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Murine DNA cytosine C^5-methyltransferase: in vitro studies of de novo methylation spreading☆

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Abstract

The preference of murine DNA (cytosine-5)-methyltransferase (Dnmt1) for single stranded DNA substrates is increased up to 50-fold by the presence of a proximal 5-methyl cytosine ($5^{me}C$). This modulation is distance-dependent and is due to an enhanced binding affinity and minor changes in catalytic efficiency. No modulation was observed with double stranded DNA. Modulation requires that the $5^{me}C$ moiety be attached to the DNA strand containing the CpG methylation target. Our results support a model in which $5^{me}C$ binding by the enzyme occurs to at least one site outside the region involved in CpG recognition. No modulation in response to $5^{me}C$ is observed with the bacterial enzyme M.*Sss*I, which lacks the large N-terminal regulatory domain found in Dnmt1. We suggest that this allosteric modulation involves the N-terminal domain of Dnmt1.

Keywords: DNA cytosine methyltransferase; Methylation spreading; Single stranded DNA; N-terminal domain; Dnmt1

In eukaryotes, cytosine methylation by the enzyme DNA cytosine C^5 methyltransferase (DCMTase EC 2.1.1.37) is the predominant modification of bases occurring after replication. This methyl group transfer to the C^5 position of cytosine requires the cofactor *S*-adenosyl-L-methionine (AdoMet) and occurs primarily in cytosyl–guanosyl (CpG) dinucleotides in tissue-specific patterns [1,2]. DNA methylation has been implicated in regulatory DNA transposition [6], DNA repair [12], recombination [14], genetic imprinting [18], and chromatin organization [31]. The inheritance of the correct DNA methylation patterns is critical in early development, as shown by Dnmt1 gene knockouts in mice [17]. DNA methylation can also serve as a protection mechanism against invading DNA sequences through hypermethylation and transcriptional silencing [5]. Upon integration into the mammalian genome, viral DNA becomes hypermethylated in a sequence independent manner at discrete loci and spreads to neighboring regions [5,22]. Similar de novo methylation occurs in gene regulatory sequences during X-chromosome inactivation [11], cellular immortalization [28,30], cellular senescence [29], and in the final stages of the Fragile X syndrome [21]. Interestingly, DCMTase inhibitors increase the effectiveness of transgene expression in retroviral gene therapy strategies [13]. The processes that lead to such localized methylation are not understood. A common initiation step most likely involves the selective recruitment of Dnmt1 (or other Dnmts), or increased accessibility of the enzyme to particular chromatin structures. The methylation spreading of newly integrated DNA could result from an inherently processive Dnmt1. Alternatively and possibly coupled with processivity, the presence of a $5^{me}C$ proximal to a target CpG could increase the catalytic efficiency of the enzyme. Our primary interest was to provide quantitative, mechanistic insights into how the activity of Dnmt1 is modulated by the presence of $5^{me}C$ moieties.

Five animal DCMTase cDNAs have been cloned and sequenced [mouse; human; chicken; frog; and sea urchin] [23]. These are relatively large proteins, ranging from 1490 to 1622 amino acids in length, and are

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Table 1
Single stranded DNA molecules used in this study[a]

Name	Sequence	^{m}C base distance from CpG
$POS1_a$	5′-GCAGCAGCAGCAGCAGCAG**CG**ATAGGT-3′	—
$POS2_a$	5′-G**^{m}C**AGCAGCAGCAGCAGCAGCAG**CG**ATAGGT-3′	21
$POS3_a$	5′-GCAG**^{m}C**AGCAGCAGCAGCAGCAG**CG**ATAGGT-3′	18
$POS4_a$	5′-GCAGCAG**^{m}C**AGCAGCAGCAGCAG**CG**ATAGGT-3′	15
$POS5_a$	5′-GCAGCAGCAG**^{m}C**AGCAGCAGCAG**CG**ATAGGT-3′	12
$POS6_a$	5′-GCAGCAGCAGCAG**^{m}C**AGCAGCAG**CG**ATAGGT-3′	9
$POS7_a$	5′-GCAGCAGCAGCAGCAG**^{m}C**AGCAG**CG**ATAGGT-3′	6
$POS8_a$	5′-GCAGCAGCAGCAGCAGCAG**^{m}C**AG**CG**ATAGGT-3′	3
POS_b	5′-ACCTAT**CG**CTGCTGCTGCTGCTGCTGCTGC-3′	—
EPOS	5′-GGCAGCAGCAGCAG**CG**ATAGGTGCAGCAGG- 3′	—
ODN	5′-TC**CG**GATC**CGCG**ATC- 3′	—
CRE_b	5′-CTGGATCCTTTTGA**CG**TCATTTGAATTCCC-3′	—
9mer	5′-GCAG**^{m}C**AGCA-3′	—
7mer	5′-CAG**^{m}C**AGC-3′	—
5mer	5′-AG**^{m}C**AG-3′	—

