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Primary DNA Sequence Determines Sites of Maintenance and De Novo Methylation by Mammalian DNA Methyltransferases

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Analysis of the enzymatic methylation of oligodeoxynucleotides containing multiple C-G groups showed that hemimethylated sites in duplex oligomers are not significantly methylated by human or murine DNA methyltransferase unless those sites are capable of being methylated de novo in the single- or double-stranded oligomers. Thus, the primary sequence of the target strand, rather than the methylation pattern of the complementary strand, determines maintenance methylation. This suggests that de novo and maintenance methylation are the same process catalyzed by the same enzyme. In addition, the study revealed that complementary strands of oligodeoxynucleotides are methylated at different rates and in different patterns. Both primary DNA sequence and the spacing between C-G groups seem important since in one case studied, maximal methylation required a specific spacing of 13 to 17 nucleotides between C-G pairs.

Isolated mammalian DNA methyltransferases can transfer a methyl group from *S*-adenosylmethionine (AdoMet) to the 5′ position of a cytosine located in a C-G pair in DNA. These enzymes will methylate single-stranded or double-stranded DNA at previously unmethylated C-G sites, in a de novo reaction (13, 15, 16). The same enzymes will also methylate hemimethylated sites in duplex DNA at rates that are 10 to 30 times the rate of de novo methylation (2, 5, 7, 13). Methylation of hemimethylated sites is considered to be important in maintaining genomic methylation patterns in newly replicated DNA (6, 14). The current mechanism of inheritance of methylation patterns from parent to daughter cells is based on the assumption that methylated sites on both strands of DNA are conserved by maintenance methylation at the hemimethylated sites which arise during semiconservative replication.

Certain single-stranded oligodeoxynucleotides containing multiple copies of the dinucleotide C-G have been shown to be suitable substrates for the DNA methyltransferases in vitro. In the one example analyzed, the location of a C-G group and the primary base sequence surrounding it apparently determined the availability of that site for methylation (3). We now report the effect of positioning and spacing of C-G sites on the methylation of unmethylated single- and double-stranded oligodeoxynucleotides (de novo methylation) and on the methylation of the unmethylated strand in a hemimethylated duplex (maintenance methylation).

MATERIALS AND METHODS

Materials. [^{3}H]methyl-AdoMet was purchased from Amersham Corp. (Arlington Heights, Ill.). AdoMet was from Sigma Chemical Co. (St. Louis, Mo.). The restriction enzymes *Hae*III and *Sau*3A-I were purchased from New England BioLabs, Inc. (Beverly, Mass.). Proteinase K was from E. Merck AG (Darmstadt, Federal Republic of Germany).

Purification of DNA methyltransferases. DNA methyltransferases were prepared from the nuclei of HeLa S3 and Friend mouse erythroleukemia (MEL) cells by the procedure outlined by Bolden et al. (2). The DNA-agarose-purified enzymes, which are free of any detectable DNase activity, were used in all experiments presented in this study. Their specific activities were 50,000 U/mg (HeLa) and 42,500 U/mg (MEL). The DNA methyltransferase assay has been previously described (2). All the assays reported in these studies were linear with respect to time for at least 6 h. With the levels of enzymes used the oligonucleotide concentrations were saturating at 1 to 3 μg/ml.

Synthesis of oligodeoxyribonucleotides. All oligomers were synthesized by using the nucleoside phosphoramidite intermediates and the solid support methodology outlined by Beaucage and Caruthers (1). Oligonucleotides prepared by this method have free 3′ and 5′ hydroxyl groups.

Methylation of 27mer-F′/12mer-F′. A mixture containing 500 ng of 5′-TCGACCCCCCCCCCCCCCCGGGTCTAG (27mer-F) and 225 ng of 5′-CTAGACCCGGGG (12mer-F′) in 25 μl of 10 mM Tris hydrochloride (pH 7.5)–400 mM NaCl was incubated at 75°C for 10 min and slowly cooled to room temperature to allow the oligodeoxynucleotides to anneal. The mixture was then diluted 10-fold into assay medium containing 16 μM [^{3}H]methyl-AdoMet (containing 6,000 cpm/pmol) and HeLa or MEL methyltransferase. Incubation was for 5 h at 37°C. After extraction of the protein with phenol-chloroform (1:1, vol/vol), the aqueous phase containing the double-stranded oligonucleotide was purified on a Sephadex G-25 column. The strands were separated on a 20% polyacrylamide gel containing 7 M urea.

