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S-adenosyl methionine alters the DNA contacts of the *Eco*KI methyltransferase

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ABSTRACT

The *Eco*KI methyltransferase methylates two adenines on opposite strands of its bipartite DNA recognition sequence AAC(N_6)GTGC. The enzyme has a strong preference for hemimethylated DNA substrates, but the methylation state of the DNA does not influence its binding affinity. Methylation interference was used to compare the contacts made by the *Eco*KI methyltransferase with unmodified, hemimethylated or fully modified DNAs. Contacts were seen at or near the N7 position of guanine, in the major groove, for all of the guanines in the *Eco*KI recognition sequence, and at two guanines on the edge of the intervening spacer sequence. The presence of the cofactor and methyl donor *S*-adenosyl methionine had a striking effect on the interference pattern for unmodified DNA which could not be mimicked by the presence of the cofactor analogue *S*-adenosyl homocysteine. In contrast, *S*-adenosyl methionine had no effect on the interference patterns for either kind of hemimethylated DNA, or for fully modified DNA. Differences between the interference patterns for the unmodified DNA and any of the three forms of methylated DNA provide evidence that methylation of the target sequence influences the conformation of the protein–DNA interface, and illustrate the importance of *S*-adenosyl methionine in the distinction between unmodified and methylated DNA by the methyltransferase.

INTRODUCTION

The methyltransferase (mtase) of the type IA restriction–modification system (see 1–3 for reviews) of *Escherichia coli* K-12 (*Eco*KI) is a trimeric enzyme, M_2S_1 (4), where the M subunits each contain an *S*-adenosyl methionine (AdoMet) binding site (5). It has recently been shown that a well-conserved amino acid motif found in the N-terminal of the M polypeptide, and common to all adenine mtases, is a crucial part of the AdoMet binding site (6). The S subunit of the mtase imparts DNA sequence specificity and the sequence recognized by *Eco*KI is:

5′-A<u>A</u>C(N_6)GTGC-3′

3′-TTG(N_6)C<u>A</u>CG-5′

where the underlined adenines on opposite strands of the DNA are methylated by the enzyme at their N6 positions. The mtase has a strong preference for hemimethylated DNA and methylates unmodified DNA much more slowly (4,7). Unmodified DNA is the target for restriction by the complete *Eco*KI restriction enzyme, which contains R (restriction) subunits in addition to M and S subunits (8).

Comparison of dissociation constants measured by gel retardation showed that differences in binding affinity contribute to the distinction between specific and non-specific DNAs by the mtase, and that this distinction is increased in the presence of AdoMet (5). The binding affinities of the mtase for unmodified, hemimethylated and fully modified specific DNAs are very similar, suggesting that the preference of the enzyme for hemimethylated DNA is effected mainly at the level of catalysis (5).

In ternary complexes of mtase, DNA and AdoMet, the methylation state of the DNA dictates conformational differences resulting in changes in migration in non-denaturing polyacrylamide gels (5) and variation in susceptibility to proteolysis (9). The methylation interference experiments described here have been used to probe the DNA contacts made in complexes of the mtase with DNAs of different methylation state, and to investigate the effects of AdoMet and *S*-adenosyl homocysteine (AdoHcy) on these contacts.

Interference methods have been used to show major and minor groove DNA contacts for many DNA binding proteins (10–14). Our methylation interference results provide the first evidence that the *Eco*KI mtase makes contacts in the major groove of its substrate DNA; there was no evidence for contacts in the minor groove. The presence of the cofactor and methyl donor, AdoMet, had a striking effect on the interference pattern for unmodified DNA but not for hemimethylated and fully modified DNA. The AdoMet analogue AdoHcy had no effect on any of the interference patterns. The results show that methylation of the target sequence influences the conformation of the protein–DNA interface and that AdoMet plays an important role in the distinction between unmodified and methylated DNA by the mtase.

MATERIALS AND METHODS

Chemicals

AdoMet was obtained from New England Biolabs and AdoHcy from Sigma Chemical Company. [γ-^{32}P]ATP (3000 Ci/mmol)

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was obtained from Amersham and T4 polynucleotide kinase (PNK) was supplied by S. Bruce (Institute of Cell and Molecular Biology, University of Edinburgh). Forty percent acrylamide stock solution (19:1 acrylamide to bis-acrylamide) and 30% acrylamide stock solution (37.5:1 acrylamide to bis-acrylamide) were purchased from NBL Gene Sciences. Bromophenol blue, xylene cyanol, tris (hydroxymethyl) aminomethane (Tris), formic acid and β-mercaptoethanol were purchased from Sigma. Glycerol was obtained from Fisons and dimethyl sulphate from Aldrich.

*Eco*KI mtase was purified and stored as described previously (4). The mtase was prepared for use in the binding reactions as described previously (5). Protein, AdoMet and AdoHcy concentrations were determined by UV absorption spectrum measurements, also as described previously (5).

Oligonucleotides and end-labelling

Synthetic oligonucleotides were prepared by OSWEL DNA (University of Edinburgh). DNAs used for the methylation interference experiments were unmodifed, hemimethylated or fully methylated specific 45mers containing the recognition sequence:

5′-TGTCTAGATATCGGCCTA**A**CCACGTGG**T**GCGTACGA-GCTCAGGCG-3′

and its complementary sequence (the *Eco*KI recognition sequence is shown in bold letters; the underlined A denotes the adenine in the trinucleotide part of the recognition sequence that is methylated, and the underlined T is opposite the adenine of the complementary sequence which is the other substrate for methylation). DNA end-labelling using [γ-^{32}P]ATP and T4 polynucleotide kinase, DNA duplex preparation and DNA concentration determination were all as described previously (5).

