Murine DNA Cytosine-C 5 Methyltransferase: Pre-Steady- and Steady-State Kinetic Analysis with Regulatory DNA Sequences †

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Murine DNA Cytosine-C^5 Methyltransferase: Pre-Steady- and Steady-State Kinetic Analysis with Regulatory DNA Sequences†

James Flynn, J. Fraser Glickman, and Norbert O. Reich*

Department of Chemistry and Program in Biochemistry and Molecular Biology, University of California, Santa Barbara, California 93106

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ABSTRACT: We present the first description of K_m^{DNA}, K_d^{DNA}, k_{cat}, and $k_{methylation}$ for a mammalian DNA methyltransferase. Homogeneous, 190 000 M_r DNA (cytosine-5-)-methyltransferase isolated from mouse erythroleukemia cells has turnover constants of 0.15−0.59 h^{-1} with single-stranded and unmethylated double-stranded oligonucleotides containing a single CpG dinucleotide. These substrates were designed to mimic DNA transcriptional cis elements previously reported to have cytosine C-5-methylated regulation. The rate-limiting step for these substrates is the methylation step itself. In contrast, hemimethylated double-stranded substrates show burst kinetics, consistent with a rapid methylation event (3 h^{-1}) followed by a slower step which determines steady-state k_{cat}. Hemimethylated and unmethylated double-stranded DNA shows similar binding affinities; these results reveal the molecular basis for the enzyme's preference for hemimethylated DNA to be the methyl transfer step. Substrates with multiple recognition sites do not show burst kinetics and have turnover rate constants of 6 h^{-1}. Catalytic turnover for the mammalian enzyme is thus approximately 10-fold slower than that for the related bacterial enzymes. Our combined results show quantitatively that one enzyme is certainly capable of both maintenance and *de novo* methylation and that maintenance of the genomic methylation pattern is preferred over the *de novo* establishment of new patterns. Direct comparison of the mammalian enzyme with the bacterial DNA cytosine-C^5 methyltransferase, M.*Sss*I, indicates dramatic differences in preferences for single-stranded, double-stranded, and hemimethylated double-stranded substrates. Moreover, the specificity hierarchy shown for the M.*Sss*I is derived from very different changes in K_m and catalysis than those observed for the mammalian DCMTase. These results demonstrate that the M.*Sss*I, and perhaps other DNA cytosine methyltransferases from bacteria, is functionally dissimilar to the mammalian enzyme.

Methylation of DNA at cytosine (5-mC)[1] occurs in most of the biological kingdoms (Vanyushin et al., 1970). The function of cytosine methylation in mammals mainly involves positive and negative transcriptional control and appears predominantly in the minimal context of the CpG dinucleotide (Boyes & Bird, 1991). The genome-wide pattern of methylation is believed to be parentally imprinted and gene-specific. A concerted progression of maintenance methylation, demethylation, and *de novo* methylation of the entire genome is envisioned as a mechanism for the final genomic expression configuration of a terminally differentiated cell (Shemer et al., 1991).

DNA methylation is catalyzed by *S*-adenosyl-L-methionine (AdoMet)-dependent, DNA (cytosine-5-)-methyltransferase (DCMTase, EC 2.1.1.37). An essential role for the control of methylation patterns by the DCMTase has been shown by specific gene disruption in the mouse (Li et al., 1992). Amplification of DCMTase expression by an exogenous mammalian DCMTase gene induces tumorigenic transformation of NIH 3T3 mouse fibroblasts (Wu et al., 1993); correspondingly, human neoplastic cells and cells derived from different stages of colon cancer express up to 200-fold higher levels of DCMTase than normal (El-Deiry et al., 1991). Conversely, expression of antisense DCMTase mRNA in the adrenocortical carcinoma cell line Y1 inhibits tumorigenesis (MacLeod & Szyf, 1995). The anticancer agent 5-azadeoxycytidine functions by inhibiting the DCMTase (Jutterman et al., 1994), and DCMTase activity contributes substantially to tumor development in a mouse model of intestinal neoplasia (Laird et al., 1995).

Mammalian DCMTase genes have been cloned and sequenced from mouse erythroleukemia cells (Bestor et al., 1988) and from human medullary thyroid carcinoma cells (Yen et al., 1992). The predicted murine 1573-amino acid polypeptide, with a 175 164 Da mass, contains a hinged 573-amino acid carboxyl terminal domain harboring conserved regions found in bacterial type II DCMTases (Posfai et al., 1989). The amino terminal two-thirds is suspected to contain regions which regulate the catalytic activity (Bestor, 1992; Leonhardt et al., 1992). Crystal structures for two bacterial type II DCMTases−DNA complexes are available and suggest that catalysis is mediated by the extrahelical stabilization of the targeted pyrimidine ring (Klimasauskas et al., 1994; Reinisch et al., 1995). The catalytic mechanism of the related bacterial DCMTases involves nucleophilic activation of the pyrimidine C-5 position by enzyme addition to the C-6 position of the heterocycle. The transfer of a methyl group from AdoMet to C-5 is followed by proton abstraction

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* To whom correspondence should be addressed.

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[1] Abbreviations: bp, base pairs; ds, double-stranded; ss, single stranded; AdoMet, *S*-adenosyl-L-methionine; DCMTase, DNA (cytosine-5-)-methyltransferase; MEL cells, Friend murine erythroleukemia cells; HPLC, high-performance liquid chromatography.

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from C-5, and β-elimination regenerates free DCMTase (Wu & Santi, 1987).