Determination of methylation sites. The oligodeoxynucleotides 5′-CCGGCCATTACGGATCCGTCCTG GGC (26mer-C) and 5′-GCCCAGGACGGATCCGTAATG GCCGGA (26mer-C′) (400 ng) or the duplexed 26mer-C/26mer-C′ (800 ng) were methylated in the presence of 53 U of the partially purified HeLa DNA methyltransferase in a volume of 200 μl. The specific activity of [^{3}H]methyl-AdoMet was 4,400 to 4,800 cpm/pmol. After incubation, the reaction mixtures were adjusted to 10 mM EDTA and 0.2% sodium dodecyl sulfate and digested with 200 μg of proteinase K per ml for 30 min at 37°C. After extraction with phenol-chloroform, the [^{3}H]methyl-labeled oligomers were precipitated from the aqueous phase with ethanol.

Purified [^{3}H]methyl-labeled 26mer-C and 26mer-C′ (360 ng

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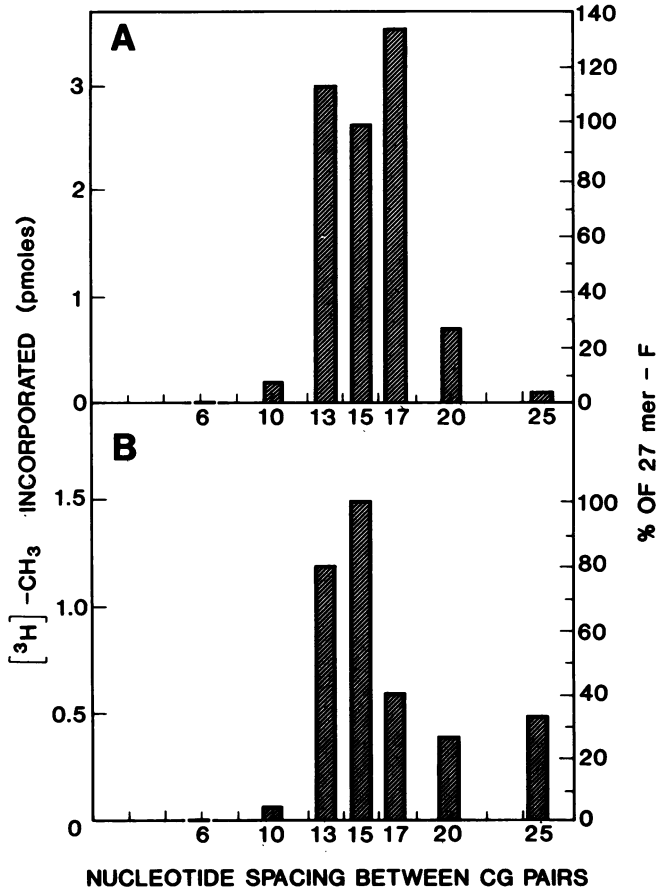

FIG. 1. Methylation of derivatives of 27mer-F. Assays (50 μl) containing 3 μg of oligodeoxynucleotide per ml and 180 U of either HeLa (A) or MEL (B) DNA methyltransferase per ml were performed for 3 h at 37°C under conditions described in Materials and Methods. The data are presented as picomoles of [^{3}H]methyl incorporated per 120 ng of oligodeoxynucleotides.

each) were annealed with their respective nonradiolabeled complementary strands at a molar ratio of 1:1 in 0.4 M NaCl–25 mM Tris hydrochloride (pH 7.5). The duplexes, at concentrations of 2 to 6 μg/ml, were subjected to double digestion by *Sau*3A-I and *Hae*III in the presence of a large excess of the enzymes (40 U/μg of oligomer). After extraction of the reaction mixtures with phenol-chloroform and incubation at 100°C for 5 min, 70 to 100 ng of the digests was loaded on an 18.5% polyacrylamide–7 M urea gel (10). After electrophoresis, the lanes were excised, sliced into 2-mm sections, and digested at 50°C for 16 h in NCS tissue solubilizer (Amersham Corp.)-H_2O mixture (9:1, vol/vol). ^{3}H counts were determined in ReadySolv (Beckman Instruments, Inc., Fullerton, Calif.) containing 35 mM acetic acid.

Methyl-26mer-C'/26mer-C and methyl-26mer-C/26mer-C' duplexes were prepared by first methylating 400 ng of either 26mer-C' or 26mer-C oligomers in the presence of 58 U of the partially purified HeLa DNA methyltransferase in a volume of 200 μl. The partially methylated oligomers were purified and annealed with equimolar amounts of their unmethylated complementary strands, as described above. The resultant hemimethylated duplexes were methylated again with [^{3}H]methyl-AdoMet in the presence of 290 U of the partially purified HeLa DNA methyltransferase per ml for 5 h at 37°C. After methylation, the duplexes were purified and *Sau*3A-I–*Hae*III digested, and the resultant restriction fragments were analyzed on 18.5% polyacrylamide–7 M urea gels.

