DNA binding and methyl transfer catalysed by mouse DNA methyltransferase

Anna REALE,*‡ Heather LINDSAY,* Hans Peter SALUZ,† Sriharsa PRADHAN,* Roger L. P. ADAMS,*||
Jean-Pierre JOST§ and Roberto STROM‡

*Institute of Biomedical and Life Sciences, Division of Biochemistry and Molecular Biology, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.,

†Hans-Knöll-Institut für Naturstoff-Forschung, Beutenbergstrasse 11, D-087745 Jena, Federal Republic of Germany, ‡Dipartimento di Biopatologia Umana,

Università degli Studi di Roma 'La Sapienza', Via Regina Elena 324, 00161 Roma, Italy, and §Friedrich Miescher-Institut, P.O.B. 2543, CH-4002 Basel, Switzerland

By using a purified fraction of mouse DNA methyltransferase we have shown, by gel-retardation analysis, that the enzyme forms a low-affinity complex preferentially with hemimethylated DNA; the complexes formed with unmethylated or with fully methylated DNA are of even lower affinity, and only very weak interaction occurs with DNA lacking CG dinucleotides. Interaction is

inhibited by *N*-ethylmaleimide. Methyl transfer from *S*-adenosylmethionine is associated with the release of the fully methylated product from the complex. Complexes formed with the intact enzyme are extremely large, but limited trypsin treatment allows a major complex to enter the gel. DNA binding is not inhibited by this limited proteolysis of the native enzyme.

INTRODUCTION

In animals, about 5% of the DNA cytosines are methylated and most of these are found in the dinucleotide mCG. Not all CGs are methylated, however, and maintenance methylation is the process whereby the pattern of methylation in the parental DNA is copied on to the two daughter molecules after replication; i.e. in maintenance methylation, the target CG must be paired with a mCG to give a hemimethylated dinucleotide (see [1] for a review). The reaction is catalysed by a DNA methyltransferase and involves the transfer of a methyl group from *S*-adenosylmethionine (AdoMet) to the 5-position on the target cytosine in DNA. The reaction is believed to involve the formation of a covalent link between the 6-position on the target cytosine and a cysteine residue at the active site of the enzyme. A methyl group is then transferred from AdoMet to the 5-position of the cytosine as the enzyme is released from the DNA [2–5].

All DNA methyltransferases purified so far from animal sources are capable of maintenance and *de novo* methylation. That is, they are able to add methyl groups on to CG dinucleotide pairs in DNA whether or not one member of the pair is already methylated. The intact enzyme shows a strong preference for hemimethylated DNA over unmethylated DNA, but the basis for this preference is unknown [1], and one of the aims of the present work was to determine whether the binding reaction formed the basis for this discrimination. However, the intact enzyme aggregates in low salt concentrations, which makes gel-retardation experiments difficult, and the enzyme used in the experiments reported here has been subjected to partial proteolysis to reduce the tendency to aggregate and to increase the activity with unmethylated target sequences [6–8]. As shown previously [6,7], limited proteolysis does not result in major dissociation of the native enzyme into small fragments, and the results presented here show that, on electrophoresis under non-denaturing conditions, one major band is seen entering the gel. This approach is open to the criticism that the proteolysis is affecting the affinity of the enzyme for its substrate and, to

counter this criticism, the digestion was carried out, in most cases, after the enzyme–DNA complex had been formed.

One of the difficulties posed for the study of DNA–methyltransferase interactions is the lack of a requirement for a specific DNA sequence (other than CG) and the presumed transient nature of the interaction. This led to concern that the retarded bands seen in gel-retardation experiments might be caused by non-specific interactions or high-affinity interactions of otherwise invisible minor protein contaminants of the enzyme. Initially, we assumed that binding might be tightest to DNA containing hemimethylated CG dinucleotide pairs, and so we compared the binding to oligonucleotide duplexes of identical sequence but differing in whether a CG dinucleotide pair was unmethylated, hemimethylated or fully methylated. Later, we obtained an oligonucleotide containing a CG pair in which the cytosine on one strand was replaced by a pyrimidinone [9,10], which forms an affinity reagent for methyltransferases. This enabled us to confirm that the bands that we observed with the normal substrates were indeed caused by binding of a methyltransferase.