Although the target of extensive enzymological studies, the mammalian DCMTase has yet to be understood mechanistically. Past studies have used enzyme preparations ranging from crude nuclear extracts (Christman et al., 1995) to partially pure or proteolyzed enzyme from mouse erythroid cells (Bestor et al., 1992; Bolden et al., 1986), human placenta (Smith et al., 1994), and mouse plasmacytoma cells (Hitt et al., 1988). These and other studies have used diverse DNA substrates, many of which have little or no assigned biological relevance (Carotti et al., 1986; Pedrali-Noy & Weissbach, 1986). A precise functional description of the enzyme is essential for understanding how DCMTase catalyzes the developmentally regulated patterns of DNA methylation and for the design of novel anticancer strategies based on regulation of the enzyme.

We report the first K_m^{DNA}, K_d^{DNA}, k_{cat} and $k_{methylation}$ determinations for any eukaryotic DCMTase. These parameters were determined with oligonucleotides designed to mimic transcriptional elements previously reported to be subject to regulation by cytosine C-5 methylation *in vivo*. The enzyme has a k_{cat} approximately 10-fold slower than the related bacterial DCMTases; turnover of single-stranded and double-stranded substrates is limited by the methylation event. In contrast, hemimethylated substrate turnover is limited by steps after methylation. The combined pre-steady- and steady-state analyses define DCMTase reaction mechanisms which contribute to the enzyme's substrate discrimination. Kinetic comparisons with a related bacterial DCMTase, M.*Sss*I, show markedly different substrate specificities and thereby reveal differences in the mechanisms of the observed discrimination.

EXPERIMENTAL PROCEDURES

Materials

S-Adenosyl-L-[*methyl*-^{3}H]methionine ([*methyl*-^{3}H]AdoMet) (75 Ci/mmol, 1 mCi/mL, 1 Ci = 37 GBq) was purchased from Amersham Corp. AdoMet (87% pure) was purchased from Sigma Chemical Co. and further purified by HPLC as described (Reich & Mashhoon, 1991). Routinely, 125 μM AdoMet stocks were prepared at a specific activity of 1×10^4 cpm/pmol. Two lots of poly(dI·dC:dI·dC) were purchased from Pharmacia Biotech, Inc., with different average lengths in base pairs: 280 and 6250 bp. DE81 paper was purchased from Whatman, Inc. Other standard chemicals and reagents were purchased from Sigma Chemical Co. or Fisher Scientific.

Mouse erythroleukemia cell DNA (cytosine-5-)-methyltransferase (DCMTase) was purified as previously described (Xu et al., 1995), and two separate preparations, with concentrations of 380 and 820 nM, were confirmed to have equivalent activities with the substrates studied. *Sss*I DNA (cytosine-5-)methyltransferase (M.*Sss*I) was purchased from New England Biolabs. This enzyme preparation was greater than 90% pure as determined by SDS−PAGE.

DNA Substrate Preparation

Six oligonucleotides were synthesized for use as substrates and are shown in Table 1. Oligonucleotides were prepared on a Cyclone Plus DNA Synthesizer (Millipore, Inc.) using (β-cyanoethyl)phosphoramidites and purified on a PureDNA reverse-phase HPLC column (Rainin Instrument Co., Inc.). All synthesis reagents were purchased from Millipore, Inc., except for 5-Me-dC-CE phosphoramidite which was purchased from Glen Research (Sterling, VA). The concentrations of single strands were determined spectrophotometrically. Oligonucleotide purity was confirmed by ^{32}P radiolabeling, PAGE separation, and subsequent autoradiography. The thermal stability of hybridized, double-stranded DNA was determined spectrophotometrically. The percentage of double-stranded DNA in annealed DNA samples was confirmed to be greater than 99.9% by ^{32}P radiolabeling, PAGE separation, and subsequent autoradiography and densitometry using a CCD camera and the SW5000 analysis package from Ultra Violet Products, Inc. (San Gabriel, CA).

Table 1: Synthetic DNA Substrates Mimicking Transcriptional *Cis*-Regulatory Elements[a]

CRE a:	5'-GGGAATTCAAATGA**CG**TCAAAAGGATCCAG-3'
CRE b:	5'-CTGGATCCTTTTGA**CG**TCATTTGAATTCCC-3'
CRE a^{met}:	5'-GGGAATTCAAATGA^{m}**CG**TCAAAAGGATCCAG-3'
GC-box a:	5'-GGGAATTCAAGGGG**CG**GGGCAAGGATCCAG-3'
GC-box b:	5'-CTGGATCCTTGCCC**CG**CCCCTTGAATTCCC-3'
GC-box b^{met}:	5'-CTGGATCCTTGCCC^{m}**CG**CCCCTTGAATTCCC-3'

[a] Six deoxyoligonucleotides were synthesized for use as substrates (^{m}C = C-5 methylcytosine). Oligonucleotides were prepared on a Cyclone Plus DNA Synthesizer (Millipore, Inc.) using (β-cyanoethyl)phosphoramidites and purified on a PureDNA reverse-phase HPLC column (Rainin Instrument Co., Inc.). The single, centrally located CpG dinucleotide is underlined, and the appropriate consensus sequence is in bold type. The complementary a, a^{met}, b, and b^{met} strands were annealed to produce unmethylated and hemimethylated double-stranded substrates.

