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5-Methyl-2′-deoxycytidine in single-stranded DNA can act in cis to signal *de novo* DNA methylation

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ABSTRACT **Methylation of cytosine residues in DNA plays an important role in regulating gene expression during vertebrate embryonic development. Conversely, disruption of normal patterns of methylation is common in tumors and occurs early in progression of some human cancers. In vertebrates, it appears that the same DNA methyltransferase maintains preexisting patterns of methylation during DNA replication and carries out *de novo* methylation to create new methylation patterns. There are several indications that inherent signals in DNA structure can act *in vivo* to initiate or block *de novo* methylation in adjacent DNA regions. To identify sequences capable of enhancing *de novo* methylation of DNA *in vitro*, we designed a series of oligodeoxyribonucleotide substrates with substrate cytosine residues in different sequence contexts. We obtained evidence that some 5-methylcytosine residues in these single-stranded DNAs can stimulate *de novo* methylation of adjacent sites by murine DNA 5-cytosine methyltransferase as effectively as 5-methylcytosine residues in double-stranded DNA stimulate maintenance methylation. This suggests that double-stranded DNA may not be the primary natural substrate for *de novo* methylation and that looped single-stranded structures formed during the normal course of DNA replication or repair serve as "nucleation" sites for *de novo* methylation of adjacent DNA regions.**

In vertebrate cells, ≈3% of cytosine (C) residues in DNA have a methyl group on carbon 5, and 5-methylcytosine (5MeC) is the only naturally occurring modified base detected in DNA (1). Enzymatic methylation of C residues in DNA occurs postreplicatively and primarily involves C residues in CpG dinucleotides, although methylation has been observed at C residues 5′ of other nucleotides (2). The extent and pattern of methylation of genomic DNA is species- and tissue-specific (3), which implies that the pattern of methylation is faithfully inherited in all cells of common lineage within a tissue. Analysis of methylation patterns of specific genes during development suggests that patterns established in sperm and oocytes are lost during early development, that regions other than CpG islands become almost fully methylated, and that loss of methylation occurs at specific sites in tissues where a gene is expressed (4–6).

Although not all genes are regulated by methylation, hypomethylation at specific sites or in specific regions in a number of genes is correlated with active transcription (7–9). DNA methylation *in vitro* can prevent efficient transcription of genes in cell-free systems or transient expression of transfected genes; methylation of C residues in some specific cis-regulatory regions can also block or enhance binding of transcription factors or repressors (7–11). DNA methylation is involved in inactivation of one of the two X chromosomes in female mammalian somatic cells (12), and allele-specific methylation has been proposed as a factor in genomic imprinting (13). The most direct evidence for the importance of DNA methylation in development is the demonstration that homozygous mutation in murine DNA 5-cytosine methyltransferase (5-C-MTase) leads to impaired embryonic development (14).

Conversely, disruption of normal patterns of DNA methylation has been linked to development of cancer. The 5MeC content of DNA from tumors and tumor-derived cell lines is generally lower than in normal tissues (15), although increased methylation of CpG sites occurs in some genes and chromosome regions (16). While these observations support the concept that methylation patterns are established in the embryo and altered during carcinogenesis by a combination of *de novo* methylation and loss of methylation in a time-, sequence-, and tissue-specific manner, the mechanism(s) by which these changes occur and are regulated with such apparent precision has not been defined.

The processes involved in regulating *de novo* methylation are particularly puzzling. As would be predicted for an enzyme that maintains established patterns of methylation during DNA replication, mammalian DNA MTases have a much greater capacity for methylating hemimethylated CpG sites in double-stranded (ds) DNA than completely unmethylated sites. However, since the gene encoding mammalian DNA 5-C-MTase is present as a single copy per haploid genome (17) and there is no direct evidence for the existence of a separate *de novo* DNA MTase, it appears that the same enzyme must carry out both functions.

On the basis of previous reports that single-stranded (ss) oligodeoxyribonucleotides (ODNs) have widely differing capacities to serve as substrates for murine DNA 5-C-MTase (18), we designed a series of 24-nt-long ODNs with multiple CpG sites flanked by restriction endonuclease cleavage sites to allow analysis of the extent of methylation at each site. Here, we describe the effect of replacement of specific C residues with 5MeC on the rate of methylation of CpG sites in these ODNs and present evidence that some 5MeC residues in ssDNA can direct methylation of CpG sites in cis (on the same strand).