The present results confirm that the initial assumption was correct, but discrimination between unmethylated and hemimethylated substrates is not extensive. Affinity is highest with hemimethylated DNA but the enzyme binds weakly, even if non-specifically, to oligonucleotides lacking CGs or containing only mCGs. On incubation in the presence of AdoMet the enzyme-bound oligonucleotide accepts a methyl group and the fully methylated product is rapidly released from the enzyme. The enzyme–DNA binding is always of low affinity and is blocked by pretreatment of the enzyme with *N*-ethylmaleimide (NEM).

MATERIALS AND METHODS

DNA methyltransferase

DNA methyltransferase was isolated from mouse Krebs II ascites-tumour cells as previously described [6]. It has a specific activity of about 20000 units/mg of protein as assayed using

Abbreviations used: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; mC, methylcytosine; NEM, *N*-ethylmaleimide; d⁴H₂C, 2-pyrimidinone 1-β-D-2'-deoxyribofuranoside; DTT, dithiothreitol.

|| To whom correspondence should be sent.

poly(dI-dC)·(poly dI-dC) and shows a the major band on SDS/PAGE at about 200 kDa, though several lower-molecular-mass bands are also visible (see Figure 1c below). These minor bands arise as a result of a small amount of proteolysis that occurs during the early stages of purification. The enzyme shows an approx. 10-fold preference for hemimethylated DNA over unmethylated DNA as substrate (see Figure 1d below). It was subjected to further limited proteolysis by incubation at 37 °C with trypsin, at an enzyme/trypsin ratio of 200:1, for various times up to 15 min. Without this treatment, very little protein enters a native gel, further testifying to the high degree of purity of the enzyme. One unit of enzyme catalyses the transfer of 1 pmol of methyl group from AdoMet to (poly dI-dC)·(poly dI-dC) in 1 h at 37 °C (this is equal to 1000 c.p.m. in the assay; the reaction is linear for several hours under these conditions).

Oligonucleotides

Oligonucleotides were synthesized by the phosphoamidite method on an Applied Biosystems DNA synthesizer, model 381A. They had the sequence:

5'-ATT CTC CGC CCC ATG GCT GAC T-3'

3'-TAA GAG GCG GGG TAC CGA CTG A-5'

with, in some instances, the underlined Cs replaced with methylcytosines. The exception was the pyrimidinone-containing oligonucleotide that was generously provided by Dr. David Hornby (Krebs Institute, University of Sheffield, Sheffield, U.K.), and it had the sequence:

5'-GTA TCT AGC ATC GGC CCG GGC CGA TGC TAG AT-3'

3'-CAT AGA TCG TAG CCG GGC CCG GCT ACG ATC TA-5'

with the underlined C replaced with 2-pyrimidinone 1- β -D-2'-deoxyribofuranoside ($d^{4H}C$).

The DNA was either gel-purified or precipitated immediately from the ammonia solution. Single-stranded oligonucleotides (0.1 μ g) were end-labelled in a volume of 11 μ l, using 10 units of T4 polynucleotide kinase and 50 μ Ci (10 pmol) of [γ - ^{32}P]ATP and buffer containing 50 mM Tris/HCl, pH 8.0, 10 mM MgCl₂ and 5 mM dithiothreitol (DTT). After incubation at 37 °C for 1 h, the volume was increased to 40 μ l by the addition of water, and the labelled oligonucleotide was isolated using a Chromaspin 10 column (Clontech). Oligonucleotides were annealed with their complementary strands as described by Kadonaga and Tjian [11].

Gel-retardation assays

End-labelled oligonucleotides were incubated in the presence of purified mouse DNA methyltransferase (1–4 μ g) fraction IV (20000 units/mg) [6] in a total volume of 25 μ l of buffer M [50 mM Tris/HCl (pH 7.8)/1 mM EDTA/1 mM DTT/0.5 μ M PMSF/10% glycerol/250 μ g/ml BSA] and, when indicated, AdoMet or S-adenosylhomocysteine (AdoHcy) (107 μ M). After incubation at 37 °C, carrier DNA (5 mg of calf thymus or *Escherichia coli* DNA) was added where appropriate and samples loaded directly on to a 6% polyacrylamide gel (acrylamide/bisacrylamide, 59:1, v/v) in 0.5 $\times$ Tris/borate/EDTA buffer, pH 8.0 (1 $\times$ Tris/borate/EDTA buffer contained 89 mM Tris base, 89 mM boric acid and 2 mM EDTA, pH 8.0). The gel was run for 2 h and fixed in 7% acetic acid for 15 min, dried and autoradiographed or scanned in a Fujix BAS 1000 phosphor-imager, which was calibrated using known amounts of labelled oligonucleotides exposed beside the dried gel. When required, bands were excised and either the radioactivity was counted in a