Enzyme Assays

Steady-State and Burst Magnitude Kinetic Determinations. Duplicate 25 μL reaction volumes contained DCMTase at 20, 40, or 100 nM in MR buffer [100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 200 μg/mL BSA, and 10 mM DTT] and 30 μM radiolabeled [*methyl*-^{3}H]AdoMet (1×10^4 cpm/pmol). Saturating concentrations of AdoMet were determined using three DNA substrates. After preincubation at 37 °C for 3 min, the reaction was initiated upon addition of DNA. Single-stranded DNA was heated to 90 °C and cooled rapidly on ice prior to initiation of the reaction. The DNA concentration and substrate were varied: GC-box a ss DNA, GC-box b ss DNA, GC-box a/b ds DNA, GC-box a/b^{met} ds DNA, CRE a ss DNA, CRE b ss DNA, CRE a/b ds DNA, CRE a^{met}/b ds DNA, or poly(dI·dC:dI·dC). The reaction was stopped after 50 min by transferring 20 μL of the reaction mixture onto DE81 filter paper and the mixture processed as described (Reich & Mashhoon, 1991). All steady-state data were analyzed by double reciprocal plots, and regression analysis was done using Leonora 1.0 software [provided with Cornish-Bowden (1995)]. Residual plots show a random arrangement of positive and negative residuals.

Reaction Progress Determinations. Duplicate 15 μL samples were removed from a single reaction volume at the times listed in the figure legends. Reaction mixtures contained DCMTase at 20 or 40 nM in MR buffer [100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 200 μg/mL BSA, and 10 mM DTT] and 15 μM radiolabeled [*methyl*-^{3}H]AdoMet (1×10^4 cpm/pmol). After preincubation at 37 °C for 3 min,

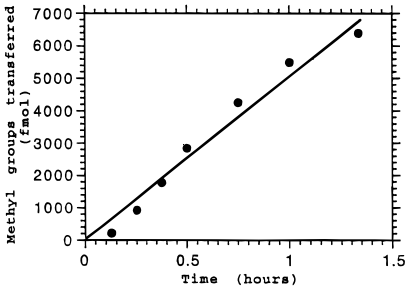

FIGURE 1: Time course for methylation of poly(dI·dC:dI·dC) catalyzed by DCMTase. A reaction mixture of 220 μL contained 1 nM DNA, 33 nM DCMTase, and 15 μM AdoMet (10 cpm/fmol) in MR buffer [100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 200 μg/mL BSA, and 10 mM DTT]. At 7.5, 15, 22.5, 30, 45, 60, and 80 min, duplicate 15 μL samples were spotted onto DE81 paper and prepared as in Experimental Procedures. Points are plotted as the mean of the two samples at each time.

the reaction was initiated upon addition of DNA. This was performed for each DNA substrate: GC-box a/b ds DNA, GC-box a/b^{met} ds DNA, CRE a ss DNA, CRE a/b ds DNA, CRE a^{met}/b ds DNA, and poly(dI·dC:dI·dC). The samples were processed as described above. All pre-steady-state data were analyzed using a regression fit to a single exponential using KaleidaGraph 2.1.2 software.

SssI DCMTase Comparisons to Murine DCMTase. The methylation rate for M.*Sss*I was linearly dependent on enzyme concentration in the range tested. Product formation was also linear for at least 40 min at 37 °C. Product formation was measured in an assay similar to that described above for the steady-state kinetic determinations, using 0.064 μg (2 units according to New England Biolabs) of M.*Sss*I in a 25 μL reaction mixture (60 nM, based on a predicted MW of 43 567). The reaction was stopped after 30 min by transferring 20 μL onto DE81 filter paper and the mixture processed as stated above. The concentrations of CRE substrates were varied in order to determine K_m and V_{max}/K_m values.

RESULTS

Poly(dI·dC:dI·dC). Poly(dI·dC:dI·dC) was previously shown to undergo methylation at rates comparable to those of some hemimethylated DNA substrates (Xu et al., 1995) and is thus an excellent substrate for monitoring the activity of DCMTase during protein purification and for defining the final specific activity of the enzyme. Each molecule of poly(dI·dC:dI·dC) provides a large number of potential sites for methylation. Figure 1 shows that methylation of poly(dI·dC:dI·dC) by the DCMTase increases linearly over 1 h, during which time each enzyme molecule is undergoing multiple catalytic turnovers. The rate of methylation is linearly dependent on enzyme concentration (1−100 nM, data not shown). Velocity measurements at various DNA concentrations are shown in Figure 2, and the data were fit to the Michaelis−Menten equation to obtain the steady-state parameters shown in Table 2. The data shown in Table 2 were obtained with poly(dI·dC:dI·dC) having an average length of 280 or 6250 bp. The turnover numbers and specificity constants for the two lengths are similar and reproducible. These parameters provide a valid basis for comparison of one enzyme preparation to another.

Design of DNA Substrates and Development of Enzyme Activity Assays. Our goals were to define suitable kinetic methods to investigate structure−function questions relating to the mammalian DCMTase, to apply such methods to quantify the enzyme's discrimination between related DNA sequences as well as between single-stranded and hemimethylated DNA, and to identify the underlying binding and catalytic contributions to any observed discrimination. The primary focus of this study is on the two DNA sequences shown in Table 1. The two sequences are thought to differentially regulate gene expression, depending on the methylation state of a single CpG dinucleotide. One sequence contains the cyclic AMP responsive element (5′-TGA**CG**TCA-3′, CRE), and the other contains a GC-box, which is an Sp1 transcription factor recognition element (5′-GGGG**CG**GGGC-3′, GC-box). In each case, the regulatory element was imbedded within a 30-nucleotide sequence with an identical flanking sequence. This length was selected on the basis of our DNA footprinting data showing that 30 base pairs should be adequate to satisfy all DCMTase−DNA interactions. Each oligonucleotide was purified by reverse-phase HPLC. Annealings were based on spectrophotometric determination of ss DNA concentrations and optimized so that less than 0.1% of the DNA was in the single-stranded form.