MATERIALS AND METHODS

Synthesis of ODNs. The phosphoramidite of 5-fluorodeoxycytidine and unmodified, 5MeC- and 5-fluorocytosine (5FC)-containing ODNs were synthesized and purified as described (19). 5-Methyldeoxycytidine phosphoramidite was from Peninsula Laboratories. ss ODNs were heated to 90°C for 10 min and quickly chilled on ice immediately prior to assay. The ss

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Abbreviations: C, cytosine; 5FC, 5-fluorocytosine; 5MeC, 5-methylcytosine; 5-C-MTase, 5-cytosine methyltransferase; ODN, oligodeoxyribonucleotide; ss, single stranded; ds, double stranded; AdoMet, *S*-adenosylmethionine.

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ODNs were not substrates for *Hpa* II or *Hha* I DNA MTases at 37°C, indicating an absence of stable intermolecular ds regions. For ds ODNs, an equimolar mixture of complementary ODNs was heated to 90°C for 10 min and slowly cooled to room temperature. These ds ODNs were susceptible to quantitative cleavage by *Hpa* II or *Hha* I when their recognition sites contained no 5MeC (data not shown).

Preparation of Murine DNA 5-C-MTase. The DNA 5-C-MTase used in these studies was the 100,000 × *g* supernatant of a 0.3 M NaCl extract of Friend erythroleukemia cell nuclei. All procedures were described in ref. 20 except for the addition of 5 μg each of antipain dihydrochloride, leupeptin, chymostatin, and pepstatin (Boehringer Mannheim) to the extraction buffer.

Methylation Assay. Reaction mixtures (50 μl) in 0.1 M imidazole, pH 7.4/20 mM EDTA/0.5 mM dithiothreitol contained 0.5 μg of each ODN indicated, ≈0.6 unit of DNA 5-C-MTase (1 unit transfers 1 pmol to A·A′M_p per min; see Table 1), and 2.8 μCi (1 Ci = 37 GBq) of [*methyl*-^{3}H]AdoMet (AdoMet = *S*-adenosylmethionine) (8 μM). Substrate C sites are in excess in the reaction, and methyl transfer is linear for >45 min. For accuracy, methylation rates <5 pmol/30 min were measured in quadrupled reaction mixtures (200 μl). After incubation for 30 min at 37°C, ODNs were processed for quantitation of radiolabel as described (21) with 25 μg of salmon sperm DNA added as carrier prior to perchloric acid precipitation.

RESULTS

ODNs A and A′ (Table 1) were tested for their ability to act as substrates for methylation by murine DNA 5-C-MTase by measuring the initial rate of methyl transfer from [*methyl*-^{3}H]AdoMet to these ODNs with substrate in excess. Neither A nor A′ was efficiently methylated in the ss form, although A was methylated at almost three times the rate of A′ (Table 1). Since A and A′ have the same number and spacing of CpG sites, this indicates that the density and spacing of CpG sites are not sufficient to establish the rate of methylation. When A and A′ were annealed to form an unmethylated ds ODN substrate, the rate of methylation was no higher than that obtained with A′ alone, even though twice as many sites per mole of substrate were available for methylation.

Table 1. Comparison of the rates of methylation of ss and ds ODNs by murine DNA C-5-MTase

ODN	Initial rate, pmol/30 min	RA
Unmethylated substrates (*de novo* methylation)		
1. A	0.14 ± 0.01	1
2. A′	0.05 ± 0.01	0.35
3. A·A′	0.05 ± 0.01	0.35
Hemimethylated substrates (maintenance methylation)		
4. A·A′M_p	18.6 ± 2.6	133
5. AM_p·A′	19.2 ± 1.2	138
6. AM_p·AM_p	ND	—
7. A·A′M_x	0.05 ± 0.005	0.35
8. AM_x·A′	0.09 ± 0.01	0.64

A is 5′-ATTGCGCATTCCGGATCCGCGATC-3′; A′ is 3′-TACGCGTAAGGCCTAGGCGCTAG-5′. M_p and M_x indicate 5MeC in place of all boldfaced or italicized C residues, respectively. All assays were performed in duplicate. Values shown are the average incorporation in three assays ± SD and represent the initial rate of methylation—i.e., incorporation of [^{3}H]CH_3 into DNA, during a 30-min incubation carried out and quantitated as described in *Materials and Methods*. With hemimethylated substrates (lines 4 and 5), ≈10% of available CpG sites were methylated in 30 min. Background incorporation in the absence of substrate was <500 dpm. RA, rate of methylation relative to A; ND, not detected.