DMS methylation of DNA

DMS methylation was adapted from the method of Siebenlist and Gilbert (15). 100 µl of 'G buffer' (50 mM sodium cacodylate pH 8.0, 0.1 mM EDTA) was mixed with 4 µl of 1 mg/ml sonicated calf thymus DNA and 1 µl DMS (10.6 M) and chilled on ice for 5 min before the addition of 10–14 µl of the appropriate DNA duplex, 5′ end-labelled on one strand, containing ~3 × 10^6 c.p.m. (~10 pmol DNA). The sample was then incubated for 13.5 min on ice, before the addition of 'G stop' solution (1.5 M sodium acetate, 1.0 M Tris–acetate pH 7.5, 1.0 M β-mercaptoethanol, 1.0 mM EDTA), followed by the addition of 750 µl of ethanol at 14 min after the start of the incubation. This was incubated for 20 min at –20°C and then spun for 10 min in the benchtop centrifuge to precipitate the DMS-methylated DNA. The supernatant was removed and ^{32}P monitored to check for the efficient recovery of DNA in the pellet. The pellet was washed with 70% ethanol, respun for 2 min and dried in a centrifugal evaporator (Gene-Vac CE100), then resuspended in 80 µl of 20 mM Tris–HCl pH 8.0, 100 mM NaCl and transferred to a fresh tube. 0.5 µl of this was spotted on a DE81 filter (Whatman), dried, and 2.5 ml of Ecoscint (National Diagnostics) added prior to scintillation counting using a Beckmann LS 7000 scintillation counter. 350 000 c.p.m. of DMS-methylated DNA was used in each binding reaction.

Preparative band-shift electrophoresis assay

Binding reactions contained 0–1 µM *Eco*KI mtase and 10–30 nM DMS-methylated DNA in 20 mM Tris–HCl pH 8.0, 100 mM NaCl, 5% glycerol (100 µl total volume), and were incubated for 10 min at 0 or 22°C, prior to electrophoresis at 4 or 22°C, respectively. The cofactor AdoMet or its analogue AdoHcy, if present, were used at 100 µM (both protein and DNA were preincubated with this concentration for 10 min prior to use in the binding reactions). Five percent (w/v) polyacrylamide non-denaturing gels, with 90 mM Tris–borate, 2 mM EDTA, pH 8.3 (TBE) running buffer, were used to separate the free DNA and DNA–protein complexes as described previously (5). The gels and running buffer were pre-equilibrated and run at either 4 or 22°C. Following electrophoresis the wet gels were wrapped in plastic film (Saran wrap), marked with Tracker-tape (Amersham) and autoradiographed using 30 min to 1 h exposure.

Elution of DNA from gel

After developing the autoradiograph, the bands of interest were excised from the gel and transferred to siliconized microtubes. 500 µl of elution buffer (0.5 M ammonium acetate pH 7.5, 1 mM EDTA, 0.1% SDS, 10 mM $MgCl_2$) (16) was added. After overnight incubation (37°C on rotating wheel) the elution buffer was removed from the gel slices and filtered through Acrodisc 0.22 µm filters into non-siliconized microtubes, and the filters washed with a further 100 µl of elution buffer, before the addition of 2 µl of 1 mg/ml sonicated calf thymus DNA and 1 ml of ethanol. The samples were chilled at –20°C for ~30 min, spun for 30 min in a microcentrifuge and the supernatant removed. The pellets were washed with 70% ethanol and dried, then piperidine cleavage was carried out as described below.

Piperidine cleavage of DNA

For the G cleavage reactions, 150 µl of 1 M piperidine (freshly diluted with distilled water from 10 M stock) was added to the DNA and the tubes were sealed with parafilm and lidlocks. The tubes were heated at 90°C for 30 min then quenched on ice before the piperidine was removed by evaporation in the centrifugal evaporator. The DNA was resuspended in 100 µl H_2O and transferred to a fresh tube before drying again for 30 min. The samples were resuspended and dried again twice in order to ensure that all traces of piperidine were removed, as piperidine interferes with the subsequent analysis. After the final drying step 10 µl of sequencing gel loading buffer [90% (v/v) formamide, 10 mM EDTA, 0.025% (w/v) bromophenol blue and 0.025% (w/v) xylene cyanol FF) was added to each sample and 0.5 µl was used for scintillation counting.

For cleavage at both A and G an extra step was included prior to the piperidine cleavage step. After ethanol precipitation of the DNA recovered from the preparative band-shift gel, the pellets were resuspended in 100 µl of 20 mM ammonium acetate pH 7.5, 10 mM EDTA and heated for 15 min at 90°C. Following this 10 µl of 10 M piperidine was added and piperidine cleavage carried out as described above.

Preparation of A+G sequencing ladder

A rapid method for preparation of an A+G sequencing ladder was as described by Papavassiliou (16).

Separation of cleavage products on sequencing gel

Equal counts of the DNA samples to be compared (e.g. free and bound samples with appropriate A+G standards) were loaded on a 12% polyacrylamide, 7.1 M urea, 0.5 × TBE sequencing gel and run for 1.5 h at 38 W. The gels were dried for 1 h at 80°C and the bands visualized by autoradiography.