The integrity of the DCMTase and its performance in our assays were studied and optimized. The rate of methylation is directly proportional to enzyme concentration (1−100 nM), when assayed with several substrates listed in Table 1 (data not shown). For all substrates other than CRE a^{met}/b and GC-box a/b^{met} (see Table 1), the amount of methylated DNA produced as a function of time is linear (Figure 3). CRE a^{met}/b and GC-box a/b^{met} show an initial burst of product

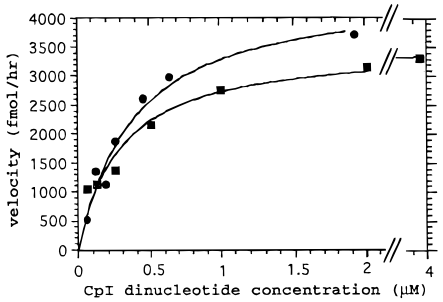

FIGURE 2: Steady-state kinetics using poly(dI·dC:dI·dC). Reaction mixtures contained either 280 bp poly(dI·dC:dI·dC) (■) at 0.062, 0.12, 0.25, 0.50, 1.0, 2.0, and 3.9 μM in CpI dinucleotide or 6250 bp poly(dI·dC:dI·dC) (●) at 0.062, 0.12, 0.19, 0.25, 0.44, 0.63, and 1.9 μM in CpI dinucleotide, 30 nM DCMTase, and 20 μM AdoMet (10 cpm/fmol) in MR buffer [100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 200 μg/mL BSA, and 10 mM DTT]. This represents macromolecular concentrations of 0.22−14 and 0.01−0.3 nM for the 280 and 6250 bp DNA, respectively. Reaction mixtures (25 μL) at each point were mixed without DNA and preincubated for 3 min at 37 °C; catalysis was initiated by the addition of DNA. After 50 min at 37 °C, 20 μL was spotted onto DE81 paper, prepared as in Experimental Procedures, and the resultant velocities were plotted using Kaliedagraph 2.1.2 software.

Table 2: Steady-State Kinetic Parameters of Murine DCMTase Using ds Poly(dI·dC:dI·dC)[a]

substrate	k_{cat} (h^{-1})	K_m^{DNA} (μM)	k_{cat}/K_m^{DNA} (h^{-1} M^{-1} × 10^6)
280 bp poly(dI·dC:dI·dC)	4.9 ± 0.5	0.27 ± 0.07	18.4
6250 bp poly(dI·dC:dI·dC)	6.4 ± 0.3	0.37 ± 0.06	17.2

[a] Initial velocity data were fit to the Michaelis−Menten steady-state equation by nonlinear regression.

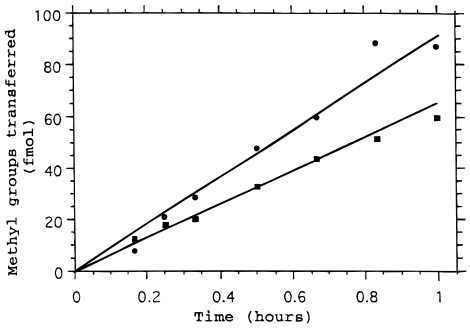

FIGURE 3: Progress curves for ss CRE a and CRE a/b. Reaction mixtures of 320 μL contained 20 μM CRE a/b (●) or 160 μM ss CRE a (■), 40 nM DCMTase, and 15 μM AdoMet (10 cpm/fmol) in MR buffer [100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 200 μg/mL BSA, and 10 mM DTT]. At 10, 15, 20, 30, 40, 50, and 60 min, duplicate 10 μL samples, containing 400 fmol of DCMTase, were prepared as in Experimental Procedures. Points are plotted as the mean of the two samples at each time.

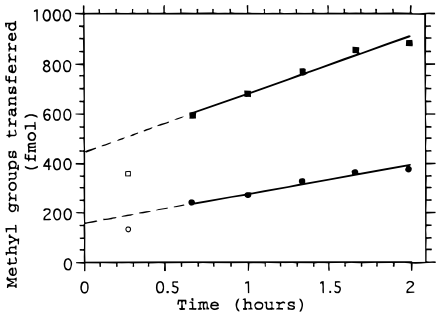

FIGURE 4: Progress curve showing the steady-state period of DCMTase catalysis of CRE a^{met}/b. Reaction mixtures of 350 μL contained 15 μM DNA, 20 nM (●) or 40 nM (■) DCMTase, and 15 μM AdoMet (10 cpm/fmol) in MR buffer [100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 200 μg/mL BSA, and 10 mM DTT]. At 40, 60, 80, 100, and 120 min, duplicate 10 μL samples, containing either 400 or 200 fmol of DCMTase, were prepared as in Experimental Procedures. Points are plotted as the mean of the two samples at each time using Kaliedagraph 2.1.2 software. Linear least square extrapolation (− −), excluding the data at 15 min (open symbols), was used to determine the burst size.

formation followed by a linear increase; the linear region extends over a period of approximately 3 h (Figure 4). No degradation of the 190 kDa DCMTase polypeptide was detected by Western blots under the conditions of the assay (data not shown). The K_m^{AdoMet} is insensitive to the DNA substrate used and equal to 3.2 ± 0.3 μM using saturating concentrations of CRE a/b and GC-box a/b and 1.7 ± 0.3 μM using poly(dI·dC:dI·dC).