[a] Fifteen deoxynucleotides were synthesized for use as substrates for DCMTase (5^{m}C: C-5 methylcytosine). Oligonucleotides were ordered from and purified by gel filtration by Midland Certified Reagent. The C-5 methylcytosine and the site of methylation are both underlined and in bold face type. Complementary a and b strands for the POS series were annealed to produce double stranded substrates with varying 5^{m}C to CpG distances.

composed of a large amino-terminal domain and a smaller carboxy-terminal domain [24]. The best-studied mammalian DCMTase, Dnmt1, shows a 10–20-fold preference for hemimethylated sequences [8]. Recently identified mammalian DCMTases Dnmt3α and Dnmt3β do not have this preference [32]. Dnmt1, as well as members of the Dnmt3 group are involved in de novo DNA methylation [32]. Due to the shared homology with the highly characterized prokaryotic DCMTases, it is most likely that the catalytic activity of the animal DCMTases resides in the C terminus [16]. The N terminus contains a replication foci targeting domain, a nuclear localization signal [15], a cysteine rich zinc binding domain [1], a phosphorylation site [10], and an allosteric DNA binding site (Reich, unpublished observations). However, the functional significance of these features of the N-terminal domain remains poorly understood [7,17].

Dnmt1 is capable of methylating both single and double stranded DNAs [4,8,19]. Single stranded DNA with one or more 5^{me}C moieties shows enhanced methylation rates, whereas double stranded DNA of the same sequence does not [19]. The underlying molecular mechanism of this activation has not been determined [4,19]. Moreover, previous studies invoked the formation of stable and transient DNA duplexes that potentially confound any mechanistic interpretation [4].

5^{me}Cytosine leads to a 50-fold specificity increase of Dnmt1 for single stranded DNA

Eight deoxyribo-oligonucleotides were synthesized to address the positional effect of 5^{me}C on the activity of Dnmt1 with single stranded DNA. These oligonucleotides are identical in sequence with a single CpG and a 5^{me}C at variable distances from the site of methylation (Table 1). Dnmt1 shows good activity with single stranded substrates of this size [7], and footprinting studies show that these substrates are sufficiently large to accommodate the enzyme (Reich, unpublished observations). Sequences were chosen to minimize the probability of intramolecular (e.g., hairpins) and intermolecular structures. The 5^{me}C was inserted into non-CpG contexts to avoid the introduction of multiple CpGs.[1] Methylation rates were measured at various DNA concentrations and the data were fit by non-linear regression using the Michaelis–Menten equation to generate k_{cat} and K_m^{DNA} (data not shown). All of the 5^{me}C containing substrates show a decrease in K_m^{DNA}, and thus higher specificity constants than the control, which lacks any 5^{me}C ($POS1_a$). The $POS3_a$ substrate has a specificity constant 50-fold higher than the unmethylated control (Fig. 1A). This effect results from a 15.6-fold decrease in K_m^{DNA} and a 3.2-fold increase in k_{cat}.

Distance-dependent modulation by 5^{me}C in single stranded DNA was previously observed [4,19]. These authors proposed that small stem-loop structures were stabilized through interactions with Dnmt1 or other interacting proteins [4,19]. The substrates shown in Table 1 are not self-complementary, are not predicted to form fold-back structures, and no evidence for unusual structures was observed by PAGE analysis (data not shown). Moreover, the modulation with $POS2_a$ in which the 5^{me}C is only three nucleotides away from the CpG is unlikely to derive from the stabilization of stem-loop structures. While we cannot exclude the stabilization of

[1] This complication could have been avoided if the sequences were altered, resulting in eight substrates with differing sequences. This would leave open the concern that any observable effect was due to the sequence differences.

