants obtained for murine Dnmt1 on poly(dG-dC)-poly(dG-dC) are very close to those of human Dnmt1 using CGG triplet repeats (Pradhan *et al.*, 1999).

It was hypothesized that the N-terminus of the mammalian Dnmt1 suppresses *de novo* methylation (Bestor, 1992), rendering it a maintenance MTase, and that the presence of this domain allows the enzyme to exhibit a higher turnover number on hemimethylated over unmethylated substrates (Kho *et al.*, 1998; Pradhan *et al.*, 1999). To address the involvement of the N-terminus of the hybrid enzymes in maintenance methylation, we tested Dnmt1-*Hha*I on unmethylated and hemimethylated oligonucleotide substrates. The rates of methylation for unmethylated and hemimethylated substrates were ob-

tained at various CG (DNA) concentrations to determine the steady-state kinetic parameters. The derived k_{cat} and K_m^{CG} are shown in Table II. k_{cat} for unmethylated and hemimethylated DNAs were similar, 2.4 h^{-1} . K_m^{CG} as well as K_m^{AdoMet} varied almost 3-fold between unmethylated and hemimethylated DNA. The catalytic efficiency, determined by k_{cat}/K_m^{CG} , is thus ~2.5 times higher for hemimethylated DNA. In contrast the parental *M.Hha*I shows 0.4 times lower catalytic efficiency for the hemimethylated DNA as compared with the unmethylated DNA.

Discussion

The domain organization of the mammalian methyltransferases suggests that they may have evolved by fusion of a

Fig. 7. Intercept and slope replots of the methylation rate in poly(dG-dC)-poly(dG-dC). Replot of the y-axis intercepts and slopes. (A) $1/V_{\max\text{app}}$ versus $1/\text{CG}$; (B) slope versus $1/\text{CG}$; (C) $1/V_{\max\text{app}}$ versus $1/\text{AdoMet}$; (D) slope versus $1/\text{AdoMet}$. (A) and (B) were plotted based on the fixed CG (DNA) primary plot. (C) and (D) were plotted based on the fixed AdoMet primary plot. Error bars are indicated. The kinetic constants k_{cat} , $K_{\text{m}}^{\text{AdoMet}}$ and K_{m}^{CG} were calculated from the plots.

Table I. Comparison of steady-state kinetic parameters of hybrid murine Dnmt1-*HhaI* with parental enzymes

DNA methyltransferase	k_{cat} (h^{-1})	$K_{\text{m}}^{\text{AdoMet}}$ (μM)	K_{m}^{CG} (μM)	$k_{\text{cat}}/K_{\text{m}}^{\text{CG}}$ ($10^6 \times \text{M}^{-1} \text{h}^{-1}$)	Reference
Dnmt1- <i>HhaI</i> hybrid	4.38 ± 0.03	0.63 ± 0.006	0.675 ± 0.04	6.49	present work
<i>M.HhaI</i>	214.00 ± 13	0.0230 ± 0.010	0.0018 ± 0.0008	6.64×10^4	present work
<i>M.HhaI</i> ^a	78	0.0147 ± 0.0018	0.0023 ± 0.0003	3.39×10^4	Wu and Santi (1987)
Recombinant Dnmt1 ^b	4.48 ± 0.5	2.50 ± 0.53	1.86 ± 0.07	2.62	Pradhan <i>et al.</i> (1997)

^aSteady-state kinetic data for *M.HhaI* using poly(dG-dC)-poly(dG-dC) substrate.

^bSteady-state kinetic data for murine Dnmt1 using poly(dI-dC)-poly(dI-dC) substrate.

Table II. Steady-state kinetic parameters of hybrid Dnmt1-*HhaI* methyltransferase and *M.HhaI* on unmethylated and hemimethylated DNA