*Hinc*II assay for methylation by *Eco*KI

This assay (17) is based on the methylation sensitivity of a type II restriction enzyme *Hinc*II, which recognizes the hexanucleotide sequence GTPyPuAC. A similar assay described previously used *Hpa*I (18). Synthetic DNA substrates were designed containing a *Hinc*II site overlapping the trinucleotide part of the *Eco*KI recognition sequence. Unmodified or hemimethylated substrates were used, and in the latter case the methylated adenine was in the tetranucleotide part of the recognition sequence. Methylation by the *Eco*KI mtase at the adenine in the trinucleotide part of its recognition sequence (A<u>A</u>C) renders the DNA resistant to cutting by *Hinc*II. Synthetic oligonucleotides for this assay were 45mers:

5′-TGTCTAGATATCGG<u>GTTAAC</u>CACGTG**GTGC**GTACGA-GCTCAGGCG-3′

The *Eco*KI site is shown in bold, while the *Hinc*II site overlapping the trinucleotide part of the recognition site is underlined. Unmodified or hemimethylated substrates were obtained by hybridizing the above to an unmethylated complementary oligonucleotide, or to an oligonucleotide methylated at the adenine in the tetranucleotide part of the *Eco*KI recognition sequence. Prior to hybridization, the top strand was 5′ end-labelled with [γ-^{32}P]ATP as described for the oligonucleotides used for methylation interference (see above).

DNA (10 nM unmodified or hemimethylated) was incubated with *Eco*KI mtase (0–50 nM) in 20 mM Tris–HCl pH 8.0, 100 mM NaCl and 50 μM AdoMet (100 μl total volume), for 0, 5, 10 or 15 min, at 0 or 22°C. After the appropriate incubation times, 1 μl samples were removed and heat-inactivated at 65°C for 15 min, then cooled on ice before being digested with *Hinc*II (2 h, 37°C). The digestion products were diluted 1:1 with non-denaturing loading buffer (10% glycerol, 0.025% bromophenol blue, 0.025% xylene cyanol and 1 × TBE) and run on a non-denaturing 10% polyacrylamide gel for 1 h at 25 mA until the bromophenol blue was halfway down the gel. The gel was dried and the bands visualized by autoradiography. Methylation by the *Eco*KI mtase resulted in an uncleaved 45mer while DNA not methylated at the AAC sequence was cleaved by *Hinc*II and visualized as a 17mer product.

RESULTS

In interference methods of footprinting the effects of certain chemical modifications of DNA are assessed by their effect on the binding by protein. Dimethyl sulphate (DMS) methylates guanine at the N7 position in the major groove, and also adenine at the N3 position in the minor groove (15). ^{32}P end-labelled DNA is lightly methylated by DMS, ideally giving one modification per DNA molecule, before binding to the protein. Free and bound DNA are then separated by non-denaturing gel electrophoresis and, following autoradiography, eluted from the gel. The DNA is cleaved at methylated purine residues and the products, for both free and bound DNA fractions, are run on a denaturing polyacrylamide gel. This allows determination of the G and A positions at which DMS-methylation gives rise to interference with binding. The lack of a band corresponding to a particular G residue in the bound fraction of the DNA indicates an important major groove interaction at or near to the N7 position of the guanine in question, whereas the lack of a band corresponding to a particular adenine residue shows a contact in the minor groove at or near to its N3 position. However, it has been suggested that N3 methylation may also result in disruption of hydrogen bonding to the N7 of adenine, in the major groove (19), so some caution is advisable in interpreting interference at adenines.

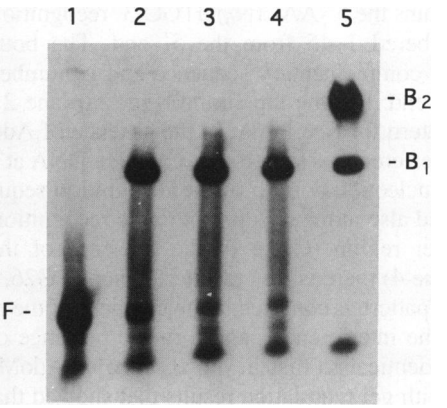

Figure 1. Preparative band-shift gel for unmodified DNA. Unmodified specific 45mer 5′ end-labelled using [γ-^{32}P]ATP and T4 PNK was incubated (10 min, 22°C) with 0, 22.5, 45, 90 or 900 nM mtase prior to loading to lanes 1–5 respectively of a non-denaturing 5% polyacrylamide gel. Electrophoresis was for 2 h at 30 mA constant current. Free DNA (F) and bound DNA (B_1 and B_2) are indicated.

Methylation interference was used to investigate DNA contacts in DNA–mtase complexes for unmodified, hemimethylated and fully modified DNA containing the *Eco*KI recognition sequence. The effects of the cofactor AdoMet and the cofactor analogue AdoHcy were observed. The use of preparative band-shift gels (Fig. 1) allowed comparison of DNA–mtase complexes of different mobilities. In Figure 1, free (F) and two bound species (B_1 and B_2) of DNA are shown where B_1 is the usual 1:1 specific complex with protein, and B_2 a species which arises at relatively high protein concentrations, and is thought to contain more than one protein per DNA molecule (5). Interference patterns produced by the modification of the N7 of guanines and the N3 of adenines were investigated, but in the latter case no interference was seen (results not shown). The G-specific cleavage reactions revealed interference at G residues in and adjacent to the DNA recognition sequence. For each DNA investigated the result of one experiment only is given. However, at least three repeats of the methylation interference experiment were done for each DNA, giving consistent interference patterns in each case, independent of the protein concentration used in the binding reactions over a range of 10–100 nM or 25–100 nM mtase in the presence or absence of AdoMet, respectively.