The activity data shown in Figures 1, 3, and 4 and the kinetic parameters in Tables 2 and 3 indicate that the DCMTase has a low turnover rate constant and that each enzyme molecule catalyzes at most six methylation events during the time of a typical assay. This is clearly not due to inactive enzyme or the presence of a contaminating 190 kDa protein; as described below, the magnitude of the burst shown in Figure 4 is consistent with the entire DCMTase sample being catalytically competent. Moreover, similar turnover numbers were measured for the recombinant murine DCMTase.[2]

Demonstration of Burst Kinetics with CRE a^{met}/b and GC-box a/b^{met}. The addition of excess CRE a^{met}/b (15 μM, 9-fold higher than K_d^{DNA}) to a preincubated solution containing DCMTase (20 nM) and a saturating concentration of AdoMet (15 μM) results in a rapid burst followed by a constant rate of product formation (Figure 4). When enzyme was similarly assayed at 40 nM, the amplitude of the burst was dependent on the concentration of DCMTase. These results are consistent with a two-step kinetic mechanism in which binding and methylation occur in the first step followed by regeneration of the catalyst in a rate-limiting second step (Reich & Mashhoon, 1991, 1993; Johnson, 1992). Similar results were also observed with GC-box a/b^{met} (data not shown). Linear least squares extrapolation back to zero time in Figure 4, excluding the reference initial burst sampling, gives a burst magnitude of 1.00 ± 0.15 mol of methylated DNA per mole of DCMTase. These data demonstrate that the methylation of CRE a^{met}/b and GC-box a/b^{met} by DCMTase is significantly faster than steady-state turnover and that the entire enzyme preparation is catalytically competent (Reich & Mashhoon, 1991, 1993; Johnson, 1992).

Table 3: Kinetic Parameters Obtained with Regulatory DNA Sequences[a]

DNA substrate	K_m (μM)	k_{cat} (h^{-1})	k_{cat}/K_m (h^{-1} M^{-1} × 10^6)
CRE a	33 ± 8	0.33 ± 0.03	0.010
CRE b	34 ± 17	0.47 ± 0.12	0.014
CRE a/b	2.8 ± 0.3	0.59 ± 0.02	0.21
GC-box a	−	<0.04	−
GC-box b	−	<0.04	−
GC-box a/b	0.80 ± 0.38	0.15 ± 0.02	0.19

DNA substrate	K_d (μM)	$k_{methylation}$ (h^{-1})	$k_{methylation}/K_d^{DNA}$ (h^{-1} M^{-1} × 10^6)
CRE a^{met}/b	1.7 ± 0.2[b]	3.0 ± 0.2[b]	1.8
GC-box a/b^{met}	0.28 ± 0.10[b]	1.2 ± 0.1[b]	4.3

[a] ± values represent standard errors. Data analysis was as described in Experimental Procedures. The substrates used in these analyses are described in Table 1. [b] The data for CRE a^{met}/b and GC-box a/b^{met} were obtained under pre-steady-state conditions (Figures 2, 4, and 7).

The linear portions of both curves shown in Figure 4 reflect methylation under steady-state conditions; the rate constants are 0.60 ± 0.02 and 0.58 ± 0.02 h^{-1} for the 20 and 40 nM curves, respectively. Since both GC-box a/b^{met} and CRE a^{met}/b contain a single CpG site, the initial burst region is due to the complete methylation of 1 equiv of DNA molecules by one molecule of enzyme, followed by the slower, linear segment encompassing all kinetic steps leading through methylation of subsequent substrates. The lack of a burst with all other substrates tested in this study (see below) suggests that the methylation step or a step prior to methylation is rate-limiting for these substrates (Johnson, 1992).

Measurement of $k_{methylation}$ for CRE a^{met}/b and GC-box a/b^{met}. By measuring the rate of CRE a^{met}/b methylation at early times under conditions similar to those in Figure 4, we were able to directly determine the methylation rate constant, $k_{methylation}$ (Figure 5). Incorporation of methyl groups into DNA was fit by nonlinear regression to a single exponential [$p = Do(1 - e^{-k_{methylation}t})$], with $k_{methylation}$ being the methylation rate constant, p the moles of methylated DNA formed, Do the maximum moles of methylated DNA formed, and t the time in hours of the reaction (Reich & Mashhoon, 1993; Johnson, 1992). $k_{methylation}$ was calculated to be 2.8 ± 0.3 h^{-1}, and a similar calculation using the data

[2] J. F. Glickman, J. Flynn, and N. O. Reich, submitted.

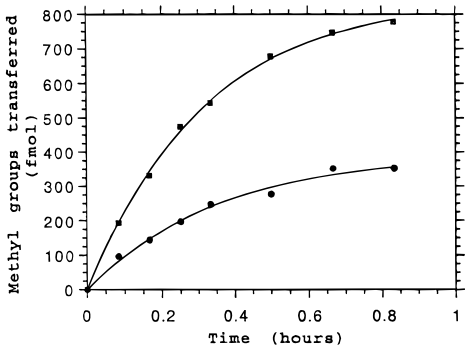

FIGURE 5: Progress curve showing the pre-steady-state period of DCMTase catalysis of CRE a^{met}/b. Reaction mixtures of 320 μL contained 15 μM DNA, 20 nM (●) or 40 nM (■) DCMTase, and 15 μM AdoMet (10 cpm/fmol) in MR buffer [100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 200 μg/mL BSA, and 10 mM DTT]. At 5, 10, 15, 20, 30, 40, and 50 min, duplicate 10 μL samples, containing either 400 or 200 fmol of DCMTase, were prepared as in Experimental Procedures. Points are plotted as the mean of the two samples at each time and fit to a first-order exponential as described (Reich & Mashhoon, 1991) using Kaliedagraph 2.1.2 software.