Enzyme	Oligonucleotide duplexes	k_{cat} (h^{-1})	$K_{\text{m}}^{\text{AdoMet}}$ (μM)	K_{m}^{CG} (μM)	$k_{\text{cat}}/K_{\text{m}}^{\text{CG}}$ ($10^6 \times \text{M}^{-1} \text{h}^{-1}$)	Relative $k_{\text{cat}}/K_{\text{m}}$ (HM/UM)
Dnmt1- <i>HhaI</i>	unmethylated	2.41 ± 0.17	0.82 ± 0.1	0.94 ± 0.04	2.6	2.35
Dnmt1- <i>HhaI</i>	hemimethylated	2.38 ± 0.04	0.26 ± 0.01	0.39 ± 0.02	6.1	
<i>M.HhaI</i>	unmethylated	214 ± 13	0.023 ± 0.01	0.0018 ± 0.8	11.5×10^4	0.58
<i>M.HhaI</i>	hemimethylated	407 ± 60	0.017 ± 0.001	0.0061 ± 0.1	6.64×10^4	

Table III. DNA primers used for construction of hybrid DNA methyltransferases

Primer	Sequence
M.HhaI FP	TGGGCGCGCTCTTCACTCACAGGATTACGCTTTATTGAC
M.HhaI RP	CGGAATTCTAATATGGTTTGAAATTTAATGATG
Dnmt1 FP	TGGGCGCGCTAGCCCCGTGAAACGCCCAAAG
Dnmt1 RP	AGTTAGCTCTTCAAGAGTTTGATGGCTGCCTCAGGCTC
M.SssI FP	CTAGCTAGCTCTTCACTTAGAGTATTTGAAGCTTTTGC
M.SssI RP	TAGCGGCCGCTAACCTCCAATTTTATCTATAATCG
Dnmt1 FP	TGGGCGCGCTAGCCCCGTGAAACGCCCAAAG
Dnmt1 RP	ATGCGGCCCGCTCTTCAAAGCTTGGGCAGTTTGATGGCTGCCTC
M.HpaII FP	CTAGCTAGCTCTTCAAAATTTACTTTTATTGATTATT
M.HpaII RP	ATGCGGCCGCTTTAACTAGTCATATAAATTCCTAATTTTTC
Dnmt1 FP	TGGGCGCGCTAGCCCCGTGAAACGCCCAAAG
Dnmt1 RP	ATGCGGCCCGCTCTTCTTTTGGGCAGTTTGATGGCTGCCTC

FP and RP represents forward and reverse primer, respectively. Restriction enzyme sites are indicated, bold = *SapI*; underlined and italic sequences represent other enzymes. Some restriction enzyme sites are overlapping.

gene specialized for DNA methylation, such as a typical prokaryotic methyltransferase gene, with one or more genes responsible for localization or regulation of the protein within the eukaryotic cell nucleus. The results presented here show that such a fusion would have been biochemically feasible without seriously impairing the catalytic activity of the methyltransferase. In each of the three hybrids that we have constructed, the enzymes show the expected specificity and promote extensive methylation both *in vivo* and *in vitro*.

While the C-terminal domain of the mouse methyltransferase is well established to be the catalytic center, much less is known about the N-terminal region, which comprises almost 75% of the protein. It has been suggested that this region may be responsible for the preference that the enzyme shows for hemimethylated over unmethylated DNA (Adams *et al.*, 1983; Bestor, 1992), although there has been no direct evidence to support this. Of the hybrid methyltransferases that we describe here, the Dnmt1-HhaI hybrid shows a 2.5-fold preference for hemimethylated DNA. This observation is based on the second-order rate constant defined by the ratio of k_{cat}/K_m values, which describes the catalytic efficiency of the enzyme. Such preference is not observed for hemimethylated DNA by the parental M.HhaI, where the catalytic efficiency for hemimethylated DNA is approximately half that of the unmethylated substrate. The parental mammalian Dnmt1 shows 3–11 times higher catalytic efficiency for hemimethylated DNA (Pradhan *et al.*, 1999). This suggests that those features of the native Dnmt1 responsible for the discrimination do not reside merely in the N-terminal region. Elements located in the C-terminal region may be just as important and it is probably the interplay between them that is key.