Methylation interference for unmodified DNA

The results of a methylation interference experiment for unmodified DNA are shown in Figure 2. The top strand of the 45mer

duplex contains the 5′-AAC(N_6)GTGC-3′ recognition sequence and is numbered 1–45 from the 5′ end. The bottom strand contains the complementary sequence and is numbered 1′–45′ from the 3′ end. For the top strand (Fig. 2a) lane 2 shows the cleavage pattern for free DNA. In the absence of AdoMet (lane 3) strong interference is seen for B_1 complex DNA at the two Gs in the tetranucleotide portion of the recognition sequence (G27 and G29) and also at the G adjacent to the recognition sequence in the spacer region (G26). In the presence of the cofactor AdoMet (lane 4) there is loss of interference at G26, so that the interference pattern is confined to the guanines of the recognition sequence. The interference pattern in the presence of AdoHcy (lane 5) is identical to that in the absence of AdoMet. This is consistent with gel retardation results that showed that AdoHcy could not mimic AdoMet as an enhancer of DNA binding by the mtase (5). The cleavage pattern for B_2 complex DNA (lane 6) is identical to that for free DNA, showing that this complex is non-specific.

A similar pattern of interference is seen for the bottom strand (Fig. 2b). In the absence of cofactor (lane 3) and in the presence of AdoHcy (lane 5) there is strong interference at three guanine residues: G30′ in the tetranucleotide part of the recognition sequence, G20′ in the trinucleotide part of the recognition sequence and G21′ in the spacer region adjacent to the trinucleotide part of the recognition sequence. In the presence of AdoMet there is loss of interference at G21′ in a manner analogous to the loss of interference at the spacer region guanine of the top strand (G26).

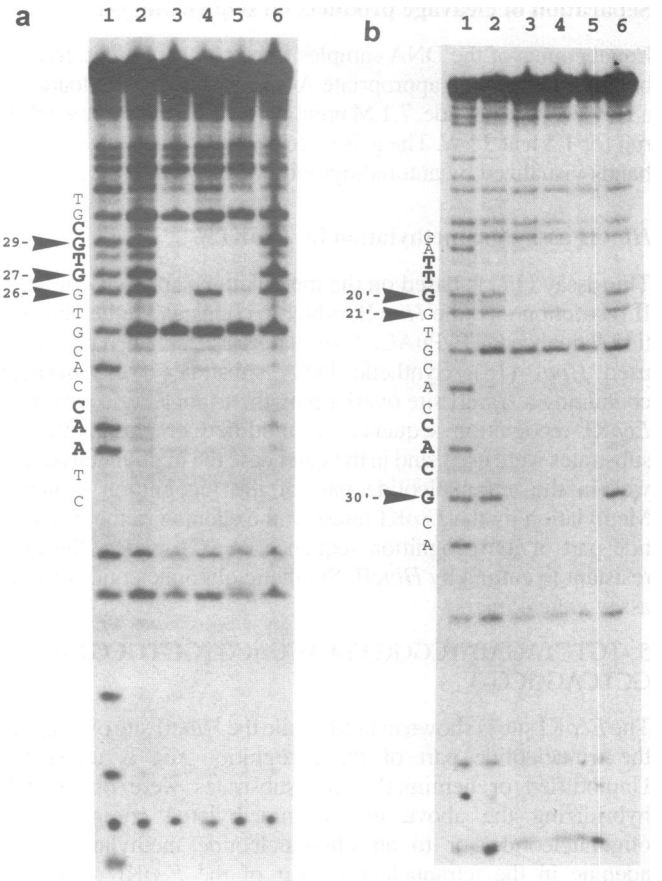

Figure 2. Methylation interference for unmodified DNA. Unmodified specific 45mer ^{32}P 5′ end-labelled on (**a**) the top or (**b**) the bottom strand respectively was treated with dimethyl sulphate. The DMS-methylated DNA (10 nM) was incubated with mtase at 22°C (as described in Materials and Methods) in a total volume of 100 µl, and free DNA (F) and mtase–DNA complex (B_1 or B_2) were separated by electrophoresis in a non-denaturing 5% polyacrylamide gel at 22°C (see Fig. 1). Following elution the DNA was treated with piperidine and the resulting cleavage products were separated on a denaturing 12% polyacrylamide–urea gel. Lane 1 for each strand contains an A+G reaction. Mtase concentrations used for each strand were 0 (lane 2, free DNA), 25 nM (lanes 3–5, B_1 DNA) or 1 µM (lane 6, B_2 DNA). AdoMet and AdoHcy were present at 100 µM for lanes 4 and 5 respectively. The arrows indicate DMS-methylated guanines that interfere with mtase binding. The *Eco*KI recognition sequence is shown in larger type than the surrounding and spacer sequences (N.B. The same interference pattern was obtained when the binding reactions were done at 0°C and the preparative bandshift at 4°C).