When either A or A′ in the ds ODN contained 5MeC residues in place of all C residues in CpG sites (M_p) forming hemimethylated sites, the rate of methylation of the unmethylated strand was increased >130-fold relative to the rate of methylation of A in the ss form (compare line 1 with lines 4 and 5 in Table 1). No methylation of completely methylated substrate could be detected (Table 1, line 6), and substitution of 5MeC for C in non-CpG sites (M_x) in the ds ODNs did not stimulate the rate of methylation significantly above that of completely unmethylated substrate (compare line 3 with lines 7 and 8 in Table 1). Thus, the interaction of murine DNA 5-C-MTase with A and A′ in ss and ds forms does not differ detectably from its interaction with longer ss- and dsDNA substrates; i.e., hemimethylated DNA is methylated much more efficiently than completely unmethylated ds- or ssDNA (22). The results also demonstrate that 5MeC in non-CpG sites fails to stimulate methylation of dsDNA, even when potential methylation sites (CpG sites) are no more than one or two base pairs distant.

In contrast, substitution of a 5MeC residue(s) for a C residue(s) in different sites in ss ODNs had widely varying effects on the rate of methylation (Fig. 1). 5MeC in all CpG sites in either A or A′ (ODN A and A′, lines 2 in Fig. 1) effectively blocked methyl transfer, which is predictable if all methylation occurs at C residues in CpG sites. However, substitution of 5MeC for all C residues next to other nucleotides had a markedly different effect on methylation of ss and ds ODNs. The rates of methylation of ss ODNs A and A′ increased dramatically, approaching or surpassing those of hemimethylated ds ODNs (compare lines 4 and 5 in Table 1 with line 3 in Fig. 1); again A was the better substrate (Fig. 1, lines 3). Since 5MeC substituted for C in non-CpG sites had no

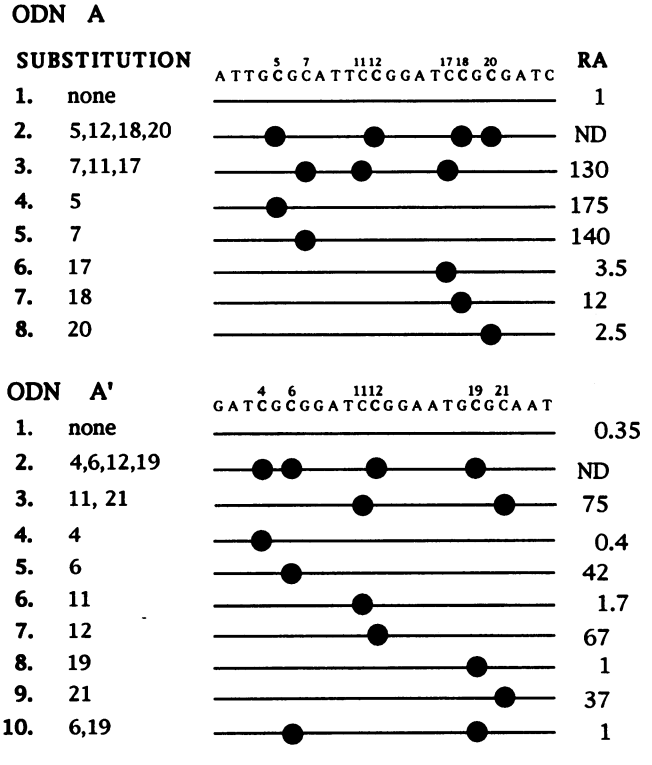

FIG. 1. Effect of substitution of 5MeC for C in specific sites on the rate of methylation of ss ODNs. The sequences of ODNs A and A′ are shown above histograms indicating the sites of substitution of 5MeC (●). All details are as in Table 1. RA, rate of methylation relative to A (0.15 ± 0.01 pmol of [*methyl*-^{3}H]AdoMet per 30 min); ND, not detectable.

effect on methylation rates of ds ODNs (Table 1, lines 7 and 8), this suggests that 5MeC in ssDNA can stimulate methylation of CpG sites in cis.