Of course, preferences observed during *in vitro* reactions need to be interpreted with caution. The native murine Dnmt1 carries out its methylation as part of the replication complex, with which it co-localizes (Leonhardt *et al.*, 1992) and its natural substrate may be quite different from the ones routinely used during *in vitro* assays. Thus, the real *in vivo* discrimination in favor of hemimethylated DNA is best judged by the fact that when Dnmt1 is expressed in an insect cell system, where methylation is normally absent and the baculovirus genome is unmethylated, the native Dnmt1 causes very little, if any,

methylation of the viral genome. The viral DNA is probably packaged before there is time for much *de novo* methylation to take place and so there is little hemimethylated DNA to serve as substrate during subsequent rounds of replication. In contrast, the three hybrids that we describe here all show extensive methylation activity *in vivo*. This means that they function well on substrates that are either unmethylated or hemimethylated. This *in vivo* behavior parallels their *in vitro* behavior. However, on the basis of rates measured *in vitro*, it seems that at least for the Dnmt1-HhaI hybrid, the rates are dramatically reduced over the rates observed for the parental M.HhaI. Nevertheless, these rates are sufficient to produce extensive, if not complete methylation of the viral and host genomes. We assume that this takes place during replication as it does in mouse cells and that the signals for targeting the enzyme to the replication foci work just as well in insect cells as they do in mouse cells.

One interesting observation from this work comes from our attempts to express the mouse Dnmt1-SssI hybrid methyltransferase in the baculovirus expression system. Surprisingly, the expression of this construct is dramatically reduced as compared with the HhaI or HpaII hybrids. We have shown that for the Dnmt1-SssI construct there is extensive methylation both in the promoter and the N-terminal coding sequences. From Western blots we know that the amount of protein is greatly reduced and we assume that this reflects a reduction in transcription. What causes this reduction is unknown. One possibility is that the extensive methylation of the CG sequences located within the viral polyhedrin promoter is responsible for a reduction in transcription of this particular construct. Insect cells have not been reported to suppress gene expression by methylation and so are expected to lack specific mechanisms for such suppression. However, we cannot exclude the possibility that there is an undiscovered methylation system. More likely though is that the location of certain CG dinucleotides within the promoter may serendipitously interfere with the binding of either a transcription factor or the RNA polymerase itself. It may also be that a particularly heavy density of CG dinucleotides within this gene may slow transcription elongation to a point where rather little mRNA is made. However, this is not a general problem as yields of the recombinant virus are normal.

Our experiments show that both the baculoviral DNA and the host genomic DNA can be extensively methylated by these hybrid constructs. Thus, it seems likely that the constructs would work well in other types of cells, including mammalian cells such as mouse cells where the hybrids should target properly to the replication machinery, and also in other cell types which do not normally experience methylation. In contrast to the native Dnmt1, the *HhaI* and *HpaII* hybrids show great sequence specificity and would be expected to methylate only a subset of the normal CG targets found in mammalian cells. Expression of these methyltransferases in systems undergoing imprinting may throw light on the mechanism and biological significance of this phenomenon. Finally, these hybrid proteins may prove suitable for crystallographic analysis, since they appear more stable than the native Dnmt1.

Materials and methods

Materials

The plasmid (pHSHW5), containing the *M.HhaI* gene, was obtained from Dr S.Kumar [New England Biolabs (NEB)]. The plasmid (pUHE25) containing the *M.HpaII* gene was obtained from Dr T.Bowen (NEB) and the *M.SssI*-containing plasmid, pCAL7, was a gift from Dr W.Jack (NEB). The mouse Dnmt1 clone, pVL1393-1, was described elsewhere (Pradhan *et al.*, 1997).

DNA substrates

Poly(dG-dC)-poly(dG-dC) with an average length of 1257 bp was obtained from Pharmacia. *HindIII*-digested λ DNA was from NEB. Synthetic oligonucleotides were prepared by the organic synthesis division of NEB and their purity was checked by 32 P-labeling and PAGE. Duplex oligonucleotides were prepared by adding equimolar amounts of the complementary strands and annealing was monitored by PAGE. The following oligonucleotides were used, where M represents 5-methylcytosine and F represents 5-fluorocytosine. The methylation sites are in bold. For steady-state kinetics oligonucleotide substrates/DNA used were without fluorocytosine.