RESULTS

Methylation of single-stranded oligodeoxynucleotides. HeLa DNA methyltransferase will methylate the oligodeoxynucleotide 5'-TCGACCCCCCCCCCCCCCG GGTCTAG (27mer-F) at position 19 (3). The methylation requires the 5' C-G group at position 2,3 which itself is hardly methylated. It became of interest to determine whether the spacing between the two C-G moieties could affect the methylation rate. For this purpose, a series of derivatives of 27mer-F were synthesized which contained 5, 9, 12, 16, 19, and 24 intervening C residues between the 5' TCGA and the C-G pair downstream. Together with the parental 27mer-F, this yielded a series of compounds in which the total nucleotide spacing between the two C-G groups was 6, 10, 13, 15, 17, 20, and 25 nucleotides. The ability of these compounds to act as substrates for the HeLa DNA methyltransferase is shown in Fig. 1A. The maximal rate of methylation was observed when the distance between the two C-G groups was 13 to 17 nucleotides. Little methylation was seen when the spacing between the C-G pairs was 10 nucleotides, and none when the spacing was 6 nucleotides. An intervening sequence greater than 17 nucleotides also diminished the enzymatic methylation process. Similarly, MEL methyltransferase also showed a preference for substrates having a spacing of 13 to 15 nucleotides (Fig. 1B). Molecules having a shorter spacing were not methylated, although a 37mer containing 25 nucleotides between the two C-G groups could be methylated to a significant extent. We conclude from these data that these two DNA methyltransferases bind to or recognize the C-G group closest to the 5' end of the oligodeoxynucleotide and then methylate a downstream C-G site located a proper distance away. For the 27mer-F series, at least, the optimum spacing between C-G sites is approximately 13 to 17 nucleotides.

To examine further the effect that positioning and spacing of C-G groups has on the substrate specificity for the methylation reaction, we tested a compound containing three C-G groups and its complementary strand. This compound, 26mer-C, has the sequence 5'-CCGGCCATTACG GATCCGTCCTGGGC and is an excellent substrate for either the HeLa methyltransferase (Table 1) (3) or the MEL methyltransferase (Table 1). The complement of 26mer-C, 26mer-C' (containing an extra A residue at the 3' end to facilitate subsequent polyacrylamide gel analyses), was only 15 to 20% as good an acceptor. Table 1 also shows that the complement of 27mer-F (27mer-F') had no activity as a methyl acceptor. The significance of the methyl-accepting ability of complementary strands of these small DNAs will be discussed later.

The availability of the complementary strand of 26mer-C permitted us to analyze the pattern of methylation obtained when 26mer-C was methylated by either the HeLa or MEL methyltransferase. The strategy for this is similar to that used previously to determine the methylation pattern of 27mer-F (3). The oligomer was first methylated by the methyltransferase with [^{3}H]methyl-AdoMet. The radiolabeled strand was annealed with its nonradioactive complement to form a double-stranded molecule and cleaved with appropriate restriction endonucleases. Cleavage of the 26mer-C duplex with *Hae*III and *Sau*3A-I led to six different restriction fragments of 4, 5, 8, 10, 12, and 14 bases in length after denaturation (Fig. 2). Three of these (4, 8, and 14) were

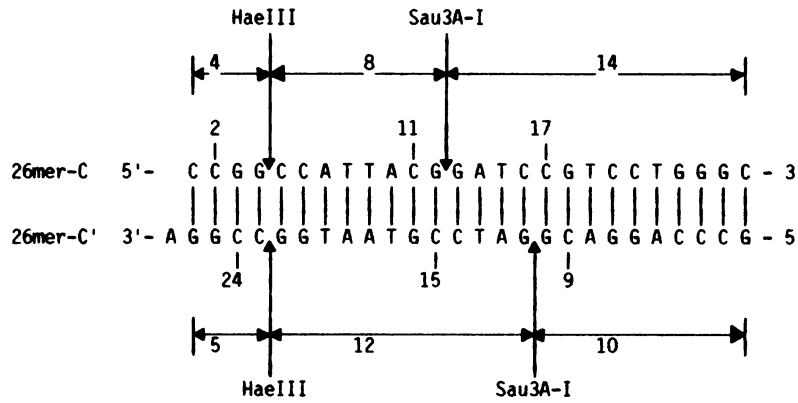

FIG. 2. *Hae*III–*Sau*3A-I restriction digestion pattern for 26mer-C/26mer-C′ duplex. The restriction site for *Hae*III is GGCC and that for *Sau*3A-I is GATC.

