Volume 320, number 3, 243–245 FEBS 12305 April 1993

What is hemimethylated DNA?

R.L.P. Adams and H. Lindsay

Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, UK

Received 2 February 1993; revised version received 26 February 1993

De novo methylation of a CG dinucleotide pair by murine DNA methyltransferase is stimulated by the presence of a single methylcytosine positioned close to the target site.

Spreading of methylation; Maintenance methylation; Methyltransferase; Methylation, de novo

1. INTRODUCTION

Vertebrate DNA methyltransferases show greater activity with hemimethylated than with unmethylated substrates and this is partly a result of the higher affinity of the enzyme for the hemimethylated target site (Reale et al., unpublished results). Following DNA replication, the daughter strand of DNA is unmethylated while the parental strand is methylated at many, though not all CGs. Thus a CG dinucleotide in the daughter strand may find itself paired with a mCG dinucleotide or with another CG dinucleotide in which case, there may be a mCG at some nearby site on the complementary strand. The results presented in this short paper indicate that a methylcytosine positioned one helix turn away from an unmethylated CG pair will stimulate de novo methylation at the target site. Unless prevented by protein binding, this could result in the spreading of methylation along the chromosome [1] particularly in the region of CG islands where the chances of finding a methylcytosine in the appropriate position relative to an unmethylated CG pair might be high.

2. MATERIALS AND METHODS

Oligonucleotide duplexes with the following sequence (derived from the SV40 promoter region) were synthesised by the phosphoamidite method on an Applied Biosystems DNA Synthesiser, model 381A. They contained methylcytosine in the positions indicated in Table I and Fig. 1 (in the upper (G-rich) or lower (C-rich) strand) and complementary strands were annaeled as described by Kadonaga and Tjian [2].

```
   1                    22
5'-AGTCAGCCATGGGGCGGAGAAT        G-rich
   TCAGTCGGTACCCCGCCACTTA-5'     C-rich
  22                    1
```

Oligonucleotide duplexes (50 ng–1 μg) were incubated for 1 h at 37°C in the presence of purified mouse DNA methyltransferase (0.3 μg) fraction IV (2000 U/mg) in a total volume of 70 μl containing Tris-HCl (50 mM), pH 7.8, EDTA (1 mM), DTT (1 mM), PMSF (0.5 mM), glycerol (20%), BSA (50 μg/ml) and tritiated *S*-adenosyl methionine (3 μM). After incubation, incorporation of tritium into DNA was measured as described previously [3]. When required, the oligoduplex (reisolated in the presence of calf thymus DNA) was digested overnight with 5 units of *Nco*I (Stratagene) prior to electrophoresis on a 12% polyacrylamide gel that was subsequently subjected to fluorography after treatment with Amplify (Amersham).

Correspondence address: R.L.P. Adams, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, UK. Fax: (44) (41) 330 4620.

3. RESULTS

From Fig. 1 it is clear that, as expected, the hemimethylated duplexes 3 and 8 are very much better substrates for the mouse DNA methyltransferase than is the unmethylated duplex 2. Moreover, there is no significant strand bias observed in the accepting activity of the two hemimethylated duplexes.

Oligonucleotide duplex 4 had been synthesised as a substrate for the pea DNA methylase. It contains two hemimethylated CNG sequences and is a good substrate for the plant enzyme [4]. However, the CG pair is unmethylated and it was expected that it would be a typical, de novo substrate for a mammalian DNA methylase. It was a surprise when it was found to be a five to tenfold better substrate than the corresponding unmethylated duplex 2 (Fig. 1), occupying a position intermediate between the unmethylated and hemimethylated duplexes.

3.1. *Slippage*

We considered the possibility that the mCCG present in oligoduplex 4 was providing a stimulus to the methylation of the cytosine in the CGG on the complementary strand. This might involve a slippage mechanism in which the methylase recognises mCNG instead of mCG on the template strand as the signal for maintenance methylation. As this sequence forms part of the *Msp*I recognition site, we constructed oligonucleotide duplexes containing two CCGG sequences.