FG oligonucleotide (Dnmt1 substrate):

5'-ATTGCGCATTCGCGATC**FG**CGATC-3'

3'-TAACGCGTAAGCCTAG**GM**GCTAG-5'

GFGC oligonucleotide (Dnmt1-*HhaI* substrate):

5'-GACTGGTACAGTATCAG**FG**CTGACCCACAACATCC-3'

3'-CTGACCATGTCATAGTCC**GM**GACTGGGTGTTGTAGG-5'

CFGG oligonucleotide (Dnmt1-*HpaII* substrate):

5'-GACTGGTACAGTATCAG**CF**GGTGACCCACAACATCC-3'

3'-CTGACCATGTCATAGTCC**GM**GACTGGGTGTTGTAGG-5'

Oligonucleotide substrates used for steady-state kinetic analysis of Dnmt1-*HhaI* were identical to the GFGC oligonucleotide except that the 5-fluorocytosine was replaced by cytosine.

Construction of hybrid cytosine-5 DNA methyltransferases

Murine Dnmt1-*M.HhaI* hybrid. A detailed scheme for the construction of the murine Dnmt1-*M.HhaI* hybrid (Dnmt1-*HhaI*) enzyme is presented in Figure 1C. Briefly, amplification of the *M.HhaI* gene used a pair of *M.HhaI* specific primers. The forward primer corresponds to the conserved motif I (Kumar *et al.*, 1994) of the MTase and contained two different overlapping restriction enzyme sites (*Bss*HII and *Sap*I). The reverse primer was designed for the C-terminal nucleotide sequence of the protein and contained the stop codon and an *Eco*RI site. The sequences of the primers are shown in Table III. Amplification of the *M.HhaI* gene used a Perkin Elmer GeneAmp PCR System 2400 and Vent exo⁻ DNA polymerase (NEB). Standard DNA amplification procedures were followed using 2 mM MgSO₄, 15 cycles of amplification at 95°C for 30 s, 60°C for 30 s and 72°C at 95°C for 5 min, followed the initial denaturation step for 60 s. The final extension used one cycle at 72°C for 7 min. The amplified DNA was phenol-chloroform extracted and digested with *Bss*HII and *Eco*RI. The digested products were run on a low melting point agarose gel and the band of interest was purified using GeneClean II (Bio 101) and ligated into pLit29, which had been predigested with *Bss*HII and *Eco*RI (NEB). The resulting clone,

pLit29Δ*HhaI*, contained a truncated *M.HhaI* gene and the correct construct was verified by sequencing.

A part of the mouse cDNA for Dnmt1 (amino acids 956–1137) was amplified using mouse cDNA specific primers (Table III). The forward primer has *Bss*HII and *Nhe*I overlapping sites and the reverse primer had a *Sap*I site. A plasmid, pVL1393-1, containing the mouse Dnmt1 cDNA (Pradhan *et al.*, 1997) was used as template. Ten PCR cycles were carried out using the above parameters. The PCR product was digested with *Bss*HII and *Sap*I and the purified band was cloned into pLit29Δ*HhaI*, which had previously been digested with *Bss*HII and *Sap*I, to create pLit29ΔDnmt1-*HhaI*. This plasmid contains DNA sequences representing amino acids 956–1137 of the mouse Dnmt1 and the complete catalytic domain of *M.HhaI*.

To shuffle the hybrid ORF into the baculovirus transfer vector, pLit29ΔDnmt1-*HhaI* was digested with *Bss*HII and *Eco*RI, and the 1550 bp fragment was ligated to plasmid pVL1393-1 (Pradhan *et al.*, 1997) that had been predigested with *Bss*HII and *Eco*RI to create pVL1393ΔDnmt1-*HhaI*. To complete the reconstruction of the N-terminus of the Dnmt1, the *Dnmt1* cDNA from pVL1393-1 was digested with *Bss*HII and *Nhe*I and the ~2280 bp fragment was ligated to *Bss*HII and *Nhe*I predigested pVL1393ΔDnmt1-*HhaI*. This plasmid is pVL1393MH. All the ligation junctions were verified by restriction enzyme digestion followed by DNA sequencing.

Murine Dnmt1 hybrids with *M.HpaII* and *M.SssI*. A scheme similar to the Dnmt1-*HhaI* construction (Figure 1C) was used to construct the hybrid MTases between mouse Dnmt1 and *M.HpaII* (Dnmt1-*HpaII*) and mouse Dnmt1 and *M.SssI* (Dnmt1-*SssI*). The constructs expressing the fusion MTase are pVL1393MHP (Dnmt1-*HpaII*) and pVL1393MS (Dnmt1-*SssI*). The amino acids of the hybrid motif I are shown in Figure 1D. A phosphoglycerate kinase polyadenylation signal was placed downstream of each hybrid MTase construct. All PCR primers (with restriction enzyme sites) used to make the hybrid genes are listed in Table III.