liquid-scintillation counter or the DNA was processed for Maxam–Gilbert sequencing [12].

RESULTS

Effect of trypsin treatment on complex aggregation

On native gel electrophoresis, whether in agarose or acrylamide with concentrations as low as 5%, the mouse DNA methyltransferase fails to enter the gel. This can be shown by staining the gel, and is a phenomenon largely independent of the presence of complexing DNA. Indeed, no protein staining is seen except at the top of the gel, showing that the enzyme preparation is free of visible contaminants. The large size is a result of aggregation occurring under the low-salt conditions that are a requirement of (a) the electrophoresis and (b) maintenance of the DNA–enzyme complexes that are the subject of the present study. Electrophoresis of the enzyme loaded in the presence of up to 300 mM NaCl does not affect the appearance of the stained gel (results not shown).

Using centrifugation analysis we have previously shown such aggregation to occur [7] and have shown that aggregates can be disrupted by limited proteolysis. Figure 1 shows a similar effect analysed by native gel electrophoresis. The enzyme–oligonucleotide complex was formed with either an end-labelled, hemimethylated oligonucleotide (Figure 1a) or with an end-labelled oligonucleotide containing $d^{4H}C$ in place of the cytosine in a CG dinucleotide pair (Figure 1b) (see the Materials and methods section for the sequences of the oligonucleotides). After incubation of the enzyme and oligonucleotide at 37 °C in the presence of AdoHcy for 1 h, the complex was incubated with a limited amount of trypsin (enzyme/trypsin ratio, 200:1) for various times up to 15 min prior to electrophoresis.

Even the briefest treatment with trypsin has a dramatic effect on the entry of the complex into the gel, and after 3–5 min trypsin treatment there is present a single retarded band which apparently does not change with further trypsin treatment. In some experiments two retarded bands can be seen. The $d^{4H}C$ -containing DNA forms a stable complex with methyltransferase and confirms that, in both cases, the retarded band results from the association of the mouse methyltransferase with the oligonucleotides. (The increased intensity of the band formed with the $d^{4H}C$ -containing DNA simply reflects the higher specific radioactivity of this oligonucleotide.)

Because the complex between the methyltransferase and the pyrimidinone-containing oligonucleotide is stable in SDS (though it is disrupted by boiling in SDS), we were able to run an SDS gel of this complex (Figure 1c). This shows that, initially, the complex consists of several high-molecular-mass species and that, after 1 min of trypsin treatment, a single band of just under 200 kDa predominates. This band is also lost on further trypsin treatment and is not replaced by any obvious band that can be distinguished from the unretarded oligonucleotide. Clearly, although stable native complexes remain after 15 min of trypsin treatment, these have suffered so much proteolytic nicking that no denatured complex can be resolved. Figure 1(d) shows the changes in enzymic activity occurring over this time period and indicates that, although *de novo* activity rises for the first 3 min of trypsin treatment, after 5 min there is a steady fall in both *de novo* and maintenance activity commensurate with the continued nicking of the protein.

As a result of these preliminary experiments we decided to carry out all subsequent experiments using complexes that had been treated for 3–5 min with trypsin under the conditions established.

Figure 1 Effect of trypsin treatment on complex formation

Complexes were formed by incubating 80 units of enzyme for 60 min at 37 °C with AdoHcy and 2 ng of end-labelled hemimethylated oligonucleotide duplex (HM, **a**) or d⁴Hc-containing duplex (P, **b** and **c**) and then subjected to trypsin treatment for 0–15 min as indicated. The reaction was terminated by addition of trypsin inhibitor, and carrier DNA and the samples were immediately loaded on to the gel for electrophoresis. The first zero-time sample was not exposed to trypsin at all. Electrophoresis was on native gels (**a** and **b**) or on an SDS-containing gel (**c**). (**d**) Shows the enzymic activity remaining when complexes formed with hemimethylated (HM) or unmethylated (UM) DNA were trypsin-treated for the indicated times.