To determine which specific 5MeC residues stimulate methylation, derivatives of A and A′ were synthesized with single substitutions of 5MeC for C. A single 5MeC near the 5′ end of ODN A, replacing either the C residue in position 5 (C5) or position 7 (C7), stimulated methylation as effectively as the three 5MeC residues replacing C7, C11, and C17 of ODN A (Fig. 1, compare lines 3–5; ODN A). In contrast, A strands with 5MeC residues substituted for C residues in other sites were only marginally better substrates than unmethylated A strands. The exception was a 5MeC in position 18, which increased the rate of methylation of A ≈12-fold (Fig. 1, line 7; ODN A). These results confirmed that 5MeC residues in ss ODN substrates do not have to be in a CpG site to activate methylation. Introduction of 5MeC residues into A′ demonstrated that the presence of a 5MeC residue near the 5′ end of an ODN is not in itself sufficient to increase the initial rate of methylation (Fig. 1, line 4; ODN A′) and that 5MeC residues in the middle (lines 5 and 7; ODN A′) or at the 3′ end (line 9; ODN A′) of an ODN can also activate methylation. Clearly, factors other than density of 5MeC residues or their position relative to the 5′ end are important in determining which 5MeC residues can serve as activators of methylation.

Substitution of C residues with 5FC was used to determine which sites become substrates for enzymatic methylation in A strands containing 5MeC. It has been shown that both bacterial and mammalian DNA 5-C-MTases form stable covalent linkages with 5FC residues in DNA during the process of methylation (23–27). Under conditions of substrate excess, this leads to rapid inactivation of the enzymes. We have found that (*i*) stable covalent complexes between murine DNA 5-C-MTase and 5FC residues are only formed when 5FC residues are in substrate CpG sites, and (*ii*) our DNA MTase extracts contain only one species of protein (≈190 kDa) that forms covalent complexes in an AdoMet-dependent manner with ODNs with 5FC in substrate sites (ref. 27 and unpublished data). 5FC substitution for C residues in all CpG sites (C residues at positions 5, 12, 18, and 20) of AM_x completely inhibited methylation, reconfirming that C residues in CpG dinucleotides are substrates (Fig. 2, line 3). Single substitutions of 5FC for C5, C12, and C20 had little effect on the rate of methylation of AM_x, whereas substitution of 5FC for C18 almost completely inhibited methylation (Fig. 2, line 6). The

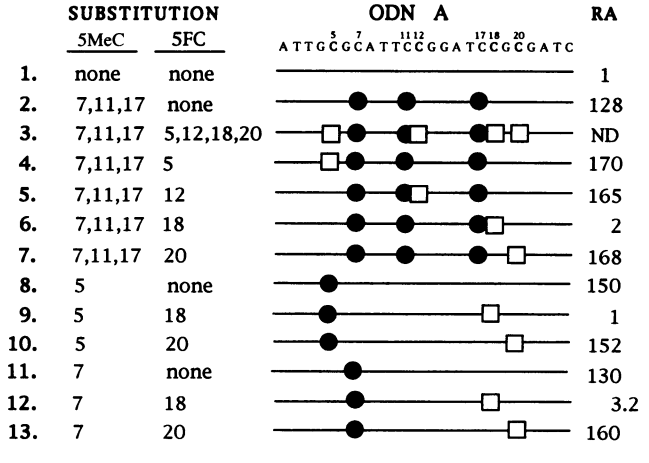

FIG. 2. Localization of substrate C residues in methylated ss ODNs through substitution of C residues with 5FC residues. Inhibition of methylation occurs only in those ODNs where 5FC is in a substrate site (27). ODNs were synthesized with 5MeC (●) or 5FC (□) in the indicated positions. All details are as in Table 1. RA, rate of methylation relative to A (0.15 ± 0.01 pmol of [*methyl*-^{3}H]AdoMet per 30 min); ND, not detectable.

same result was obtained with A ODNs containing a single 5MeC in position 5 or 7; i.e., 5FC in position 18 completely inhibited methylation while 5FC in position 20 had no effect on the rate of methylation (Fig. 2, lines 9, 10, 12, and 13). Thus, 5MeC in position 5 or 7 activates DNA 5-C-MTase to methylate C18 while failing to activate methylation of a C residue only two bases downstream. Since the distance between C5 and C18 and between C7 and C20 is the same, this result suggests a very specific relationship between DNA structure and/or sequence and the recognition of substrate sites in ssDNA that is not strictly related to distance between sites. It can also be concluded (*i*) that 5MeC residues can activate methylation of both ss- and dsDNA and that in both cases the substrate C residue is in a CpG site; (*ii*) that in completely dsDNA, 5MeC residues must be located in CpG sites either to block the use of DNA as a substrate or to activate methylation (Table 1); and (*iii*) that in ssDNA, 5MeC residues must be in CpG sites to block methylation (compare ODN A′, lines 5 and 10 and ODN A and A′, lines 2 in Fig. 1) but not to serve as activators of methylation.