Cotransfection and expression of hybrid C-5 DNA methyltransferase

The transfer vectors, pVL1393MH, pVL1393MHP, pVL1393MS and BaculoGold DNA, a modified linearized AcNPV DNA (Pharminogen), were co-transfected into a monolayer of SF9 insect cells as described previously (Pradhan *et al.*, 1997). Transfection supernatants were screened for recombinant baculoviruses using PCR. Polyhedrin forward and reverse primers were used to amplify the insert. The viral titer was determined by the agarose overlay technique followed by neutral red staining. Detailed techniques are described elsewhere (O'Reilly *et al.*, 1992). Each purified plaque was amplified to reach a viral titer of $>2 \times 10^8$ p.f.u./ml.

High Five insect cell lines were routinely used for the expression of recombinant protein. Cells were grown at 27°C on a Bellco steering platform at 70 r.p.m. and maintained in Excell-400 serum free medium (JRH Biosciences) supplemented with an antibiotic-antimycotic solution with a final concentration of 5 U/ml penicillin, 50 µg/ml streptomycin and 0.125 µg/ml amphotericin B. For initial expression studies 25 cm² flasks were used. However, for routine purification of protein, spinner culture flasks were used. High Five cells at a density of $1.4\text{--}1.6 \times 10^6$ were infected at a multiplicity of infection (m.o.i.) of 10. The cells were incubated with a setting of 60 r.p.m. on a stirring platform (Bellco). Cells were harvested 48 h post-infection unless otherwise stated. Infected cells were pelleted at 1200 g, 4°C. The pelleted cells were washed once with phosphate-buffered saline.

Purification of recombinant hybrid cytosine-5 DNA methyltransferases

Typically 6.0×10^8 infected High Five cells were resuspended in 15 ml of lysis buffer M (20 mM Tris-HCl pH 7.4, 1 mM Na₂EDTA, 10% sucrose, 10 µg/ml TPCK, 10 µg/ml TLCK, 5 µg/ml E64, 5 µg/ml leupeptin, 7 µg/ml phenylmethylsulfonyl fluoride, 1 mM β-mercaptoethanol) supplemented with 250 mM NaCl. The cell suspension was sonicated on ice for 30 s using a model W-225R (Heatsystem-Ultrasonics, USA) sonicator in pulsed mode with 50% duty cycle. The extract was incubated on ice for 30 min and then centrifuged at 11 000 g in a JA18 rotor at 4°C for 45 min. The supernatant was collected and an equal volume of buffer M, without NaCl, was added to bring the final concentration of NaCl to 125 mM. This mixture was centrifuged for a further 30 min at 11 000 g to remove the precipitate. The supernatant was loaded onto a 70 ml Q Sepharose HR (Pharmacia) column pre-equilibrated with buffer A (M with 100 mM NaCl) at 1 ml/min using a superloop. The column was washed with 200 ml of buffer A. The bound

proteins were eluted with a linear gradient of 0–1.0 M NaCl in 300 ml of buffer B (buffer M supplemented with 1.0 M NaCl). Fractions containing MTase activity were pooled and the final salt concentration of the protein solution was brought to 100 mM NaCl by the addition of buffer M.

This protein solution was loaded onto a 5 ml heparin–Sephacrose Hitrap (Pharmacia) column. The column was washed with buffer A containing 100 mM NaCl and the bound protein was eluted with 90 ml of a linear gradient from 0.1 to 1.5 M NaCl in buffer M. Fractions containing MTase were pooled and dialyzed against buffer M containing 100 mM NaCl and protease inhibitors. The dialyzed MTase was loaded onto a 1 ml Source 15Q (Pharmacia) column. The unbound proteins were washed with buffer M containing 100 mM NaCl and the bound proteins were eluted with 60 ml of a linear gradient from 0.1 to 1.0 M NaCl in buffer M.

If required, the MTase fraction was further purified by gel filtration on a Hiload Superdex 200 prep grade 16/60 (Pharmacia) column equilibrated with buffer M containing 100 mM NaCl at 0.2 ml/min. The peak fractions were collected and concentrated using a Centricon 20 dialyzing against buffer M containing 50% glycerol and stored at -20°C until further use.