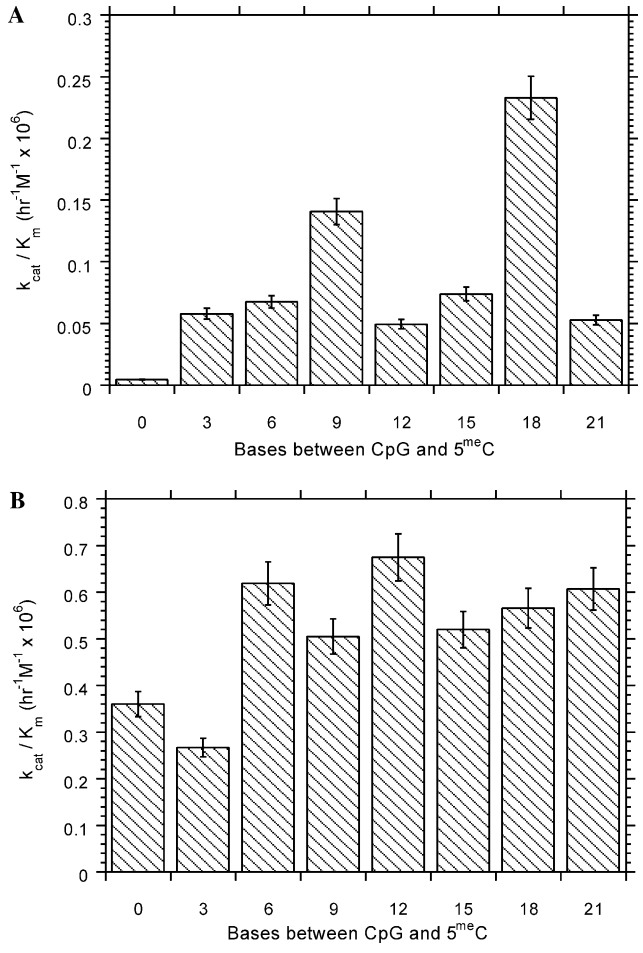

Fig. 1. k_{cat}/K_m^{DNA} as a function of the 5^{me} cytosine distance from the site of methylation using: (A) single stranded DNA and (B) the complementary double stranded DNA forms. Reaction mixtures contained 100 nM DCMTase (purified as described in [8]), DNA oligonucleotide concentrations at 0.5. 1.0, 4.0, 10.0, 15.0, and 30.0 μM, and 10 μm AdoMet (8.0 cpm/fmol, Sigma) in MR buffer [100 mM Tris–HCl (pH 8.0), 10 mM EDTA, 10 mM DTT, and 1 mg/mL BSA]. Reaction mixtures (20 μL, in triplicate) were incubated for 1 h at 37 °C. Following incubation, a portion of the reactions (17.5 μL) were stopped by subsequent spotting onto DE81 filter papers and processed as described in [25]. k_{cat} and K_m^{DNA} values were determined and calculated using Kaliedagraph 3.09 software.

transient structures by the Dnmt1, our highly homogeneous enzyme preparation argues against the involvement of other interacting proteins.

No dramatic modulation was observed when the single stranded positional substrates were annealed with their complementary strand to create double stranded substrates (Fig. 1B). Just as with the single stranded DNA substrates, methylation rates were determined at various DNA concentrations. The data were fit by nonlinear regression using the Michaelis–Menten equation to generate k_{cat} and K_m^{DNA} (data not shown). No effect greater than 2-fold was observed with double stranded DNA (Fig. 1B).

Modulation may involve the large N-terminal domain of Dnmt1

Highly conserved motifs found in all bacterial DCMTases and the C-terminal third of Dnmt1 are involved in AdoMet binding, substrate binding, and catalysis. M.*Sss*I is typical of many bacterial DCMTases (42 kDa) and lacks the N-terminal domain found in Dnmt1. Comparison of the activities of Dnmt1 and M.*Sss*I provides an indirect measure of the role this N-terminal domain plays in regulating Dnmt1 function (Fig. 2). M.*Sss*I does not reveal the modulation exhibited by Dnmt1 using the same set of single stranded DNA substrates (Fig. 2), suggesting that the $5^{me}C$ binding area is located on the N-terminal domain of Dnmt1. As these are two different enzymes, however similar, any mechanistic interpretation is purely suggestive.