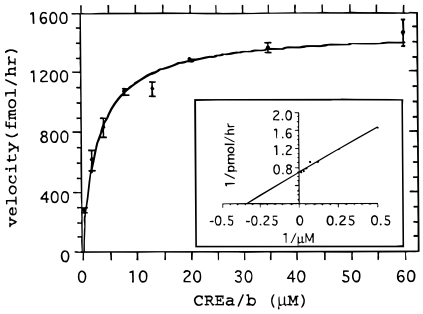

FIGURE 6: Steady-state kinetics of murine DCMTase with ds CRE a/b. Reaction mixtures contained DNA (0.5, 2, 4, 8, 13, 20, 35, or 60 μM), 100 nM DCMTase, and 30 μM AdoMet (10 cpm/fmol) in MR buffer [100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 200 μg/mL BSA, and 10 mM DTT]. Duplicate 25 μL reaction mixtures at each point were mixed without DNA and preincubated for 3 min at 37 °C, catalysis was initiated by the addition of DNA. After 50 min at 37 °C, 20 μL was spotted onto DE81 paper, prepared as in Experimental Procedures, and the resultant velocities were plotted as the mean with standard errors using Kaliedagraph 2.1.2 software. The inset is the corresponding Lineweaver−Burke plot.

obtained with 40 nM DCMTase gave a value of 3.1 ± 0.1 h^{-1}; these values are approximately 5-fold larger than the rate constant obtained for the steady-state region of the reaction (Figure 4). $k_{methylation}$ was shown to be independent of DNA concentration in the range of 0.5−2 μM DNA (data not shown). Similar experiments with GC-box a/b^{met} gave a $k_{methylation}$ of 1.2 ± 0.1 h^{-1} (Table 3).

Kinetic Analysis of Single-Stranded and Unmethylated Double-Stranded Substrates. Methylation rates of single-stranded and unmethylated substrates (Figure 3) were obtained at various DNA concentrations to determine k_{cat} and K_m^{DNA}. Curves of the observed velocity were plotted against DNA concentrations (Figure 6) and were fit by nonlinear regression to the Michaelis−Menten equation. The derived k_{cat} and K_m^{DNA} values are shown in Table 3. The single-stranded GC-box a and GC-box b oligonucleotides showed less than the detectable activity, 0.04 h^{-1}. k_{cat} for CRE a, CRE b, CRE a/b, and GC-box a/b varied at most by 4-fold, and the values are comparable to or smaller than the constants measured for the linear steady-state portion of the CRE a^{met}/b and GC-box a/b^{met} curves (Figure 4 and Table 3). The lack

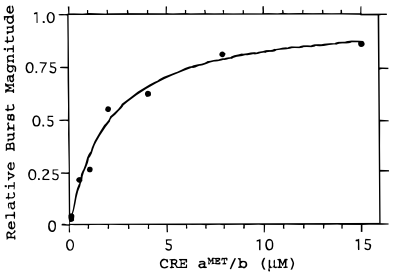

FIGURE 7: Burst magnitude versus CRE a^{met}/b concentration. Reaction mixtures contained DNA (0.05, 0.1, 0.5, 1, 2, 4, 8, or 15 μM), 100 nM DCMTase, and 30 μM AdoMet (10 cpm/fmol) in MR buffer [100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 200 μg/mL BSA, and 10 mM DTT]. Duplicate 25 μL reaction mixtures at each point were mixed without DNA and preincubated for 3 min at 37 °C; catalysis was initiated by the addition of DNA. After 50 min at 37 °C, 20 μL was spotted onto DE81 paper and prepared as in Experimental Procedures.

Table 4: Relative Specificity of DCMTase and M.*Sss*I[a]

	K_m (μM)		relative V_{max}		relative V_{max}/K_m	
	M.*Sss*I	mouse	M.*Sss*I	mouse	M.*Sss*I	mouse
CRE a	15	33	1	0.1	0.08	0.006
CRE a/b	0.03	2.8	0.02	0.2	1	0.1
CRE a^{met}/b	1.6	1.7	0.08	1	0.06	1

[a] The relative specificity is defined as a normalized V_{max}/K_m within each enzyme source.

of any detectable burst with CRE a, CRE b, CRE a/b, and GC-box a/b is consistent with a mechanism where methylation or a preceding step limits turnover, and thus, these constants can be viewed as turnover constants (k_{cat}) (Johnson, 1992).

Binding of CRE a^{met}/b and GC-box a/b^{met} Measured Using Burst Analysis. Under the conditions of the experiment in Figure 4, the burst magnitude is equivalent to the moles of functional DCMTase−AdoMet−DNA complex upon initiation of the reaction (Reich & Mashhoon, 1990; Rose, 1980). Measurement of the burst magnitude as a function of DNA concentration can therefore be used to determine the dissociation constant for this complex (Reich & Mashhoon, 1990; Rose, 1980). The bursts shown in Figure 4 were determined at different concentrations of CRE a^{met}/b by measuring the amount of product formed at 50 min, which is within the linear, steady-state region. The dissociation constant was determined to be 1.7 μM by replotting the burst magnitude versus CRE a^{met}/b concentration (Figure 7) (Reich & Mashhoon, 1990; Rose, 1980); a similar analysis using GC-box a/b^{met} gave a dissociation constant of 0.28 μM. These values are similar in magnitude to the K_m^{DNA} values obtained for the unmethylated CRE a/b and GC-box a/b and significantly lower than the values obtained for single-stranded CRE a and CRE b (Table 3).

Comparison of Mammalian and Bacterial DCMTases. A comparison of kinetic parameters of the murine DCMTase and M.*Sss*I, a bacterial DCMTase which similarly catalyzes the methylation of CpG dinucleotides (Renbaum & Razin, 1992), is shown in Table 4. We report relative V_{max} and V_{max}/K_m values, since, although shown to be greater than 90% pure by SDS−PAGE, this preparation was obtained from a commercial source. No burst was detected for the M.*Sss*I with any substrate. The M.*Sss*I shows much wider variation in K_m and V_{max} values for single-stranded, double-stranded,

and hemimethylated CRE than the murine DCMTase. Comparison of the relative V_{max}/K_m values demonstrates that the bacterial and murine DCMTases display entirely different preferences for single-stranded, double-stranded, and hemimethylated CRE.