Methyltransferase assay

To screen hybrid MTase expression, crude extracts were prepared from infected cells and assayed *in vitro* for methyltransferase activity. The cells were washed with phosphate-buffered saline, briefly sonicated and the cell debris was removed by centrifugation. The supernatant was used to assay for the incorporation of [^3H]methyl groups into substrate DNA. The reaction mix (25 μl) contained 5 μM [^3H]AdoMet (15 Ci/mmol), 1 μg DNA and cell extract in buffer M. The reaction was incubated at 37°C , for 30 min, cooled in ethanol/dry ice and processed as described earlier (Pradhan *et al.*, 1997; Bacolla *et al.*, 1999).

To study the steady-state kinetic parameters of the purified murine Dnmt1–HhaI enzyme, poly(dG–dC)–poly(dG–dC), unmethylated and hemimethylated oligonucleotides were used as substrate. For kinetic analysis, the CG concentration was calculated from either substrate oligonucleotides or poly(dG–dC)–poly(dG–dC). One nanomolar double-stranded oligonucleotide with 1 CG site is 2 nM CG. For poly(dG–dC)–poly(dG–dC), an average length of 1250 bp was used to determine the concentration of CG. The reaction mix contained 10 nM of the purified enzyme, varying amounts of AdoMet (0.5, 0.75, 1.0, 1.5, 2.0, 3.0 μM) and varying amounts of CG (0.5, 0.625, 0.835, 1.25, 2.5, 5.0 μM) in reaction buffer (50 mM Tris–HCl pH 7.5, 10 mM Na_2EDTA , 5 mM β -mercaptoethanol). For both unmethylated and hemimethylated oligonucleotide duplexes the CG concentrations were varied (2.5, 3.125, 4.175, 6.25, 12.5 and 25 μM). AdoMet concentration was also varied (0.5, 0.75, 1.0, 1.5, 2.0 and 3 μM). For M.HhaI assay CG concentrations were varied (1, 1.25, 1.67, 2.5, 5 and 10 nM) and AdoMet concentration was varied (7, 9, 15 and 25 nM). [^3H]methyl incorporation was measured and double reciprocal plots were constructed as described (Bacolla *et al.*, 1999). The concentration of protein was estimated using the Bradford reagent with bovine serum albumin as the protein standard.

5-fluoro-2'-deoxycytidine (FdC) assay

Duplex oligonucleotides containing 5-fluoro-2'-deoxycytidine (FdC) were ^{32}P end-labeled using polynucleotide kinase and [γ - ^{32}P]ATP. Hybrid MTase (10 nM) was used with 5 nM fluorocytosine-containing oligonucleotide duplex in the presence of 50 μM cold AdoMet in $1\times$ reaction buffer at 37°C for 30 min. The reaction was stopped by adding SDS gel loading dye (one-third of the reaction volume) and boiling the sample at 100°C for 5 min. The boiled protein/DNA mixtures were loaded and resolved on a 4–20% Tris–glycine–SDS gradient gel. The gel was dried and autoradiographed.

Methylation protection assay

Plasmid DNA (1 μg) was incubated with 1–10 nM hybrid MTase with or without AdoMet in $1\times$ reaction buffer at 37°C for 60 min. The plasmid DNA was extracted with phenol–chloroform and ethanol precipitated. The precipitated DNA was dissolved in water and used as substrate for the cognate restriction enzymes. The digested DNAs were compared on an agarose TBE gel.

DNA isolation and Southern blot analysis

Genomic DNA was isolated from High Five cells 48 h post-infection with recombinant virus using the Easy DNA Kit (Invitrogen). The purified DNA was digested by restriction enzymes. Digestion of a cocktail of genomic DNA plus plasmid or phage DNA was performed in parallel (control to monitor complete digestion). The digested DNA fragments were separated on a 1% agarose TBE gel. The DNA in the gel was blotted onto Hybond N+ and probed with a random-primed BamHI 2100 bp or

NsiI 2200 bp fragment of mouse Dnmt1 clone. The blot was washed using a standard protocol and autoradiographed (Sambrook *et al.*, 1989).