Effect of time of incubation and enzyme concentration

Although some complex is formed almost instantaneously on mixing the enzyme with the end-labelled oligonucleotide, there is about a 2-fold increase with time of incubation up to 60 min in the amount of complex observed. This is surprising, since one might expect that equilibrium would be reached almost instantaneously; however, this is assumed to be a consequence of the relatively weak association coupled with a rapid rate of dissociation of the complex.

There is a steady, almost linear, increase in the amount of the retarded band as the amount of enzyme protein in the incubation is increased to 10 µg (results not shown).

Affinity of the enzyme for hemimethylated oligonucleotide

Figure 2 shows the effect of increasing the concentration of hemimethylated or unmethylated oligonucleotide on the amount of the retarded band. In this experiment, samples of increasing amounts of the same labelled oligonucleotides were processed along with the dried gel to allow quantification of the amounts of the retarded bands.

The affinity of the hemimethylated oligonucleotide for the methyltransferase is very low and the K_d can be shown by Scatchard analysis to be about 7×10^{-7} M. This is much higher than the values obtained for transcription factors or prokaryotic DNA methyltransferases and helps to explain some of the difficulties encountered in gel-retardation experiments using enzymes, such as mammalian DNA methyltransferase, that have very short target sequences that occur frequently in DNA. It is,

however, not a surprising value given that the function of the enzyme is to 'scan' the newly replicated DNA without necessarily methylating every target CG dinucleotide. The K_d value for the corresponding unmethylated duplex is even higher, at 1.4×10^{-6} M. Whether this 2-fold difference in the value of the dissociation constant is sufficient to allow the enzyme to discriminate between unmethylated and hemimethylated substrates seems doubtful.

The relative affinity of the enzyme for different oligonucleotides was established by incubating, for 10 min, 10 units of enzyme with AdoHcy or AdoMet and 2 ng of end-labelled hemimethylated oligonucleotide in the presence of 100 ng or 1 µg competing oligonucleotide duplex (Figure 3). Addition of the competitor after the incubation (as opposed to during the incubation) did not affect the results (not shown). Duplexes containing one or two hemimethylated sites are the best competitors but, in the presence of AdoHcy, unmethylated or symmetrically methylated duplexes are no better competitors than is a duplex lacking a CG dinucleotide. There thus appears to be a considerable preference for the enzyme to bind hemimethylated DNA.

In the presence of AdoMet, however, the unmethylated and symmetrically methylated duplexes are significantly better competitors than a duplex lacking a CG dinucleotide, and they approach hemimethylated DNA in their ability to compete for binding to the enzyme.

Effect of competing DNAs on the methyl-transfer reaction

Differences are also apparent when considering enzymic activity using a hemimethylated duplex in the presence of excess, com-

Figure 2 Affinity of the enzyme for hemimethylated and unmethylated oligonucleotide duplex

End-labelled hemimethylated (HM) or unmethylated (UM) oligonucleotide (5 ng), together with appropriate amounts of unlabelled but otherwise identical DNA was incubated with AdoHcy and the enzyme (10 units) for 10 min prior to trypsin treatment (3 min) and electrophoresis on a native gel. The gel was fixed and dried and scanned on the phosphorimager, along with known amounts of the radioactive oligonucleotides. The values for K_d were derived from Scatchard plots.

peting unmethylated or doubly methylated duplexes of the same sequence (Figure 4). Although the enzyme binds increasing amounts of hemimethylated duplex as the DNA concentration rises to micromolar levels (Figure 2), the enzymic reaction, in the presence of AdoMet, is almost saturated at 0.1 μ M (100 ng/assay). When, in addition to the near-saturating level of hemimethylated duplex, increasing amounts of unmethylated or fully methylated duplex were added to a standard methyltransferase assay, a reduction in methyl transfer is observed (Figure 4). By themselves these duplexes show accepting activity of 5 and 2% respectively that of hemimethylated DNA and so contribute very little to the overall activity. (This contribution has been subtracted to obtain the data shown in Figure 6 below). A 5-fold excess of doubly methylated duplex is required to reduce enzymic activity to 50% of that seen with 100 ng of hemimethylated duplex alone, whereas it takes a 19-fold excess of unmethylated duplex to achieve the same level of reduction. This shows that, when the overall methyl-transfer reaction is studied with AdoMet present, the enzyme shows higher affinity for the doubly methylated duplex than for the unmethylated duplex, but that the affinity for the hemimethylated substrate is greatest.