derived from the 26mer-C strand, and the other three (5, 10, and 12) were derived from the complementary 26mer-C′ strand. These fragments were separated by polyacrylamide gel electrophoresis, and the distribution of [^{3}H]methyl in the fragments was analyzed (Fig. 3). Restriction of methylated 26mer-C led to radioactivity in the 8mer and 14mer, whereas restriction of methylated 26mer-C′ led to [^{3}H]methyl only in the 12mer. The small amount of radioactivity found in fragments larger than the 14mer reflects incomplete digestion by the restriction endonucleases. A summary of these data is shown in Table 2. It is clear that 26mer-C is methylated at positions 11 and 17 and that 26mer-C′ is methylated only at position 15. In both cases the C-G group which is closest to the 5′ end of the molecule is not methylated. Although the data shown in Table 2 and Fig. 3 were obtained with the HeLa methyltransferase, the same patterns of methylation were obtained with the MEL methyltransferase (data not shown).

Methylation of double-stranded oligodeoxynucleotides. It is important to know whether the double-stranded forms of these oligomers are methylated in the same patterns as are the single-stranded molecules. Moreover, the results presented in Tables 1 and 2 raise the question of why the single-stranded complementary strands of 27mer-F and 26mer-C are methylated at different rates and in different patterns than 27mer-F and 26mer-C. To this end, we prepared the double-stranded oligomers by annealing the complementary pairs and tested them as substrates for the HeLa or MEL methyltransferase.

Although 27mer-F was an effective substrate for the HeLa and MEL methyltransferases, its complementary strand was not methylated. The duplex formed between the two strands was not a substrate for the HeLa enzyme but could be slowly methylated by the MEL enzyme (Table 3). Of greater interest was the observation that a hemimethylated duplex formed between the methylated 27mer-F and a nonmethylated complementary strand was a poor substrate for both the HeLa and MEL enzymes. These results demonstrate that a hemimethylated site need not be an efficient substrate for the methyltransferases. The inability of the 27mer-F duplex to accept methyl groups may be due in part to the stretch of 15 G residues in the 27mer-F complement. In a control experiment, we found that the 27mer-F complement, at the concentrations used in these experiments (2 μg/ml), partially inhibited (70%) the methylation of hemimethylated ϕX174 DNA by the HeLa methyltransferase (data not shown). Oligo-$dG_{(12-18)}$ at a concentration of 10 μg/ml has been found to partially inhibit the HeLa DNA methyltransferase (2). However, the observed inhibition by 27mer-F′ cannot fully explain the inability of the 27mer-F duplex to be methylated. To further test this point, we prepared a partial complement of 27mer-F which covered the 3′ part of 27mer-F, including the C-G group at position 19,20. This molecule, 5′-CTAGACCCGGGG (12mer-F′), was not methylated by the HeLa or MEL methyltransferase (Table 3, line 6). When annealed to 27mer-F, the resulting partial duplex was also a poor substrate for both enzymes relative to the single-stranded 27mer-F (Table 3, line 7). Analysis of the separate strands for methyl incorporation revealed that approximately 80% of the counts incorporated by the HeLa enzyme were in the 27mer-F strand. The MEL methyltransferase could also methylate 27mer-F/12mer-F′ oligomeric duplex

TABLE 1. Methylation of complementary single-stranded oligodeoxynucleotides[a]

Substrate	DNA methyltransferase			
	HeLa		MEL	
	pmol of [^{3}H]methyl incorporated	Relative activity	pmol of [^{3}H]methyl incorporated	Relative activity
27mer-F (5′-TCGACCCCCCCCCCCCCCCCGGGTCTAG)	6.5	100	8.2	100
27mer-F′ (5′-CTAGACCCGGGGGGGGGGGGGGTCGA)	0	0	0.1	1
26mer-C (5′-CCGGCCATTACGGATCCGTCCTGGGC)	7.5	100	19.7	100
26mer-C′ (5′-GCCCAGGACGGATCCGTAATGGCCGGA)	1.5	20	3.2	16

[a] Methylation of the single-stranded oligodeoxynucleotides was carried out as described in Materials and Methods. The concentration of oligodeoxynucleotides in each assay was 2 μg/ml. The reactions with the HeLa enzyme contained 130 U of enzyme per ml and were carried out for 5 h at 37°C; those with the MEL enzyme contained 230 U of enzyme per ml and were carried out for 3 h at 37°C.