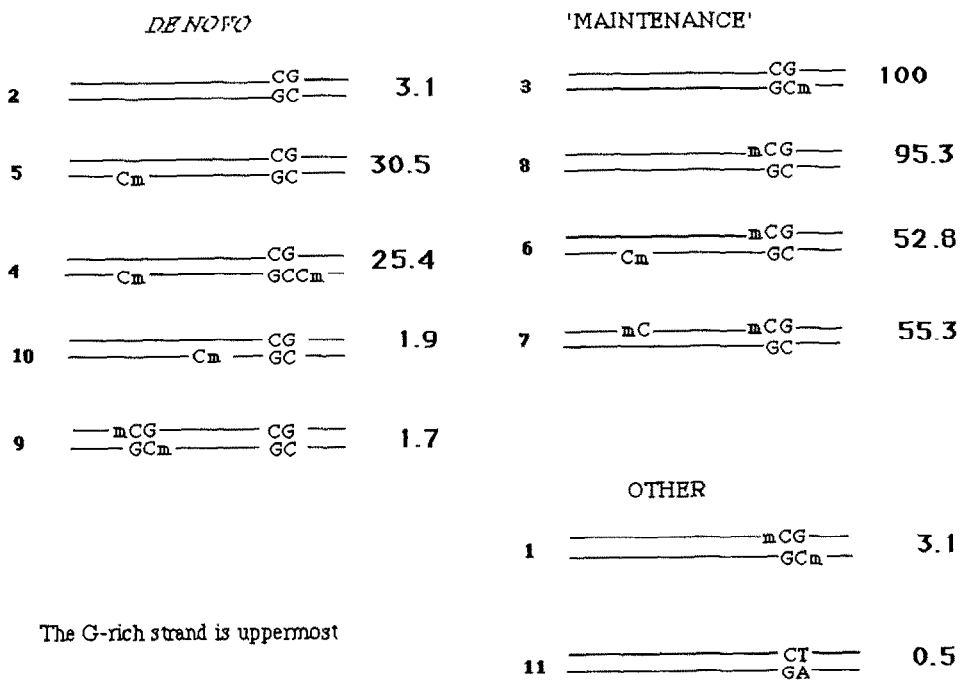

Fig. 1 Influence of the GHRP-Dansyl peptide on the anticlotting effect of the D_H fragment Panel A represents the inhibition of the polymerization of fibrin by increasing amounts (up to 0 4 mg/ml) of the D_H fragment. Panel B demonstrates progressive elimination of the anticlotting effect of D_H fragment (0.4 mg/ml) when first incubated with various amount of the GHRP-Dansyl peptide; the X axis represents molar ratio

Complete methylation of these oligonucleotides with M.*Msp*I (as judged by resistance to the corresponding restriction enzyme) did not affect the ability of such molecules to accept methyl groups when they were incubated with murine DNA methylase. This appears to rule out the above, slippage proposal.

However, when M.*Msp*I was present *during* an incubation with the mouse enzyme there was a fifteen-fold stimulation of incorporation (Table II).

As M.*Msp*I acts independently on each strand then, particularly at early stages of methylation, there will be many duplex molecules that will contain methyl groups on one strand only, i.e. they will resemble oligo 4 in that they will contain hemimethylated mCCG target sites.

This indicates that, to be a good substrate for the mouse enzyme, DNA need not necessarily contain hemimethylated CG target sites but that it may be sufficient to contain methylcytosine on one strand.

3.2. *Hemimethylation?*

To further investigate this problem, several other oligonucleotide duplexes were synthesised with the same sequence as oligoduplex 2 but with methylcytosine substituting for cytosine as shown in Table I and Fig. 1.

Oligoduplex 5 contains an unmethylated CG base pair but also has a single methylcytosine 10 bp away on one strand only. It is the same as duplex 4 but lacks the mC in mCCG that we had initially considered of prime importance. As shown in Fig. 1, duplex 5 is a much better substrate than is the unmethylated duplex (oligo 2) and is equally as good as duplex 4 indicating that the hemimethylated mCCG is irrelevant to the stimulation.

Table I

Oligonucleotide duplexes used in this study (C and G in the second column refer to C-rich and G-rich strand)

Duplex 1	C7 + G15	CG pair fully methylated
Duplex 2		unmethylated
Duplex 3	C-7	hemimethylated (mC on C-rich strand)
Duplex 4	C6 + C17	CG pair unmethylated but two mCNGs on C-rich strand
Duplex 5	C17	CG pair unmethylated but one mC on C-rich strand
Duplex 6	C17 + G15	hemimethylated (mC on G-rich strand) with an extra mC on C-rich strand
Duplex 7	G4 + G15	hemimethylated (mC on G-rich strand) with an extra mC on G-rich strand
Duplex 8	G15	hemimethylated (mC on G-rich strand)
Duplex 9	G7 + C15	unmethylated but with a mCG pair created towards the distal end of the duplex
Duplex 10	C12	unmethylated but with a mC five bases away from the CG pair
Duplex 11		CG pair replaced by CT/AG pair

Duplex 10 is similar to duplex 5 except that the mC is only 5 nucleotides away from the CG pair (i.e. on the opposite side of the DNA duplex) yet this duplex shows similar substrate activity to the unmethylated duplex 2 (Fig.1).