Smith *et al.* (28, 29) have previously noted that in dsDNA substrates or in ssDNAs where the substrate CpG site is present in the ds stem of a long-stemmed stem–loop structure, the substrate C residue for mammalian DNA 5-C-MTase must be in a CpG site but need not be base paired to a G residue in the complementary strand (Fig. 3*A*). A search for similar recognition sites in potential inter- and intramolecular hydrogen-bonded structures that can be formed by ODN A was made using the STEMLOOP function of the Genetics Computer Group package (Version 7.3.1-UNIX; September 1993) and the self-complementarity function of OLIGO (Version 3.4; National Biosciences, Hamel, MN). The structures generated by these programs indicated that ODN A could form a homodimer with 32 hydrogen bonds (Fig. 3*B*, structure 1) and a variety of stem–loop structures. Of the six potential stem–loops stabilized by at least 3 adjacent base pairs, only those with more than 9 hydrogen bonds or those with C7 positioned to direct methylation at C18 or C20 are shown (Fig. 3*B*, structures 2–5). Neither 5MeC in position 5 nor 5MeC in position 7 is in a base-paired region in homodimer structure 1 or stem–loop structures 3 and 4. Structure 2, which contains a 10-bp-long stem stabilized by 19 hydrogen bonds has a base-paired CpG site in the stem. When 5MeC is substituted for C5 in this site, a hemimethylated recognition site for DNA 5-C-MTase is formed with C18. No methylation of the C20 in structure 2 would be predicted, since C20G21 is paired with T3G4 and, thus, is not in a recognition site. Theoretically, a 5MeC in position 18 in structure 2 or 3 could activate methylation at C5 or C12, respectively. In this regard it is of interest that 5MeC in position 18 does activate methylation of A, although with less efficiency than 5MeC in position 5 or 7. Structures 1–4 do not, however, explain how 5MeC in position 7 can activate methylation at C18. Structure 5 is the only stem–loop found in which C18 is positioned in a substrate recognition site containing 5MeC in position 7. It has a 3-bp-long stem, with only 9 hydrogen bonds, and has the substrate C18 in a non-base-paired position. X-ray diffraction studies of the structure of DNA in the active site of a bacterial DNA 5-C-MTase (HhaI) have recently been reported (30). They demonstrate that hydrogen bonds between the substrate C residue and the G residue in the complementary strand are broken; the C residue is swung out of the helix, allowing methylation to occur. This may explain why substrate C residues that cannot be base paired are particularly good methyl acceptors, since their rotation out of the helix requires less energy than rotation of normally hydrogen-bonded C residues. In this regard, it should be noted that 5MeC in position 5 in structure 5 could potentially direct methylation at C20. However, no catalytic interaction was detected between murine DNA 5-C-MTase and A with 5MeC in position 5 and