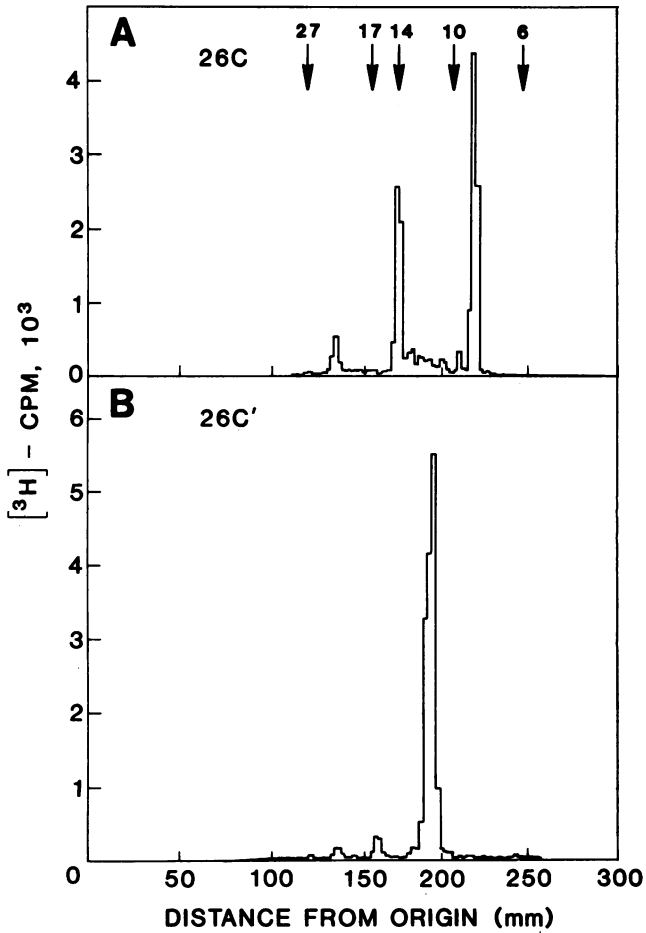

FIG. 3. Polyacrylamide gel analysis of *Sau*3A-I–*Hae*III restriction digests of ^{3}H-methylated 26mer-C and 26mer-C'. The enzymatic methylation, restriction enzyme digestion, and polyacrylamide gel electrophoresis are described in Materials and Methods. (A) The recovery of counts per minute placed on the gel (32,500 cpm) was 53%. Of the recovered ^{3}H counts, 8% migrated as uncut duplex and partially digested molecules. (B) The recovery of counts per minute placed on the gel (28,128 cpm) was 63%. Of the recovered ^{3}H counts, 9% migrated as uncut duplex and partially digested molecules. The migration of marker oligonucleotides in the gel is denoted with vertical arrows.

(Table 3, line 7) with more than 90% of the [^{3}H]methyl incorporated in the 27mer-F strand (data not shown). It should be noted that the incorporation of methyl groups into 12mer-F' in this experiment represents only a small percentage of the total methylation that can occur in the duplex. These experiments indicate that the methylation of hemimethylated sites as well as sites on single- or double-stranded DNA may be modulated by the DNA sequence surrounding the site.

The influence of the position of C-G sites within the DNA molecule upon maintenance methylation is further illustrated with 26mer-C and its complement. 26mer-C is methylated at two of the three C-G groups, whereas its complementary strand is methylated only at the middle C-G (position 15,16) (Table 2). Table 4 (lines 1, 2, and 3) shows the amount of [^{3}H]methyl incorporated into 26mer-C, 26mer-C', and a duplex of the two complementary strands. It is worth noting that, at the relatively high enzyme levels used in these experiments, 76% of the two available C-G pairs in 26mer-C were methylated, indicating that 50% or more of the 26mer-C had two methyl groups incorporated into the same molecule. The 26mer-C/26mer-C' duplex was methylated at a rate intermediate between that of 26mer-C and 26mer-C', and the incorporated methyl groups were found to be located at positions 11 and 17 of the 26mer-C strand and only at position 15 of the 26mer-C' strand. Thus, the methylated 26mer-C/26mer-C' duplex contains one fully unmethylated C-G duplex at position 2 of the 26mer-C, a fully methylated C-G duplex at position 11 of 26mer-C, and a hemimethylated site at position 17 of 26mer-C.