Modulation requires the covalent attachment of $5^{me}C$ to methylatable substrate

A series of $5^{me}C$ containing "modulating" analogs were designed to determine if the modulator and the substrate are required to be covalently attached and to facilitate possible mechanistic studies [26]. The modulator analogs mimic the modulating portion of the $POS3_a$ substrate and are five, seven, and nine nucleotides in length (Table 1). We also designed CRE_b, EPOS,

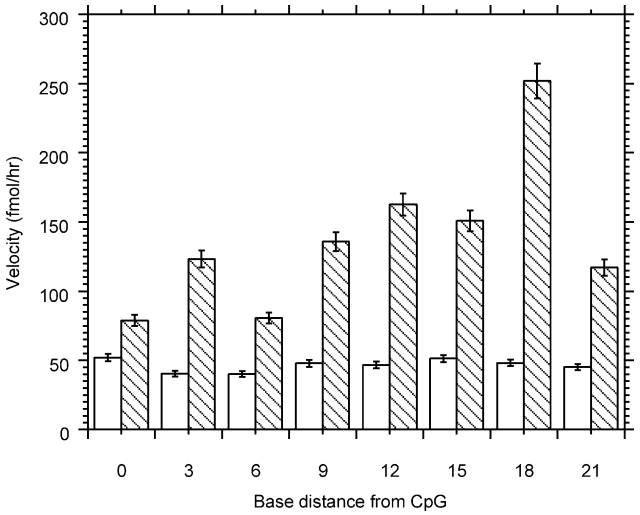

Fig. 2. Comparison of the observed velocities for Dnmt1 (lines) and M.*Sss*I (open) as a function of 5^{me} cytosine distance from the site of methylation using single stranded DNA substrates. Reaction conditions for Dnmt1 are identical to those mentioned in Fig. 1. Reaction mixtures for M.*Sss*I (purchased from New England Biolabs) contained 69.5 pM DCMTase, 30 μM single stranded DNA oligonucleotide, and 10 μM AdoMet (8.0 cpm/fmol) in MR buffer, and velocities were determined as described in Fig. 1.

and ODN substrates with which to test these potential modulators. The design of three new substrates to be used with these modular analogs was necessary as to maintain only one modulating $5^{me}C$ per molecule of CpG in the reaction. The addition of modular analogs to a reaction containing substrates already containing similar modulating portions would confuse mechanistic interpretations.

CRE_b was chosen because it shows good activity with Dnmt1 [7], EPOS contains the substrate portion of $POS3_a$ and eight additional bases to the 3′ end of the CpG (Table 1). The ODN DNA substrate was designed based on the findings by Christman et al. [4] in which a similar substrate showed $5^{me}C$ induced activation. We modified their substrate by removing the modulating portion and retaining the methylation target and flanking bases (Table 1). These three novel substrates were used in conjunction with varying concentrations of the modulator analogs to assay Dnmt1 activity.

No modulation of Dnmt1 activity with the three substrates was observed upon the addition of any activator up to 100 μM (data not shown). Dissociation constants of Dnmt1 for single stranded DNA substrates are in the low micromolar range [9]. Therefore, the concentrations used here are likely to be sufficient enough to ensure binding and modulation. A minor 5% inhibition was detected, perhaps resulting from the modulator binding to the active site in competition with substrate binding.

Conclusion

Dnmt1 is dramatically responsive to the presence of $5^{me}C$ outside the target CpG. Our observation that all single stranded DNAs containing a $5^{me}C$ exhibit a decreased K_m^{DNA} suggests that multiple enzyme–DNA interactions outside the CpG dinucleotide are involved in the modulation. The enzyme broadly prefers a $5^{me}C$ within its DNA substrate, at least when it is in the single stranded form. Covalent attachment is required to reveal the modulation, because an addition of the $5^{me}C$ portion from these oligonucleotides in *trans* does not result in any positive modulation.[2] This requirement for covalent attachment may simply result from a much higher effective activator concentration when attached to the substrate.