Peptide competition experiments

The experiments with the pyrimidinone made us confident that the retarded bands were formed by interaction of a methyltransferase with the labelled oligonucleotides. To confirm this and to eliminate further the possibility that the retarded band

Figure 3 Competition with different oligonucleotides

Enzyme (10 units) and end-labelled hemimethylated oligonucleotide (2 ng) were incubated with cofactor as indicated and the indicated competitor oligonucleotide duplex (1 μ g, except where stated) for 10 min and then electrophoresed after trypsin treatment, but without addition of further competitor DNA. (An experiment where the competitor was added after the incubation gave similar results.) No competitor was present in the first lane of each set. The competitors used were identical hemimethylated duplex (HM), identical fully methylated duplex (MM), identical unmethylated duplex (UM) and unrelated duplexes containing two hemimethylated CG dinucleotide pairs (2 $\times$ HM) or no CG dinucleotides (No CG). The relative intensity (RI) of the retarded bands (as measured using the phosphorimager) is shown beneath the photograph and is the sum of the material in both retarded bands.

might be caused by association with some minor contaminant in the enzyme preparation, we have used competitor peptides corresponding to various regions of the mouse DNA methyltransferase [5,8]. Of the synthetic peptides tested, only peptide 157 inhibits methyltransferase activity (Figure 5), and this also

Figure 4 Effect of competing oligonucleotide duplexes on enzyme transfer

Enzyme (10 units) was incubated for 1 h with 100 ng of hemimethylated substrate (over 80% of a saturating level) in a standard methyltransferase reaction mixture to which had been added increasing amounts of competitor oligonucleotide duplex. $\circ$, Fully methylated competitor; $\bullet$, unmethylated competitor. The results are plotted semilogarithmically and show activity relative to the that in the absence of competitor.

Figure 5 Effect of competing peptides

Peptide 157, derived from region X of the mouse methyltransferase, was added at the indicated concentration to a methyltransferase assay mixture using a poly(dI-dC) · poly(dI-dC) substrate. The assay contained 8 units of enzyme at a concentration of approx. 23 nM. The active-site peptide (156) had no inhibitory effect on the assay.

inhibits, by 87%, the formation of the retarded band seen on native gel electrophoresis. A different peptide, namely peptide 156, that contains the active site (region IV) of the enzyme, does not inhibit the enzymic activity even when present in vast molar excess (Figure 5). The inhibitory peptide 157 has the sequence DRHRQVGNAV and corresponds to a hydrophilic region containing conserved region X [5,8]. These results indicate that this region of the enzyme plays a fundamental role in binding DNA and they indicate that there is competition between the peptide and region X of the enzyme for binding to DNA. This observation suggests a mechanism that might be used to control DNA methylation *in vivo*, namely making use of transcription factors that also incorporate this peptide into their sequence and thereby

Table 1 Involvement of SH groups in the binding reaction

Reactions were set up using 20 units of enzyme. NEM (20 μM) was added next, or after addition of 1 ng of hemimethylated oligonucleotide and/or AdoHcy, and the mixture incubated at 37 °C for 5 min prior to further additions. The final incubation was for a total of 60 min. Prior to electrophoresis, the samples were treated with trypsin for 3 min, and calf thymus carrier DNA was added. The dried gel was scanned in the phosphorimager and the results are expressed as the amount of oligonucleotide retarded as a percentage of the amount retarded in the absence of NEM.