Methylation of hemimethylated 26mer-C. The pattern of methylation obtained with the duplex 26mer-C/26mer-C' oligomer was identical to that obtained when each of the strands was independently methylated by the HeLa methyltransferase. This led to the formation of a hemimethylated site that was not further methylated by the enzyme despite the high levels of enzyme used in these 5-h incubations. To confirm this observation, we prepared duplexes which contained either an enzymatically methylated 26mer-C molecule duplexed to a nonmethylated 26mer-C' strand or, conversely, a methylated 26mer-C' molecule duplexed to a 26mer-C strand (Table 4, lines 4 and 5). The hemimethylated duplexes were reincubated with the HeLa enzyme and [^{3}H]methyl-AdoMet, and the newly incorporated methyl groups were located by restriction analysis. Table 4 (lines 4 and 5) shows that a hemimethylated duplex containing methyl groups at positions 11 and 17 of 26mer-C was further methylated only at position 15 of the 26mer-C' strand. These data confirm that position 9 of 26mer-C' cannot be methylated in either a hemimethylated or a nonmethylated duplex structure. The converse hemimethylated duplex that initially contained a methyl group at position 15 of the 26mer-C' strand was only methylated at positions 11 and 17 of the 26mer-C. Methylation of a hemimethylated site located at position 9 of 26mer-C' (the complementary C to the methylated C at position 17 of 26mer-C) was not observed. This shows again that methylation occurs only at sites that are targets for de novo methylation on the single-stranded oligomers and is consistent with the inability of the enzymes to efficiently methylate a hemimethylated site as demonstrated in the previous section with 27mer-F.

DISCUSSION

Evidence that the level of methylation of specific CpG sites located near the 5' end of certain genes can block active expression of these genes has been demonstrated in a few diverse systems (4, 8, 9, 17). In these studies, the state of

TABLE 2. Distribution of [^{3}H]methyl radioactivity in 26mer-C and 26mer-C' oligodeoxynucleotides[a]

DNA	Restriction fragments			
	Length	cpm	% of total radioactivity recovered	Position of C (base no.)
[^{3}H]methyl-26mer-C	14	6,006	35	17
	8	7,907	46	11
	4	0	0	2
[^{3}H]methyl-26mer-C'	12	14,457	82	15
	10	0	0	9
	5	92	0.4	24

[a] The preparation of [^{3}H]methyl-labeled 26mer-C and 26mer-C' and the polyacrylamide gel analysis are described in Materials and Methods and the legend to Fig. 2.

TABLE 3. Methylation of single- and double-stranded 27mer-F[a]

Substrate	Methyltransferase activity (pmol of [^{3}H]methyl incorporated)	
	HeLa[b]	MEL[c]
1. 27mer-F	6.5	8.2
2. 27mer-F′	0	0.1
3. 27mer-F/27mer-F′ 5′-TCGACCCCCCCCCCCCGGGTCTAG 3′-AGCTGGGGGGGGGGGGGCCCAGATC	0	0.4
4. Methyl-27mer-F/27mer-F′ CH_3 5′-TCGACCCCCCCCCCCCCGGGTCTAG 3′-AGCTGGGGGGGGGGGGGCCCAGATC	0.8	1.0
5. 27mer-F	4.3	4.6
6. 12mer-F′ (5′-CTAGACCCGGGG)	0	0
7. 27mer-F/12mer-F′ 5′-TCGACCCCCCCCCCCCGGGTCTAG GGGGCCCAGATC-5′	1.5	2.6

[a] The experimental details are listed in Materials and Methods.
[b] The enzyme concentration was 130 U/ml.
[c] The enzyme concentration was 180 U/ml.

methylation of specific CpG sites within the globin, adenine phosphoribosyltransferase, and adenovirus genes correlated with expression of these genes, demonstrating the importance of the methylation pattern in vivo. On the other hand, the work of Orlofsky and Chasin (11) and Ott et al. (12) on the albumin gene of rat hepatoma cells has suggested that the perpetuation of a heritable phenotype does not rely solely on maintenance methylation but may involve de novo methylation at specific CpG sites within a bounded domain.