Immunoblot analysis of hybrid cytosine-5 DNA methyltransferase

Cell extracts or purified protein samples were mixed with SDS–PAGE sample loading buffer and incubated at 98°C for 5 min. The protein mixtures were separated in a 4–20% polyacrylamide ISS miniprep SeptraGel. The protein bands were blotted on to a PVDF membrane and probed using a polyclonal rabbit antibody (Ab 334) raised against a peptide, RSPRSRPKPRGPRRSK, located 123 amino acids downstream of the initiator methionine in the full-length murine Dnmt1 protein (Pradhan *et al.*, 1997).

Acknowledgements

We thank Drs Sanjay Kumar and Jingwei Ni for suggestions and Prof. R.Jaenisch for Ab 334. This research was supported by NIH grant GM46127 to R.J.R.

References

- Adams,R.L., Burdon,R.H., McKinnon,K. and Rinaldi,A. (1983) Stimulation of *de novo* methylation following limited proteolysis of mouse ascites DNA methylase. *FEBS Lett.*, **163**, 194–198.
- Bacolla,A., Pradhan,S., Roberts,R.J. and Wells,R.D. (1999) Recombinant human DNA (cytosine-5) methyltransferase. II. Steady-state kinetics reveal allosteric activation by methylated DNA. *J. Biol. Chem.*, **274**, 33011–33019.
- Barlow,D.P. (1995) Gametic imprinting in mammals. *Science*, **270**, 1610–1613.
- Bestor,T.H. (1992) Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain. *EMBO J.*, **11**, 2611–2617.
- Bestor,T.H. and Verdine,G.L. (1994) DNA methyltransferases. *Curr. Opin. Cell Biol.*, **9**, 380–389.
- Bestor,T., Laudano,A., Mattaliano,R. and Ingram,V. (1988) Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. *J. Mol. Biol.*, **203**, 971–983.
- Chuang,L.S., Ian,H.I., Koh,T.W., Ng,H.H., Xu,G. and Li,B.F. (1997) Human DNA–(cytosine-5) methyltransferase–PCNA complex as a target for p21WAF1. *Science*, **277**, 1996–2000.
- Czank,A., Hauselmann,R., Page,A.W., Leonhardt,H., Bestor,T.H., Schaffner,W. and Hergersberg,M. (1991) Expression in mammalian cells of a cloned gene encoding murine DNA methyltransferase. *Gene*, **30**, 259–263.
- Davis,T.R., Trotter,K.M., Granados,R.R. and Wood,H.A. (1992) Baculovirus expression of alkaline phosphatase as a reporter gene for evaluation of production, glycosylation and secretion. *Biotechnology*, **10**, 1148–1150.
- Flynn,J., Glickman,J.F. and Reich,N.O. (1996) Murine DNA cytosine-5 methyltransferase: pre-steady- and steady-state kinetic analysis with regulatory DNA sequences. *Biochemistry*, **35**, 7308–7315.
- Glickman,J.F. and Reich,N.O. (1994) Baculovirus-mediated high level expression of a mammalian DNA methyltransferase. *Biochem. Biophys. Res. Commun.*, **204**, 1003–1008.
- Graessmann,M. and Graessmann,A. (1993) DNA methylation, chromatin structure and the regulation of gene expression. In Jost,J.P. and Saluz,H.P. (eds), *DNA Methylation, Molecular Biology and Biological Significance*. Birkhauser Verlag, Basel, Switzerland, pp. 404–424.
- Imbert,G., Feng,Y., Nelson,D.L., Warren,S.T. and Mandel,J.L. (1998) FMR1 and mutation in Fragile X syndrome: molecular biology, biochemistry and genetics. In Wells,R.D. and Warren,S.T. (eds), *Genetic Instability and Hereditary Neurological Diseases*. Academic Press, pp. 27–46.
- Kho,M.R., Baker,D.J., Laayoun,A. and Smith,S.S. (1998) Stalling of human DNA (cytosine-5) methyltransferase at single-strand conformers from a site of dynamic mutation. *J. Mol. Biol.*, **275**, 67–79.
- Klimasauskas,S., Kumar,S., Roberts,R.J. and Cheng,X. (1994) HhaI methyltransferase flips its target base out of the DNA helix. *Cell*, **76**, 357–369.
- Kumar,S., Cheng,X.D., Klimasauskas,S., Sha,M., Posfai,J., Roberts,R.J.