DISCUSSION

The biological importance of DNA methylation to normal mammalian development has been demonstrated (Monk et al., 1987: Kafri et al., 1992; Li et al., 1992). Investigations over the last 25 years have resulted in a limited understanding of the functional and mechanistic contributions by DCMTase toward maintaining and establishing genomic methylation. Solely on the basis of relative velocities, the mammalian DCMTase appears to prefer double-stranded over single-stranded DNA and hemimethylated over unmethylated double-stranded DNA. Beyond this, little could be said with certainty regarding the enzyme's substrate preferences and mechanism of action. Several factors have contributed to this lack of biochemical understanding. First, the relatively large mammalian DCMTase has historically been difficult to purify to homogeneity in quantities suitable for mechanistic studies. Even recent studies rely on nuclear extracts (Christman et al., 1995) or partially purified preparations (Laayoun et al., 1995; Tollefsbol & Hutchison, 1995) for enzymological characterization. Second, the enzyme is extremely sensitive to proteolytic degradation, resulting in numerous studies done with fragments of the 190 kDa DCMTase (Reale et al., 1995). Since extensive proteolysis is known to alter the enzyme's functional characteristics (Bestor & Ingram, 1985), the validity of studies using partially proteolyzed preparations remains in doubt. Third, past investigations of the enzyme's substrate specificity have relied simply on relative velocity measurements without an understanding of the enzyme's kinetic mechanism; thus, real specificity differences are obscured, and an understanding of the mechanism of any observed discrimination is precluded.

We recently described a protein purification strategy which provides 300−500 μg of homogeneous mammalian DCMTase from murine erythroleukemia cells. The 190 kDa M_r protein shows no sign of proteolysis when purified in the presence of protease inhibitors, most notably the protease inhibitor E64, *N*-[*N*-(L-3-*trans*-carboxirane-2-carbonyl)-L-leucyl]agmatine. The murine DCMTase has been extensively studied and shows significant protein sequence similarity to the human DCMTase; these are the only mammalian DCMTases for which sequence information is available.

Characterization with poly(dI·dC:dI·dC). Our initial analysis used the double-stranded synthetic polymer, poly(dI·dC:dI·dC). This substrate, while lacking biological relevance, was previously shown to have comparable or greater activity than the majority of tested substrates (Pedrali-Noy & Weissbach, 1986). Unfortunately, no previously published accounts describe even the simplest of kinetic parameters for this substrate, and the results shown in Table 2 are the first description of any steady-state parameters for a eukaryotic DCMTase. The results reveal that the enzyme has a relatively low turnover number, which is consistent with other enzymes that catalyze AdoMet-dependent methyltransfers to the C-5 position of pyrimidines. The turnover number for the only characterized bacterial DCMTase, M.*Hha*I, is 78 h^{-1} (Wu & Santi, 1987), which is within 10-fold of that of the mammalian DCMTase, and for tRNA (m^5U54) MTase 108 h^{-1} (Kealey et al., 1994).

Kinetic Analysis with regulatory DNA Sequences. The role of the mammalian DCMTase in determining and maintaining patterns of DNA methylation can only be understood if the inherent discrimination of the enzyme for various potential substrates is described. As a starting point, we sought to define suitable activity assays with substrates containing single CpG sites. This approach provides definitive data describing the enzyme's inherent substrate preferences and the underlying molecular contributions to any observed discrimination without the contribution of additional same strand CpG dinucleotides.

Our studies were initiated with two substrates whose methylation status *in vivo* is directly correlated to the differential regulation of gene expression [CRE, Iguchi-Ariga and Schaffner (1989) and Moens et al. (1993); and GC-box, Joel et al. (1993) and Jane et al. (1993); see Table 1]. The single-stranded and unmethylated double-stranded versions of these substrates are methylated at a slow but linear rate (Figure 3), whereas the hemimethylated substrates (CRE a^{met}/b and GC-box a/b^{met}) show a burst of product formation (Figures 4 and 5) followed by a linear rate of product formation. Methylation of CRE a^{met}/b and GC-box a/b^{met} must therefore be relatively rapid and followed by a slower step. Since this initial burst corresponds to a single turnover of substrate and these substrates contain a single CpG site, we suggest that the second, slower process is defined by step(s) following catalysis. Support for this is indirectly provided by the lack of any burst observed with poly(dI·dC:dI·dC) (Figure 1) which has multiple sites for methylation within each DNA molecule and undergoes several cycles of methylation during the assay. Since the catalysis of multiple methylation events on poly(dI·dC:dI·dC) does not require the enzyme to dissociate from the DNA, it may not need to go through the same slow steps following catalysis which are observed with the substrates containing a single recognition site.

The k_{cat} and $k_{methylation}$ values shown in Tables 2 and 3 are extremely slow when compared to turnover numbers of $1 - 10^6$ s^{-1} for enzymes in general (Fersht, 1985). However, the burst experiments in Figure 3 definitively show that the entire sample of protein is active. Using a gel-retardation assay, we also recently demonstrated that this enzyme preparation binds DNA sequence-specifically and that the entire protein sample is functional by this assay.[3] Also, as discussed in the case with poly(dI·dC:dI·dC), the turnover numbers are only about 10-fold lower than those observed with bacterial DCMTases. Since $k_{methylation}$ is not available for any other DCMTase, no comparison can be made with the mammalian enzyme. While $k_{methylation}$ has been measured for the M.*Eco*RI which methylates the second adenine in GAATTC (1.5×10^5 h^{-1}; Reich & Mashhoon, 1993), comparison with the mammalian DCMTase is problematic since the chemical mechanisms for the two classes of DNA MTases are most likely different.