It has been postulated that postreplicative methylation of the newly synthesized DNA strand in the cell requires a specific DNA methyltransferase that recognizes the pattern of methyl-C on the parent strand as the determinant for maintenance methylation (6, 14). This enzyme is thought to scan for hemimethylated sites on the DNA after replication and to transfer a methyl group from AdoMet to the complementary C on the newly synthesized daughter strand. In this manner, the methylation pattern of the parent genome would be conserved in subsequent generations. These authors have also suggested that another DNA methyltransferase is needed to catalyze methylation at completely unmethylated sites. However, the evidence available to date suggests that

TABLE 4. Distribution of [^{3}H]methyl radioactivity in single- and double-stranded 26mer-C and 26mer-C′[a]

Substrate	Total pmol of [^{3}H]methyl incorporated	pmol of [^{3}H]methyl incorporated into each C-G group					
		26mer-C			26mer-C′		
		Position 2	Position 11	Position 17	Position 9	Position 15	Position 24
1. 26mer-C	17.4	0	8.6	6.6			
2. 26mer-C′	11.5				0	10.3	0
3. 26mer-C/26mer-C′ 5′- CCGGCCATTACGGATCCGTCCTGGGC 3′-AGGCCGGTAATGCCTAGGCAGGACCCG	15.5	0	2.1	1.3	0.3	7.1	0
4. Methyl-26mer-C/26mer-C′[b] CH_3 CH_3 5′- CCGGCCATTACGGATCCGTCCTGGGC 3′-AGGCCGGTAATGCCTAGGCAGGACCCG	11.6	0	0	0	0	8.5	0
5. 26mer-C/methyl-26mer-C′[c] 5′- CCGGCCATTACGGATCCGTCCTGGGC 3′-AGGCCGGTAATGCCTAGGCAGGACCCG CH_3	12.3	0	5.0	4.7	0	1.0	0

[a] The experimental details are described in Materials and Methods. Data are presented as picomoles of [^{3}H]methyl incorporated per 100 ng of each strand of oligomer.
[b] Positions 11 and 17 of the 26mer-C strand of methyl-26mer-C/26mer-C′ are 46% methylated.
[c] Position 15 of the 26mer-C′ strand of 26mer-C/methyl-26mer-C′ is 58% methylated.

the known eucaryotic DNA methyltransferase functions in vitro and in both de novo and maintenance methylation (2, 13, 15).

In this study the use of oligodeoxynucleotide substrates indicated that the enzymatic methylation of C-G sites is strongly influenced by the nature of the DNA sequence surrounding the site and the position of the C-G pair relative to the 5′ end of the molecule and other C-G pairs. As previously reported, the methylation at position 19 of 27mer-F is almost eliminated if the C-G moiety at position 2,3 is replaced by a C-A group (3). This was interpreted to indicate that the methyltransferases recognized a 5′ C-G group as a binding or steering site to methylate the next available 3′ C-G group located at an appropriate distance from it. This hypothesis predicts that there should be an optimum distance between the two C-G pairs which may reflect the physical distance between a recognition site and the catalytic site of the methyltransferase protein molecule. As reported here, the experiments with 27mer-F and its derivatives seem to support this idea since the rate of methylation by both the HeLa and MEL enzymes was clearly maximal when the spacing between C-G pairs was approximately 13 to 17 nucleotides.

The pattern of methylation observed with 26mer-C or its complement, 26mer-C′, resembles that seen with 27mer-F in that the C-G pair nearest the 5′ end of the molecule is not methylated. The next C-G pair in the 3′ direction is methylated (position 11 of 26mer-C and position 15 of 26mer-C′), even though the spacing between C-G pairs is only 7 nucleotides (26mer-C) or 4 nucleotides (26mer-C′). However, one should note that the methylation of 26mer-C′ proceeds at only 15 to 20% of the rate of 26mer-C, reflecting, perhaps, the shorter nucleotide spacing between the 5′ C-G group at positions 9,10 and the methylatable C-G at positions 15,16.

The differences in rates of methylation and the patterns of methyl incorporation between complementary strands observed in both the 27mer-F and 26mer-C series remain unexplained. Nevertheless, our data suggest that the enzyme distinguishes two types of C-G pairs in DNA. One class of C-G sites can be methylated in either a single- or double-stranded configuration whether the substrate DNA molecules are unmethylated or hemimethylated. The other type of C-G site in DNA cannot be methylated when present in either an unmethylated or hemimethylated configuration. Analysis of the sites of methylation on the oligomer 26mer-C, its complement, and the duplex form and the inability of the enzyme to efficiently methylate the complementary strands of hemimethylated 26mer-C duplexes indicates that only those C-G sites that are targets for de novo methylation on the single-stranded oligomers are methylated in the double-stranded oligomers. This suggests that the pattern of methylation in hemimethylated DNA is determined by the primary sequence of the target strand and not by the pattern of methylation in the complementary parent strand. If maintenance methylation and de novo methylation share the same substrate specificity, an observation that implies that both of these processes are a manifestation of the same enzymatic mechanism, the difference between maintenance and de novo methylation becomes one of rates rather than substrate recognition. This would eliminate the need to invoke the existence of separate enzymes to catalyze these two processes, and the known DNA methyltransferase in animal cells would be sufficient for the cellular requirements during replication, development, and differentiation.