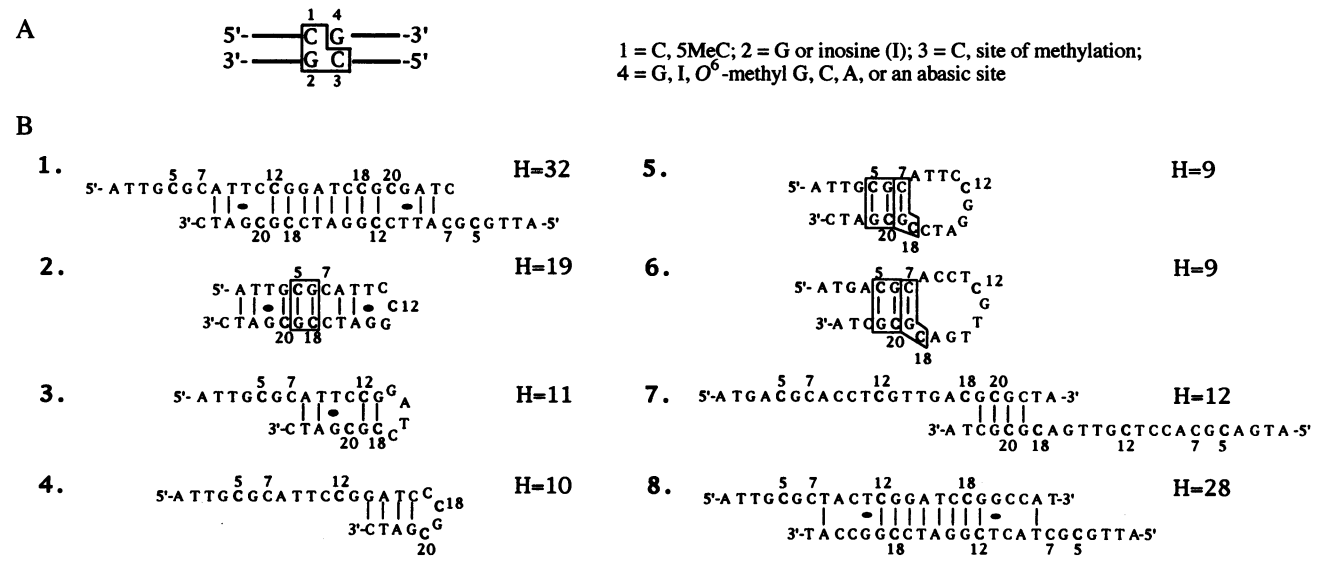

FIG. 3. Analysis of ODN A and its analogs for DNA 5-C-MTase recognition sites including either C5 or C7. (*A*) Diagrammatic representation of the three-nucleotide recognition motif of mammalian DNA 5-C-MTase postulated by Smith *et al.* (29). In hemimethylated DNA, site 1 is 5MeC and site 3 is the substrate C residue. (*B*) Potential homodimeric and stem–loop structures formed by ODN A and its analogs. The number of hydrogen bonds (H) stabilizing each structure is indicated and assumes that a single hydrogen bond (●) can be formed between T and G residues. Recognition sites that include C5 or C7 and C18 or C20 are boxed.

5FC in position 20 (Fig. 2, line 10). This suggests a preferential binding of the MTase to the "mismatched" recognition site formed by hydrogen bonding between 5MeC in position 7 and G19 over the fully base-paired recognition site formed by hydrogen bonding between 5MeC5G6 and C20G2.

Although we do not have direct evidence for formation of these looped structures and cannot rule out the possibility that the computer algorithms used failed to detect some potential substrate sites with non-Watson–Crick pairing, the structures in Fig. 3 allowed design of additional ODNs to test the hypothesis that formation of a loop with a mismatched recognition site is necessary for 5MeC in position 7 to direct methylation at C18. Two ODNs with approximately the same base composition, a 5MeC in position 7, and the same number and spacing of CpG sites as ODN A were synthesized. One ODN (Fig. 3*B*, structure 6) forms a stem–loop of the same size with the same relationship between C7 and C18 as A (structure 5). It can also form a homodimer that is stabilized by 12 hydrogen bonds (structure 7) with the substrate C18 adjacent to a ds region but not in a hemimethylated site. The other ODN forms a homodimer with 28 hydrogen bonds (structure 8) and five stem–loop structures stabilized by at least three adjacent hydrogen-bonded base pairs. C7 is not base-paired in any of these structures. However, it cannot form a stem–loop structure analogous to structures 5 and 6. This ODN is methylated at the same rate as unmethylated A, whereas the ODN that can potentially form structure 6 is methylated at >300 times the rate of unmethylated A.

DISCUSSION

Since little or no methylation of CpG sites (including C18) occurs when they are in an unmethylated ds ODN (A·A′) or a ds ODN containing 5MeC in non-CpG sites including C7 (Table 1, lines 3, 7, and 8), it can be concluded from the data presented here that a stem–loop structure may be both necessary and sufficient to allow 5MeC in position 7 to direct methylation at a site 12 bases downstream. However, it is unlikely that such a stem–loop structure containing only nine hydrogen bonds would exist in solution at 37°C unless it is stabilized by its interaction with DNA 5-C-MTase, perhaps with the aid of other nuclear proteins present in our extracts.