One model, which can account for our observations, invokes a region near the active site that is lined with hydrophobic amino acids. The single stranded DNA fits into this "hydrophobic rail," and $5^{me}C$ at various positions enhances DNA binding. The increased modulation observed with the substrates containing the $5^{me}C$ nine and eighteen bases away from the target CpG may result from a phasing of $5^{me}C$ with respect to the enzyme surface ("hyrophobic rail"). This interpretation requires that the single stranded DNA retain some helical character. However, the structure and flexibility of single stranded DNA is not well understood. The form of modulation observed here is most noticeable at the k_{cat}/K_m^{DNA} level and may not adhere to a classical definition of "activation" [26]. Nevertheless, the effect results in a dramatic 50-fold preference for CpG sites with a specifically placed proximal $5^{me}C$. This k_{cat}/K_m^{DNA} effect could manifest itself as a modulation in processivity on multisite substrates. For example, an initial methylation would result in an increased preference for methylating adjacent CpG sites, and processive methylation of multiple sites.

Modulation was not observed with the double stranded forms of our single stranded DNA molecules. This may result from duplex DNA preventing access to the modulating $5^{me}C$. Alternatively, Christman et al. [4] did observe some activation with double stranded DNA when the $5^{me}C$ was placed in a CpG context. However, this activation could have resulted from the formation of hemimethylated sites which are known to be better substrates ([4,8]). Our double stranded substrates have the $5^{me}C$ in a non-CpG context; thus it remains formally possible that we did not observe a modulation of Dnmt1 activity in double stranded DNA since our $5^{me}C$ was not in a CpG context. We are presently testing if the placement of $5^{me}C$ into a CpG context in double stranded DNA results in significant modulation.

The modulation with single stranded DNA is intriguing since there are several situations where single stranded DNA and even more complex structures are formed in vivo. For example, single stranded DNA within replication forks may form alternative DNA structures that Dnmt1 preferentially recognizes [27]. Furthermore, some mammalian viruses have single stranded DNA genomes [20], and the insertion of most viral DNA into the host genome involves significant alterations in chromatin structure which could present alternative DNA conformations preferred by Dnmt1 [22]. The expansion of the triple repeat in Fragile X syndrome is thought to involve several non-standard single stranded DNA structures [3].

Dnmt1 is a large, complex, and poorly characterized enzyme, and the modulation described here most likely involves the N-terminal domain. Support for this model comes from the observation that the bacterial enzyme M.*Sss*I, which lacks the N-terminal domain, does not display the modulation observed with Dnmt1. Our results are suggestive for either direct or indirect

[2] Alternatively, the analysis of the results with activator and substrate mixtures could be confounded by complex interactions of the activator binding to the substrate site and the substrate binding to the activator site.

interaction of the N-terminal domain in Dnmt1 with the $5^{me}C$ to achieve modulation. Cleavage of the N-terminal domain from the C-terminal of Dnmt1 increases the enzyme's activity for unmethylated DNA without a change in the rate of methylation for hemimethylated DNA [1]. This suggests that the N-terminal domain contributes to the recognition of the methylation status of the target CpG. Moreover, our results suggest that the N-terminal domain is involved in recognizing the modulating $5^{me}C$, no matter how close it is to the CpG of interest (Fig. 2), even at a distance of three bases. In contrast Lindsay and Adams [19] showed that modulation is distance dependent. These different results may be due to sequence differences between the DNA substrates. We suggest that the hydrophobic rail is in part defined by the N-terminal domain. Our work clearly shows $5^{me}C$ considerably modulates DCMTase activity within single stranded DNA and that the mechanism involves an enhanced binding of the enzyme to its substrate, with only minor improvements in activity. The role of $5^{me}C$ in duplex DNA remains uncertain, although with the set of sequences shown in Table 1, $5^{me}C$ shows no stimulation. Our observed modulation of Dnmt1 activity could become important during any cellular process that reveals single stranded DNA, such as DNA replication or viral DNA integration. Moreover, novel DCMTase inhibitors may enhance the effectiveness of retroviral-mediated gene therapy by preventing the inactivation of therapeutic genes.

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