- and Wilson,G.G. (1994) The DNA (cytosine-5) methyltransferases. *Nucleic Acids Res.*, **22**, 1–10.
- Leonhardt,H., Page,A.W., Weier,H.U. and Bestor,T.H. (1992) A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell*, **71**, 865–873.
- Li,E., Bestor,T.H. and Jaenisch,R. (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell*, **12**, 915–926.
- Modrich,P. (1989) Methyl-directed DNA mismatch correction. *J. Biol. Chem.*, **264**, 6597–6600.
- Nelson,M., Raschke,E. and McClelland,M. (1993) Effect of site-specific methylation on restriction endonucleases and DNA modification methyltransferases. *Nucleic Acids Res.*, **21**, 3139–3154.
- O'Reilly,D.R., Miller,L.K. and Luckow,V.A. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*. W.H. Freeman and Co., New York, NY.
- Okano,M., Xie,S. and Li,E. (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nature Genet.*, **19**, 219–220.
- Pradhan,S., Talbot,D., Sha,M., Benner,J., Hornstra,L., Li,E., Jaenisch,R. and Roberts,R.J. (1997) Baculovirus-mediated expression and characterization of the full-length murine DNA methyltransferase. *Nucleic Acids Res.*, **25**, 4666–4673.
- Pradhan,S., Bacolla,A., Wells,R.D. and Roberts,R.J. (1999) Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification and comparison of *de novo* and maintenance methylation. *J. Biol. Chem.*, **274**, 33002–33010.
- Redaschi,N. and Bickle,T.A. (1996) DNA restriction and modification. In Curtiss III,R., Ingraham,J.L., Lin,C.C.E., Low,K.B., Magasanik,B., Reznikoff,W.S., Riley,M., Schaechter,M. and Umberger,H.E. (eds), *Escherichia coli and Salmonella Cellular and Molecular Biology*. ASM, Washington, DC, Vol. 2, pp. 773–781.
- Roberts,R.J. and Macelis,D. (1999) REBASE-restriction enzymes and methylases. *Nucleic Acids Res.*, **27**, 312–313.
- Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schmutte,C. and Jones,P.A. (1998) Involvement of DNA methylation in human carcinogenesis. *Biol. Chem.*, **379**, 377–388.
- Singer-Sam,J. and Riggs,A.D. (1993) X chromosome inactivation and DNA methylation. In Jost,J.P. and Saluz,H.P. (eds), *DNA Methylation, Molecular Biology and Biological Significance*. Birkhauser Verlag, Basel, Switzerland, pp. 358–384.
- Stambuk,S. and Radman,M. (1998) Mechanism and control of interspecies recombination in *Escherichia coli*. I. Mismatch repair, methylation, recombination and replication functions. *Genetics*, **150**, 533–542.
- Stewart,F.J. and Raleigh,E.A. (1998) Dependence of McrBC cleavage on distance between recognition elements. *Biol. Chem.*, **379**, 611–616.
- Tollefsbol,T.O. and Hutchison,C.A.,III (1995) Mammalian DNA (cytosine-5)-methyltransferase expressed in *Escherichia coli*, purified and characterized. *J. Biol. Chem.*, **270**, 18543–18550.
- van der Ploeg,L.H., Groffen,J. and Flavell,R.A. (1980) A novel type of secondary modification of two CCGG residues in the human $\gamma\delta$ β -globin gene locus. *Nucleic Acids Res.*, **24**, 4563–4574.
- Wu,J.C. and Santi,D.V. (1987) Kinetic and catalytic mechanism of *HhaI* methyltransferase. *J. Biol. Chem.*, **262**, 4778–4786.
- Yang,A.S., Jones,P.A. and Shibata,A. (1996) The mutational burden of 5-methylcytosine. In Russo,V.E.A., Martienssen,R.A. and Riggs,A.D. (eds), *Epigenetic Mechanisms of Gene Regulation*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 77–95.
- Yen,R.W. et al. (1992) Isolation and characterization of the cDNA encoding human DNA methyltransferase. *Nucleic Acids Res.*, **20**, 2287–2291.

Received February 16, 2000; revised and accepted March 10, 2000