Effect of temperature on the methylation rate of hemimethylated DNA by mtase

In order to probe the interactions in DNA–protein complexes containing hemimethylated DNA we wanted to ensure that there was minimal DNA methylation during the incubation prior to separation of the bound and free DNA on the non-denaturing gel. AdoHcy cannot be used to mimic AdoMet in the binding reactions (5) and we therefore lowered the temperature of the incubations in order to reduce methylation in the presence of the methyl donor AdoMet. In order to test the amount of methylation resulting from incubation at 0 or 22°C we used an assay (17) based on the methylation sensitivity of a type II restriction enzyme *Hin*cII (Fig. 3). Lanes 9–16 show the susceptibility of 'hemimethylated' DNA or unmodified DNA to cleavage by *Hin*cII after 0, 5, 10 or 15 min incubation with *Eco*KI mtase at 22°C. The proportion of the hemimethylated DNA that becomes methylated during the incubation increases to almost 100% after 15 min as indicated by the increased resistance to *Hin*cII cleavage (Fig. 3, lanes 9, 11, 13 and 15), whereas the unmodified DNA substrate remains susceptible to *Hin*cII cleavage (lanes 10, 12, 14 and 16). At 0°C (Fig. 3, lanes 1–8) there is no detectable methylation of unmodified DNA (lanes 2, 4, 6 and 8), and most of the hemimethylated DNA (lanes 1, 3, 5 and 7) remains unaltered even after 15 min incubation (lane 7). Therefore, for the methylation interference experiments described below, the incubations were at 0°C prior to the preparative band-shift step, to minimize the methylation of the hemimethylated DNA substrate, and the non-denaturing gel was equilibrated and run at 4°C. It should be noted, however, that the interference patterns for fully modified and hemimethylated DNAs were unaltered when the incubations and preparative band-shift step were carried out at 22°C, instead of 0 and 4°C respectively. Similarly, the interference patterns for unmodified DNA were the same when the binding was at 0°C and the preparative bandshift at 4°C, rather than 22°C (results not shown).

Methylation interference for hemimethylated DNA and fully modified DNA

For hemimethylated DNA (Fig. 4) and for fully modified DNA (Fig. 5) the interference patterns are very similar. The results shown in Figure 4 are for hemimethylated DNA with the methyl group in the tetranucleotide part of the recognition sequence, but the same interference pattern was observed for the other hemimethylated DNA with the methyl group in the trinucleotide

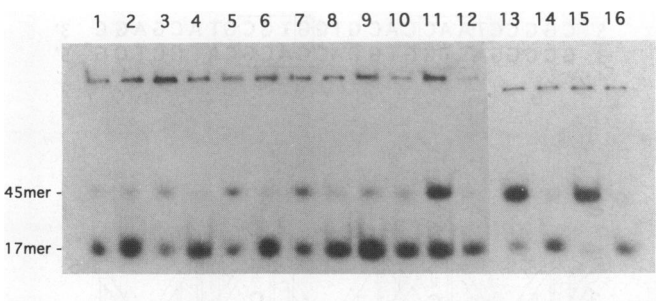

Figure 3. *Hin*cII assay for DNA methylation by mtase at 0 and 22°C. Hemimethylated or unmodified 45mers (10 nM) containing overlapping *Eco*KI and *Hin*cII recognition sites (see Materials and Methods) were incubated in 20 mM Tris–HCl pH 8.0, 100 mM NaCl, 5% glycerol with 24 nM mtase and 50 μM AdoMet (100 μl total volume) at 0 or 22°C, for 5, 10 or 15 min before 1 μl samples were removed and heat-inactivated at 65°C for 10 min. These samples were digested with *Hin*cII for 2 h, as was DNA removed before the addition of mtase (i.e. '0 min' incubation). The digested samples were loaded onto a non-denaturing 10% polyacrylamide gel which was run for 1 h at 25 mA. Hemimethylated and unmodified DNA were loaded in alternate lanes. Lanes 1–8 contained samples digested after incubation at 0°C and lanes 9–16 contained samples from 22°C incubations. Lanes 1, 3, 5 and 7 (0°C) contained hemimethylated DNA incubated for 0, 5, 10 and 15 min respectively prior to *Hin*cII digestion, as did lanes 9, 11, 13 and 15 (22°C). Lanes 2, 4, 6 and 8 (0°C) contained unmodified DNA incubated for 0, 5, 10 and 15 min respectively prior to *Hin*cII digestion, as did lanes 10, 12, 14 and 16 (22°C).