By our calculations, the relatively slow catalytic turnover of the mammalian DCMTase does not preclude its proposed

[3] J. Flynn, R. Azzam, and N. O. Reich, in preparation.

biological role. Thus, a cell containing 5×10^5 DCMTase molecules per cell (Xu et al., 1995) can methylate the 3×10^7 cytosines predicted to be methylated in a typical mammalian genome at the maximal rate observed in these studies in less than 10 h. The doubling time of mouse erythroleukemia cells is about 20 h; therefore, the DCMTase studied here *in vitro* is sufficiently active to maintain the genomic methylation pattern *in vivo*.

The discrimination of the DCMTase for various substrates is quantitatively shown by the specificity constants in Table 3. The enzyme has an approximate 2-fold preference for the GC-box a/b^{met} sequence over the CRE a^{met}/b sequence, and this preference is not observed with the unmethylated versions of these substrates. Interestingly, these specificity constants are comparable to that determined for poly(dI·dC:dI·dC). The preference for GC-box a/b^{met} derives from a 6-fold greater binding affinity for the GC-box a/b^{met} sequence, suggesting that protein−DNA interactions beyond the CpG dinucleotide can be important binding determinants. The enzyme prefers the hemimethylated CRE by 9-fold over the unmethylated double-stranded CRE, and this hierarchical preference is 23-fold in the case of the GC-box sequence. While these values are similar to previous results (Bolden et al., 1985) comparing only relative velocities, our data clearly demonstrate that this discrimination is largely manifested through changes in the methylation rate constant. Thus, the DCMTase binds to the unmethylated and hemimethylated substrates with comparable affinities, but the methyl transfer step is increased to rates superseding the rate of a subsequent event. A similar analysis of the single-stranded CRE substrates shows that the enzyme prefers the unmethylated and hemimethylated double-stranded substrates by 18- and 150-fold, respectively.

The functional parameters described above clearly support the one enzyme model for maintenance and *de novo* methylation. However, our findings do not exclude the possibility of other DCMTases capable of separate duties or specific regulators of the activities. The greater specificity shown with hemimethylated substrates suggests that maintenance methylation may well be a more dominant function relative to *de novo* methylation *in vivo*. Throughout development, even modest morphological and functional cellular changes generally occur over many cell divisions. Epigenesis, as the underlying differences between the genomic methylation states of the precursor and the daughter cell, is suspected to be minimal at any one cell division. There would appear to be a greater need, from a gene activity standpoint, to keep the majority of methyl-encoded information and to make only small expression changes at each generation. Mammalian development, thereby, occurs systematically, and as reflected in our specificity measures, the DCMTase has functionally evolved to meet the dynamic methylation demands during ontogeny.

Comparison of Mammalian and Bacterial DCMTases. Bacterial DCMTases with various DNA sequence specificities are presently used to investigate the biology of mammalian DNA methylation. The significant amino acid similarity between the C-terminal one-third of the mammalian DCMTase and most bacterial DCMTases has led to the expectation that the bacterial and mammalian enzymes are thereby functionally similar (Bestor & Verdine, 1995). Representative of this group of bacterial DCMTases is the M.*Sss*I which recognizes CpG dinucleotides. Comparison of the M.*Sss*I and mammalian DCMTases in Table 4 clearly reveals large qualitative and quantitative functional differences. The preferred substrate for the M.*Sss*I is the unmethylated CRE a/b, while the hemimethylated and single-stranded substrates show similar specifities. In contrast, the mammalian DCMTase prefers the hemimethylated substrate and shows a dramatic discrimination against the single-stranded form (Tables 3 and 4). The specificity hierarchy shown for the M.*Sss*I derives from very different changes in K_m and catalysis than those observed for the mammalian DCMTase.

These results demonstrate that the M.*Sss*I, and perhaps other bacterial DCMTases as well, is mechanistically dissimilar to the mammalian DCMTase. Furthermore, substrate specificity analyses carried out by comparing relative rates at single substrate concentrations are susceptible to significant misinterpretation. For example, various reports describe single-stranded DNA as a good substrate for mammalian DCMTases (Carotti et al., 1986; Christman et al., 1995; Smith et al., 1992a, Pfeifer et al., 1985; Hitt et al., 1988). In contrast, other studies suggest that single-stranded DNA is a poor substrate for the enzyme (Gruenbaum et al., 1982; Smith et al., 1992b). A single report suggests that a bacterial DCMTase from *Spiroplasma* strain MQ-1 has poor activity with single-stranded viral DNA (Nue et al., 1985). The results in Tables 3 and 4 clearly show how these various observations can be reconciled. For example, if assayed at high DNA concentrations, the mammalian enzyme shows only a 10-fold preference for hemimethylated DNA over single-stranded DNA, while this preference is 160-fold at low DNA concentrations. Even more dramatic is the bacterial enzyme which at high substrate concentrations shows a 50-fold preference for single-stranded DNA over double-stranded DNA, while at low substrate concentrations, this is reversed, with a 12-fold preference for double-stranded DNA. We suggest that much of the confusion in the present literature on the mammalian DCMTase is based on studies of relative rate determinations performed without consideration of the underlying kinetic components.

Changes in patterns of mammalian DNA methylation have been observed in X-chromosome inactivation, genetic imprinting, oncogenesis, and numerous developmentally regulated alterations in gene expression (Saluz & Jost, 1993). The role(s) of the mammalian DCMTase in these important biological processes can now be addressed using the homogeneous 190 kDa M_r enzyme and the assays and analyses described in this report.

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