NEM added after:	Amount of oligonucleotide retarded (% of amount retarded in the absence of NEM)	
	Oligonucleotide then AdoHcy	AdoHcy then oligonucleotide
Enzyme	7	15
Oligonucleotide	10	—
AdoHcy	—	19
All components	17	27

Table 2 Location of methylated product as free oligonucleotide

Enzyme (160 units) was incubated with [³H]AdoMet (2 μCi; 15 Ci/mmol; Amersham International) and oligonucleotide duplex (400 ng) for the indicated times. The complex was then treated with trypsin for 3 min, carrier DNA was added and the mixture fractionated by native gel electrophoresis. The free oligonucleotide (detected by ethidium staining) and all retarded material were cut out and washed separately with 5% trichloroacetic acid prior to solubilization in 0.5 M perchloric acid (70 °C for 30 min) and estimation of radioactivity using Ultima-Flo AF (Packard). No activity above the enzyme free control was detected in the retarded material. The results show the radioactivity (d.p.m.) in the free oligonucleotide. Background radioactivity seen in the absence of enzyme has been subtracted from all values.

Time of incubation (min)	Radioactivity in free oligonucleotide (d.p.m.)	
	Unmethylated substrate	Hemimethylated substrate
0	0	0
30	3300	8600
60	5800	13 500
120	10 400	24 100

interfere with the binding of DNA methyltransferase to DNA adjacent to their recognition element.

Involvement of SH groups in the binding reaction

Addition of NEM (20 μM) to the incubation inhibits by about 90% the formation of the retarded complex seen on native gel electrophoresis (Table 1). This is not surprising, since the complex formation between DNA methyltransferases and DNA has been shown to involve an active-site cysteine. Slight protection against NEM inhibition is given by the presence of either AdoHcy or oligonucleotide alone, and the two together increase the protection to the extent that inhibition now averages only about 80%. That greater protection is not observed is probably a reflection of the low affinity of the oligonucleotide for the enzyme which, therefore, exists in a ligand-free state for a considerable proportion of the incubation time (see below).

Affinity for product DNA

Table 2 reveals that, when a hemimethylated oligonucleotide is incubated with the enzyme in the presence of tritiated AdoMet, the tritiated oligonucleotide product is not bound to the enzyme but increases with time as free oligonucleotide. Although, clearly, the enzyme–DNA complex has to form in order for methyl transfer to occur, this complex is apparently only transitory and dissociates immediately upon transfer.

The same result is seen when the reaction is performed with unmethylated oligonucleotide, implying either that both strands are methylated in a concerted reaction or that even the newly hemimethylated duplex shows little affinity for the enzyme and has to be released before rebinding and methylation of the second strand.

We have also analysed the methylation status of the DNA present in various bands using Maxam–Gilbert sequence analysis (Figure 6). In this experiment we used a longer oligonucleotide containing two CG dinucleotide pairs and, in order to try to maximize *de novo* methylation, we have used enzyme subjected to limited proteolysis before, rather than after, incubation with

Figure 6 Gel-retardation using vitellogenin promoter oligonucleotide duplex

The lower strand of this oligonucleotide duplex has the sequence:

CTT TTT CCA ATC ATA AAT TCT CTT CAC GGC GAA TAA AGA AAA

containing two CG dinucleotides (underlined) present in the unmethylated (a, lane B) or methylated state (a, lane A). Hence, in the region of interest, the upper strand has the sequence CGCG where the cytosines in *italics* are targets for the enzyme, but where the central cytosine would not be expected to be methylated by a CG methyltransferase. (a) Gel retardation. The oligonucleotide duplex (upper strand kinase-treated; 1 ng; 2×10^5 c.p.m.) was incubated with 80 units of DNA methyltransferase (trypsin-treated for 3 min) and AdoMet (0.32 mM) in a volume of 70 μ l. After 3 h at 37 °C, *E. coli* carrier DNA (7.5 μ g) was added before loading on to the gel. Bands 1–8 were isolated, digested with proteinase K, twice phenol-treated and subjected to chemical sequencing [11]. (b) C-reactions of the gel-shift bands (Maxam and Gilbert [11]); only the relevant parts of the sequences are shown. Arrowheads indicate the target cytosines, which were methylated in lanes 2–4, partially methylated in lanes 1 and 8, and unmethylated in lanes 5–7. Lanes 1–8 correspond to the number of each band shown in the gel-retardation assay of (a).