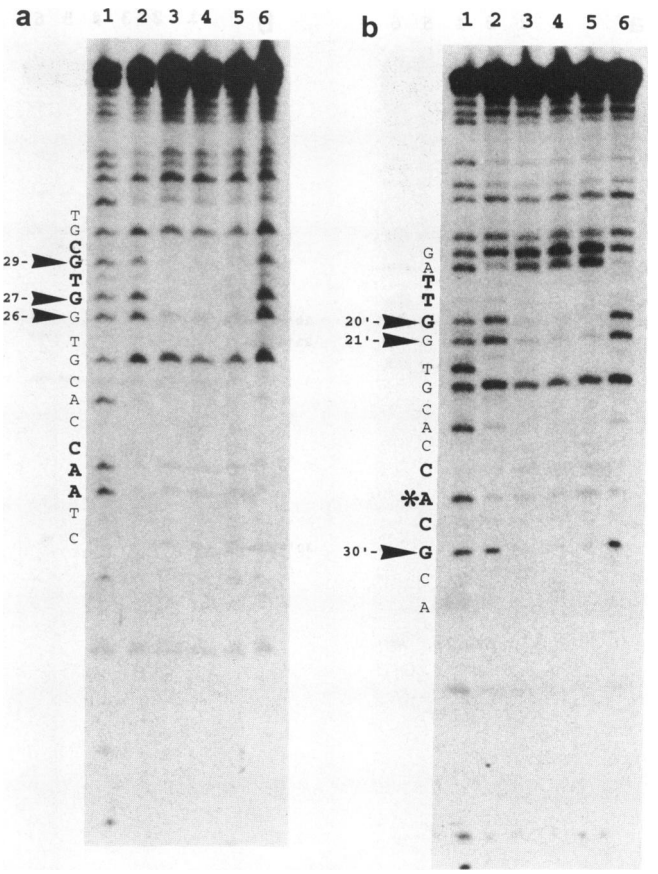

Figure 4. Methylation interference for hemimethylated DNA. Hemimethylated specific 45-mer ^{32}P 5′ end-labelled on (**a**) the top or (**b**) the bottom strand respectively was treated with DMS and the DMS-methylated DNA (12.8 nM) incubated with mtase. The binding reactions (100 μl) were incubated at 0°C to minimize methylation of the DNA in the presence of AdoMet and the preparative bandshift gel was pre-equilibrated and run at 4°C. The DNA was processed as described for Figure 2. The mtase concentrations used in the binding reactions for each strand were 0 (lane 2, free DNA), 100 nM (lane 3, B_1 DNA), 50 nM (lanes 4 and 5, B_1 DNA) or 1 μM (lane 6, B_2 DNA). AdoMet and AdoHcy were included at 100 μM for the sample for lanes 4 and 5 respectively. Lane 1 for each strand contains an A+G reaction. The hemimethylated DNA used was methylated at the adenine in the tetranucleotide part of the recognition sequence as indicated by the asterisk. The arrows indicate DMS-methylated guanines that interfere with binding.

part of the recognition sequence (results not shown). For the top strand in the 1:1 complex for either hemimethylated (Fig. 4a, lane 3) or for fully modified DNA (Fig. 5, lane 3), there is strong interference at G27 and G29 in the tetranucleotide part of the recognition sequence, but the interference is slightly less pronounced at G26 in the spacer region of the DNA and a faint band corresponding to this G can be seen. In the bottom strand there is strong interference at G30′ in the tetranucleotide part of the recognition sequence and G20′ in the trinucleotide part of the recognition sequence, but the interference is once again slightly less pronounced at G21′ in the spacer region. In the A+G tracks for the bottom strands that contain N6 methyl-adenine at position 28′ (Fig. 4b, lane 1 and Fig. 5b, lane 1), an anomalous strong band was obtained at T22′, the reason for which is not known.

In contrast to unmodified DNA, the presence of AdoMet has no effect on the interference pattern for hemimethylated DNA (lane 4 of Fig. 4a and b) or for fully-modified DNA (lane 4 of Fig. 5a and b) and there is no change in the interference at the Gs in the spacer region for either the top (G26) or the bottom strand (G21′). This is in spite of the fact that the presence of AdoMet enhances mtase binding to these substrates as it does to unmodified DNA (5). Although an AdoMet-dependent change in contacts to the DNA was observed only for the unmodified DNA, other contacts not detectable by methylation interference could be altered for the complexes with the methylated DNAs. In the same vein it is perhaps surprising that both kinds of hemimethylated DNA and fully modified DNA gave the same interference pattern, but differences in contacts could also exist in these complexes that are not detected by this interference method. The results for the slower-migrating B_2 complex (lane 6, Fig. 5a and b and Fig. 6a and b) are again the same as for free DNA, confirming that this is a non-specific complex unaffected by the methylation state of the DNA.

In addition to the loss or reduction of guanine bands in the specific (B_1) complex lanes, for hemimethylated and fully modified DNA, an increase in the intensity of two bands adjacent to the trimeric part of the recognition sequence on the bottom strand is seen at G16′ and A17′ regardless of the presence or absence of AdoMet . The significance of this is not known, but it appears that DMS-methylation at these positions is beneficial to binding. This suggests that the sequence adjacent to the recognition site can influence binding and this could in turn lead to different rates of methylation at different recognition sites depending upon flanking sequences. It has been shown that sequences flanking the recognition sites for *Eco*RI and *Eco*RV endonucleases can affect their activity (20,21 respectively). In addition bases flanking the *Dam* mtase recognition site appear to be involved in DNA recognition by this enzyme (22).