In order to determine whether such extra methylcytosines might have an effect (either positive or negative) on maintenance methylation we tested duplexes 6 and 7 and compared their activity with the hemimethylated duplex 8. We considered that an extra methylcytosine on the unmethylated strand might reduce the rate of methylation, which it does, but we unexpectedly found that an extra methylcytosine on the methylated strand also reduced substrate activity somewhat (Fig. 1).

3.3. *Site of methylation*

That little methylation is seen with the fully methylated duplex 1 nor with the non CG containing duplex 11 (Fig. 1) strongly indicates that methylation is occurring on the CG pair. However, to confirm this, we methylated duplexes 2, 5 and 8 in vitro and then digested them with *Nco*I that cleaves the CCATGG sequence yielding two fragments, the larger of which contains the CG pair while the smaller contains the cytosine at position C-17 that is modified in oligo 5. Only one tritiated fragment is produced, indicating that the enzymic methylation is occurring at the CG pair at position C-7,8 (results not shown).

4. DISCUSSION

We have investigated the ability of single methylcytosines to influence the efficiency of a 22mer oligonucleotide duplex to act as a substrate for maintenance and de novo methylation by the mouse DNA methylase. Although the best targets are those carrying a hemimethylated CG (oligos 3 and 8), oligo 5, where the methylcytosine is one helix turn away from the CG pair, is almost one-third as effective. Oligo 4, despite having two mCs and one only a single base away from a CG, is no better as a substrate than is oligo 5. Oligo 10, where the extra methyl group is on the other side of the helix, is no more effective than the unmethylated duplex.

Duplexes lacking a cytosine in a CG pair (i.e. duplexes 1 and 11) are poor substrates for the enzyme indicating that most of the methylation is normally occurring at the CG pair and this is supported by the studies with *Nco*I which show the methylation to occur on that half of the duplex.

The studies with oligos 6, 7 and 8 investigate maintenance methylation at a hemimethylated CG dinucleotide pair and they indicate that a distant methylcytosine, on either strand, causes some inhibition of the methylation reaction. We can only suggest that the enzyme binds firmly to a single methylcytosine whether or not it forms part of a CG pair (Reale et al., unpublished results) and that this limits the availability of the enzyme to act at the adjacent hemimethylated site.

These results bring into question the assumption that maintenance methylation is solely dependent on the methylation status of the complementary CG. Rather, a neighbouring, appropriately positioned, single mCG may activate the DNA methylase. In this way methylation may travel along the DNA from a focus of methylcytosine in a manner similar to that suggested by Holliday and Pugh [5]. This is proposed to result from the enhanced binding of the enzyme to a single methylcytosine (normally in a CG/mCG pair) and hence its positioning close to an adjacent, target CG.

The ability of the mouse enzyme to act on *hemimethylated* DNA when the 'template' strand carries the methylcytosine at sites other than the complementary mCG raises the possibility that maintenance methylation is not target site specific. Rather, the methylase might act on the 'substrate' strand at any CG that is not protected, provided that the 'template' strand contains methylcytosine. Such spreading is most likely to occur when there is a high density of CGs, as in CG islands and, once under way, it could lead to the rapid conversion of an unmethylated island into a methylated island in the absence of protection. However, the spreading effect would be limited to the periods following replication and would not occur at other times as shown by the finding that a pair of mCGs one turn of the helix away from the unmethylated CG pair (oligo 9) does not generate a substrate with enhanced accepting activity. We have shown elsewhere (Reale et al., unpublished results) that the enzyme has very low affinity for DNA with paired mCG dinucleotides.

Table II

Stimulation of methylation by *Msp*I

Time (h)	Methylcytosine synthesised (pmol) using enzyme from			Stimulation
	mouse	M.*Msp*I	mouse + M.*Msp*I	
0.5	0.4	3.4	10.2	16-fold
2.0	0.8	9.9	22.4	15-fold

Acknowledgements: We would like to thank the Wellcome Trust for the provision of funds to carry out this work and Prof. Fewson and the University of Glasgow for the provision of facilities.

REFERENCES

[1] Orend, G., Kuhlmann, I. and Doerfler, W. (1991) J. Virol. 65, 4301–4308.
[2] Kadonaga, J.T. and Tjian, R. (1986) Proc. Natl. Acad. Sci. USA 83, 5889–5893.
[3] Adams, R.L.P., Gardiner, K., Rinaldi, A., Bryans, M., McGarvey, M. and Burdon, R.H. (1986) Biochim. Biophys. Acta 868, 9–16.
[4] Houlston, C.E., Lindsay, H., Pradhan, S. and Adams, R.L.P. (1993) Biochem. J. (in press).
[5] Holliday, R. and Pugh, J.E. (1975) Science 187, 226–232