Our results with oligodeoxynucleotides provide a new approach to obtain specific information regarding the effects of flanking sequences on the pattern of methylation within a defined region of the DNA. It remains to be seen whether the same patterns of methylation are seen in naturally occurring DNA where other factors, such as DNA-binding proteins or the structure of the DNA in chromatin, may alter the targeting of the DNA methyltransferase.

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LITERATURE CITED

1. **Beaucage, S. C., and M. H. Caruthers.** 1981. Deoxynucleoside phosphoramidite—a new class of key intermediates for deoxynucleotide synthesis. Tetrahedron Lett. **22**:1859–1862.
2. **Bolden, A., C. Ward, J. A. Siedlecki, and A. Weissbach.** 1984. Inhibition of *de novo* and maintenance methylation *in vitro* by RNA and synthetic polynucleotides. J. Biol. Chem. **259**: 12437–12443.
3. **Bolden, A. H., C. M. Nalin, C. A. Ward, M. S. Poonian, W. W. McComas, and A. Weissbach.** 1985. DNA methylation: sequences flanking C-G pairs modulate the specificity of the human DNA methylase. Nucleic Acids Res. **13**:3479–3494.
4. **Busslinger, M., J. Hurst, and R. A. Flavell.** 1983. DNA methylation and the regulation of globin gene expression. Cell **34**: 197–206.
5. **Gruenbaum, Y., H. Cedar, and A. Razin.** 1982. Substrate and sequence specificity of a eukaryotic DNA methylase. Nature (London) **295**:620–622.
6. **Holliday, R., and J. E. Pugh.** 1975. DNA modification mechanisms and gene activity during development. Science **187**: 226–232.
7. **Jones, P. A., and S. M. Taylor.** 1981. Hemimethylated duplex DNAs prepared from 5-azacytidine-treated cells. Nucleic Acids Res. **9**:2933–2947.
8. **Kruczek, I., and W. Doerfler.** 1982. The unmethylated state of the promoter/leader and 5′ region of integrated adenovirus genes correlated with gene expression. EMBO J. **1**:409–414.
9. **Langer, K. D., L. Vardimon, D. Renz, and W. Doerfler.** 1984. DNA methylation of three 5′-CCGG-3′ sites in the promoter and 5′ region inactivate the E2a gene of adenovirus type 2. Proc. Natl. Acad. Sci. USA **81**:2950–2954.
10. **Maniatis, T., A. Jeffrey, and H. V. DeSande.** 1975. Chain length determination of small double and single-stranded DNA molecules on polyacrylamide gel electrophoresis. Biochemistry **14**:3787–3794.
11. **Orlofsky, A., and L. A. Chasin.** 1985. A domain of methylation change at the albumin locus in rat hepatoma cell variants. Mol. Cell. Biol. **5**:214–225.
12. **Ott, M.-O., L. Sperling, D. Cassio, J. Levilliers, J. Sala-Trepat, and M. C. Weiss.** 1982. Undermethylation at the 5′ end of the albumin gene is necessary but not sufficient for albumin production by rat hepatoma cells in culture. Cell **30**:825–833.
13. **Pfeifer, G. P., S. Grunwald, T. L. J. Boehm, and D. Drahovsky.** 1983. Isolation and characterization of DNA cytosine 5-methyltransferase from human placenta. Biochim. Biophys. Acta **740**:323–330.
14. **Riggs, A. D.** 1975. X-inactivation, differentiation and DNA methylation. Cytogenet. Cell Genet. **14**:9–25.
15. **Roy, P. H., and A. Weissbach.** 1975. DNA methylase from HeLa cell nuclei. Nucleic Acids Res. **2**:1669–1684.
16. **Simon, D., F. Grunert, U. V. Acken, H. P. Düring, and H. Kröger.** 1978. DNA methylase from regenerating rat liver: purification and characterization. Nucleic Acids Res. **5**: 2152–2167.
17. **Stein, R., A. Razin, and H. Cedar.** 1982. *In vitro* methylation of the hamster adenine phosphoribosyltransferase gene inhibits its expression in mouse L cells. Proc. Natl. Acad. Sci. USA **79**: 3418–3422.