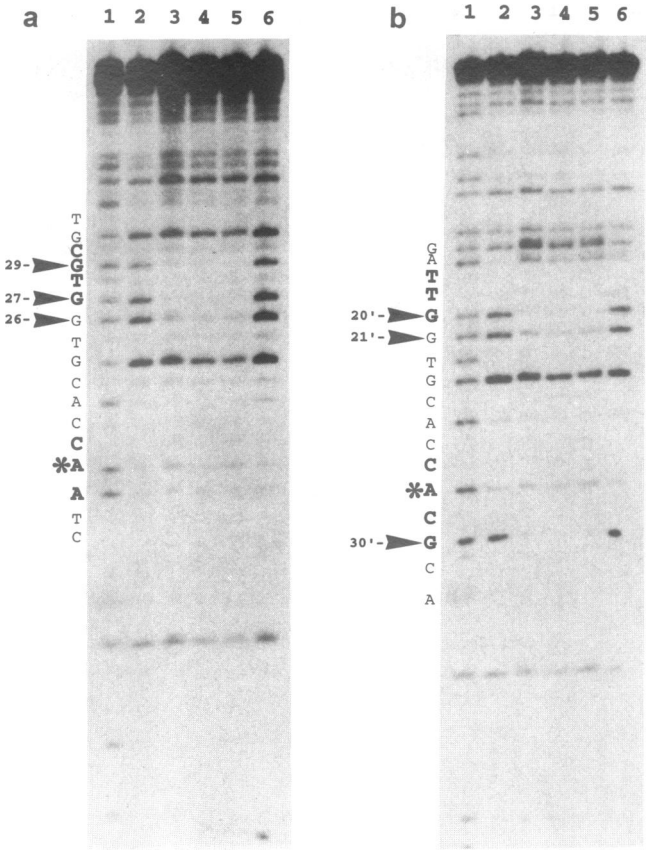

Figure 5. Methylation interference for fully modified DNA. Fully-modified specific 45mer ^{32}P 5′ end-labelled on (**a**) the top or (**b**) the bottom strands respectively were treated with DMS and then incubated with mtase at 0°C prior to loading onto a 5% non-denaturing gel pre-equilibrated and run at 4°C. The DNA was processed as described for Figure 2. Mtase concentrations used in the binding reactions (100 µl) for each strand were 0 (lane 2, free DNA), 100 nM (lane 3, B_1 DNA), 50 nM (lanes 4 and 5, B_1 DNA) or 1 µM (lane 6, B_2 DNA). DNA was at 12.8 nM. AdoMet and AdoHcy were included at 100 µM for the samples for lanes 4 and 5 respectively. Lane 1 for each strand contains an A+G reaction. The methylated adenines in the tri- and tetranucleotide parts of the recognition sequence are marked with asterisks. The arrows indicate DMS-methylated guanines that interfere with mtase binding.

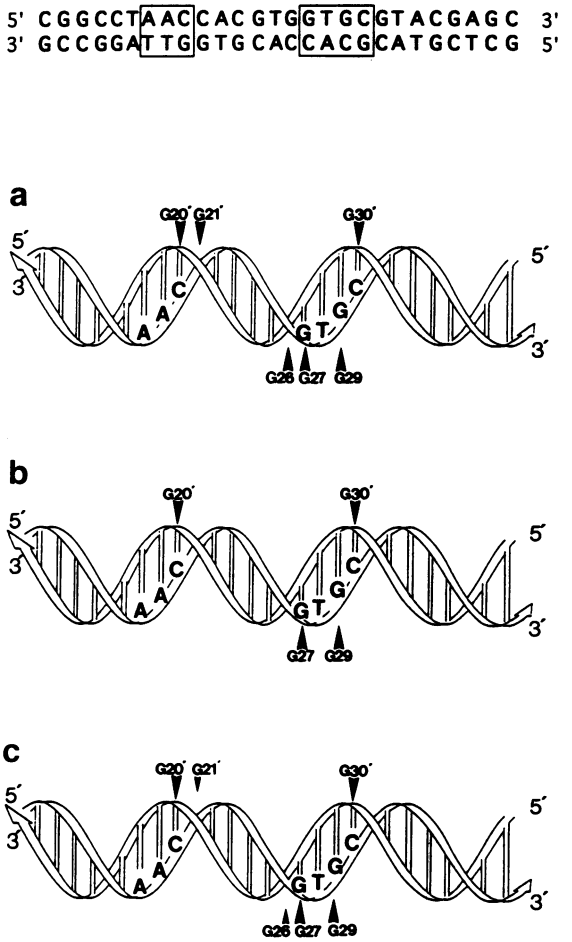

Figure 6. Summary of methylation interference results. Schematic diagram showing the DMS-methylated guanines that interfere with protein binding for (**a**) unmodified DNA in the absence of AdoMet, or in the presence of AdoHcy, (**b**) unmodified DNA in the presence of AdoMet or (**c**) hemimethylated or fully modified DNA in the presence or absence of AdoMet, or the presence of AdoHcy. The larger arrows illustrate strong interference at the G indicated, while the smaller ones show weaker interference. The sequence for base pairs 12–38 of the 45mer duplexes used is shown at the top of the diagram, with the bipartite recognition sequence outlined. The recognition sequence is also indicated on the B-form DNA.

DISCUSSION

*Eco*KI methyltransferase contacts its substrate DNA via the major groove in a manner affected by DNA methylation

Our results for the methylation interference experiments with unmodified, hemimethylated and fully modified DNAs (Figs 2, 4 and 5) show that the *Eco*KI mtase interacts with its recognition sequence via contacts in the major groove, and demonstrate a clear distinction between unmodified and modified DNAs. However, this method reveals no differences in contacts between hemimethylated and fully modified DNAs. There are contacts at or near the N7 of all the guanines in the *Eco*KI recognition sequence regardless of the methylation state of the DNA. This is true even for fully methylated DNA which is not a substrate for either the *Eco*KI methyltransferase or the complete restriction endonuclease. This is in contrast to results for the *Eco*RI endonuclease where methylation interference was seen with a duplex containing an unmodified *Eco*RI site but not with a methylated site (23). For the *Eco*KI mtase, in addition to the recognition sequence contacts, there are contacts at two Gs in the spacer region (G26 of the top strand and G21′ of the bottom) for complexes with unmodified DNA in the absence of AdoMet, and slightly weaker contacts at these two Gs for hemimethylated and fully modified DNA in the presence or absence of AdoMet (see Fig. 6 for summary). The presence of AdoMet only affects the contacts for unmodified DNA. The contacts in the spacer region are not seen for mtase–unmodified DNA complexes in the presence of AdoMet, and therefore AdoMet enhances the distinction between unmodified and methylated DNA. The cofactor analogue AdoHcy has no effect on the contacts for any of the DNAs, consistent with gel retardation results that showed AdoHcy could not substitute for AdoMet in its role as an enhancer of DNA binding by the mtase (5). When methylation interference was used

to probe mtase contacts to the N3 position of adenines in the sequence, i.e. minor groove contacts, none was revealed. Contacts by the *Eco*KI mtase to the major groove of the two halves of the bipartite recognition sequence are consistent with the postulated S subunit structure.