AdoMet and DNA for 3 h. Under these conditions, two retarded bands are present together with material that fails to enter the gel (Figure 6a; lane A, bands 1–3 and lane B, bands 5–7). When the oligonucleotide is initially unmethylated (lane B), little methylation can be detected by this method, and all the bands contain mainly unmethylated cytosine, though some methylation of the cytosine in the free DNA is apparent (Figure 6b, lanes 5–8). However, when the oligonucleotide is initially hemimethylated at both CG pairs (Figure 6a, lane A), it is almost completely methylated at the end of the incubation with only band 1 and, to a lesser extent, band 2 showing evidence of unmethylated cytosine (Figure 6b). The loading in lanes 2 and 3 is less than for the other lanes, as can be judged from the intensity of the central cytosine band; nonetheless, faint bands are visible in lane 2 corresponding to the two target cytosines, showing these to be incompletely methylated.

DISCUSSION

The results reported here indicate that the mouse DNA methyltransferase interacts particularly with hemimethylated CG dinucleotide target sites in DNA. The complex is non-covalent, of low affinity and transient. ('Artefactual' covalent complexes have been reported to form on continued incubation of the enzyme with DNA, even when that DNA is devoid of CG target dinucleotide pairs [14].) Estimates of K_d are in the micromolar range; i.e. two or three orders of magnitude greater than those seen with *EcoK* methyltransferase [13]. They indicate only a 2-fold difference between the affinity of the enzyme for hemimethylated and unmethylated DNA, and this is borne out by competition experiments using gel retardation. On this basis

one might conclude that the 20-fold difference in the ability of hemimethylated and unmethylated DNA to act as substrate for the enzyme might be attributed to differences in catalysis. However, a 19-fold excess of unmethylated DNA is required to bring about a 50% reduction in the methylation of hemimethylated DNA in a standard assay. It is possible, therefore, that the difference in binding is adequate to account for the preference of the enzyme for hemimethylated substrates, but this cannot be taken as proven.

Relative to hemimethylated DNA, fully methylated DNA interacts less well with the enzyme and dissociates in the presence of a continued supply of hemimethylated substrate and AdoMet. The enzyme does appear, however, to interact more strongly with fully methylated DNA than with unmethylated DNA (Figure 4) and this may indicate the presence on the enzyme of a site that reacts with mCG (an interaction that forms part of the preference of the enzyme for hemimethylated DNA). This interaction with methylated DNA may also account for some of the problems encountered in attempts to obtain 100% methylation of unmethylated DNA using the mouse DNA methyltransferase.

The enzyme shows significant non-specific interaction with any DNA, and this will aid the initial interaction with DNA and allow the enzyme to process along the DNA duplex behind the replication fork. The relative affinity of the enzyme for hemimethylated compared with completely unmethylated DNA may be enough to explain the action of the enzyme as a maintenance methyltransferase. *In vivo* this would have the consequence that the methyltransferase would pause when it encountered a hemimethylated site and move on only once methyl transfer had been effected. As the affinity for the unmethylated site is lower, the probability is increased that the enzyme would process before *de novo* methyl transfer had been achieved. However, it still remains a possibility that binding and catalysis are not coupled.

Although the briefest trypsin treatments have little effect on enzymic activity or on the size of the enzyme polypeptide, they have a dramatic effect on the aggregation of native enzyme–DNA complexes. This effect could be brought about by the removal of a small terminal peptide required for aggregation or by action on some accessory protein that serves to cross-link enzyme–DNA complexes. Inhibition of enzymic activity and DNA binding by a peptide corresponding to conserved region X implies that this region plays a part in the binding reaction. In the bacterial methyltransferase *HhaI*, region X forms part of the AdoMet binding pocket and also part of the hinge joining the two domains of the catalytic unit [15]. It is possible that the free peptide interacts with the enzyme to prevent it from assuming the correct conformational form for DNA binding and enzymic activity. Less likely is the possibility that it interacts directly with the DNA to prevent binding.

NEM might be expected not to interfere with non-covalent DNA binding, but it has been shown for methyltransferase *HhaI* that a conformational change occurs on DNA binding that relocates the active-site cysteine. Such a change may not be possible following interaction with NEM. What is more surprising is that little protection is provided when the oligonucleotide and/or the AdoHcy is added before the NEM, but this may reflect the equilibrium nature of the reaction studied.

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