The model proposed in Fig. 4 is consistent with our results. This model posits that the active site in mammalian DNA 5-C-MTase contains both a regulatory region and a catalytic site. The regulatory site limits the rate of methyl transfer at the catalytic site. Interaction between 5MeC and the regulatory region relieves this inhibition, leading to an increased rate of methylation of a substrate C residue. In dsDNA, activation occurs primarily at hemimethylated recognition sites in which the substrate C need not be hydrogen bonded to a G in the complementary strand (29). Our results indicate that 5MeC in a looped ssDNA can also activate methylation of a substrate C residue in instances that allow DNA to form a structure in the active site analogous to the recognition sites for DNA 5-C-MTase in dsDNA (Fig. 3*B*, structures 2, 5, and 6). Based on this model, our results would further suggest that a short ds region of 3 bp including a base pair between the 5MeC residue and the G residue in the substrate CpG site is sufficient to activate

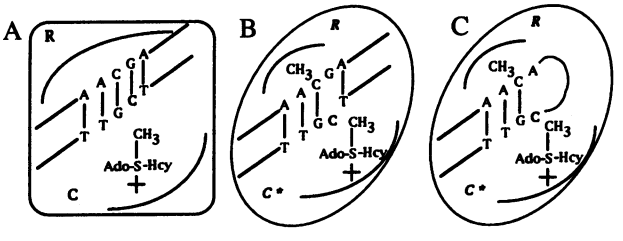

FIG. 4. Model for activation of DNA 5-C-MTase by interaction with different ODN substrates. (*A*) An unmethylated ds ODN occupies the substrate binding (recognition) site. Because there is no interaction between a 5MeC residue in the substrate and the regulatory region (R), methylation (C) proceeds inefficiently. (*B*) A methylated ds ODN occupies the substrate-binding site. Interaction between the 5MeC in the recognition motif and the regulatory region leads to activation of the catalytic function of the enzyme (C*). The ODN is depicted with the substrate C residue (C) rotated out of the helix. (*C*) A methylated ss ODN occupies the substrate-binding site and is stabilized by its interaction with the DNA MTase into a structure analogous to that of a ds ODN with the substrate C rotated out of the helix. The model suggests that interaction between 5MeC and the regulatory region mediates an allosteric change in the active site of the enzyme to optimize methyl transfer. There is as yet no experimental data supporting this mechanism. Hcy, homocysteine.

methylation but only when the C to be methylated is *not* hydrogen bonded to a G.

If one assumes that ss regions in larger DNA molecules can form similar looped structures in the active site of DNA MTase, it is evident that this mechanism could account for methylation of CpG sites at some distance from an established methylation site. Methylation would occur in cis through formation of recognition sites in stem–loops and might also occur in trans when ss loops from different DNA molecules or regions are brought close enough in the nucleus to form recognition sites. ss regions in DNA occur during the course of normal DNA replication and repair and may also be available as a result of "melting out" of DNA regions through protein binding or through formation of cruciform structures (31–33). When a ss region is converted back to dsDNA, through reannealing with its complement or through replication, a hemimethylated recognition site is formed, which is then a substrate for maintenance methylation. While the proposed mechanism is supported by observations that *de novo* methylation of integrated viral genomes or repeat elements can spread from a founder site (34–36), further studies will be required to confirm that 5MeC in ss regions of DNA can actually activate methylation at distant sites in DNA of living cells. It will also be of interest to examine the possibility that the small percentage of 5MeC residues that are not in CpG sites in mammalian DNA influence the extent of methylation at adjacent CpG sites and to determine whether these 5MeC residues indicate the existence of a directive mechanism for *de novo* methylation mediated by additional DNA MTases or simply random errors introduced by normal DNA 5-C-MTase with relaxed specificity due to posttranslational modification or partial proteolytic degradation.

In summary, our results provide evidence for a mechanism whereby the single DNA 5-C-MTase found in mammalian cells can be as active in directing *de novo* methylation as it is in maintaining established patterns of methylation. They also suggest a rationale for hypothesizing that the specificity required for establishing tissue-specific patterns of methylation is determined by a combination of inherent factors that include the ability of particular sequences to form the required stem–loop structures in ssDNA, the lifetime of the single-stranded state, and the availability of proteins to stabilize or destabilize particular looped structures.

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