The specificity of DNA recognition by the *Eco*KI mtase (in common with all type I restriction–modification systems) lies within two long target recognition domains (TRDs) in the S polypeptide, where the amino TRD recognizes the trinucleotide part of the recognition sequence and the carboxy TRD recognizes the tetranucleotide part (24). The S polypeptide also has two shorter regions that are well-conserved within the type IA family (25). Repeat sequences found in the conserved regions of type IA, IB and IC S polypeptides (26) have been implicated in interactions with M subunits for the type IC mtases *Eco*DXXI (27) and *Eco*R124I (28). Limited proteolysis of the *Eco*KI mtase has indicated interactions between the central conserved region of the S polypeptide and the carboxyl domain of the M polypeptide (9).

The structure of a type I mtase has been postulated to be a symmetrical structure by way of a 'circular' organization of the S polypeptide (6,9,27,29) with the two TRDs spaced appropriately by the conserved regions. The M subunits are placed on either side of the conserved regions of the S subunit, one interacting with the repeat sequences in the central conserved region and the other with the carboxyl conserved region of the S polypeptide. Our results are consistent with the two TRDs of the specificity subunit contacting the major groove of the DNA at their respective halves of the bipartite recognition sequence and UV crosslinking results have recently shown that the amino target recognition domain of the S polypeptide contacts the major groove of the trinucleotide part of the recognition sequence (30). The tri- and tetranucleotide parts of the recognition sequence are separated by one turn of the DNA, and therefore the N6 positions of the substrate adenines project into the major groove on the same face of the DNA helix, on either side of the minor groove (1,31). Therefore one would envisage the conserved regions of S covering the intervening minor groove between the two TRDs.

It has been implied previously that the specificity subunit of the mtase recognizes the DNA recognition site via the major groove (1), but a model has also been proposed in which sequence recognition is in the minor groove to allow space for methylation by the M subunits at the adenine N6 position in the major groove (31). The spatial difficulties in major groove recognition of the sequence by S, and methylation at sites in the major groove by M may be reconciled if the *Eco*KI mtase can flip its target adenines out of the DNA helix, while still using the major groove for sequence recognition, in the same way as the *Hha*I C5 cytosine mtase (32).

Crystal structures recently determined for the type II mtases *Hha*I (32) and *Taq*I (33) show that these enzymes consist of an AdoMet-binding catalytic domain and a DNA recognition domain. In the case of the *Hha*I mtase, substrate DNA is bound in a cleft between these two domains, which close around the DNA, and the DNA recognition domain makes all but one of the specific protein–DNA contacts in the major groove. The catalytic domain is placed on the minor groove to interact with the substrate cytosine base which is dramatically flipped out of the DNA helix. Flipping out a base was proposed to be a general mechanism for DNA-modifying enzymes including N6-adenine mtases (32). It has been suggested that a type I mtase resembles two type II mtases stacked together (6,9). One could imagine the M subunits interacting via their carboxyl domains with the conserved regions of the S polypeptides allowing the positioning of the M polypeptide catalytic domains on the minor groove of the DNA, in a position to methylate a flipped-out adenine, after closing around the DNA. This is consistent with the recent finding that DNA binding induces a large change in the quaternary structure of the type IC mtase M.*Eco*R124I as shown by small angle X-ray scattering (34). Near UV circular dichroism measurements showed an accompanying distortion in the DNA tertiary structure. No differences were observed between complexes containing unmodified DNA and those with hemimethylated DNAs for this major structural transition, but the authors suggest that DNA methylation may cause smaller, more localised conformational effects in the mtase. Our results for the *Eco*KI mtase reveal changes in DNA contacts between unmethylated and methylated DNA that could be related to such localized conformational effects in the mtase subunits. These differences are particularly clear in the presence of the cofactor and methyl donor AdoMet.

Effects of cofactor AdoMet on DNA–protein contacts

The presence of the cofactor AdoMet has a striking effect on the interference pattern for unmodified DNA (Fig. 2) while it does not affect the interference pattern for hemimethylated or fully modified DNAs. In the presence of AdoMet, the interference at G26 and G21′ in the spacer sequence is lost for unmodified DNA, resulting in an apparent narrowing of the important major groove contacts to bases of the recognition sequence. The interference patterns for complexes of the *Eco*KI mtase with hemimethylated and fully modified DNAs are unchanged in the presence of AdoMet, indicating interactions with the DNA major groove of the spacer region both in the presence and absence of the cofactor.

The results demonstrate that AdoMet is important for discrimination between unmodified and modified DNA by the mtase. Discrimination between DNAs of different methylation state is also important for the complete *Eco*KI restriction endonuclease. The restriction enzyme contains R (restriction) subunits in addition to the M and S subunits needed to make the mtase (8), and has different activities depending upon the methylation state of the DNA (31,35). The restriction enzyme with AdoMet bound forms a recognition complex with DNA containing the *Eco*KI recognition site regardless of its methylation state (35). Following ATP binding, if the DNA substrate is fully modified there is no reaction and the enzyme dissociates; if the substrate is hemimethylated, methylation occurs at the unmethylated adenine, while unmodified DNA is cleaved at a site distant from the recognition sequence, in a reaction requiring Mg^{2+}, ATP and AdoMet. For the complete restriction endonuclease AdoMet acts as an allosteric effector in the restriction reaction (36) and as both allosteric effector and methyl donor in the methylation reaction (18). It has been suggested that the *Eco*KI enzyme uses the methyl group of bound AdoMet to probe for methyl groups on its substrate DNA in order to detect the methylation state of the DNA in the recognition complex (31). The importance of AdoMet in the distinction between methylated and unmodified DNAs demonstrated by the methylation interference results suggests the mtase may use AdoMet in this way. Extrapolating these results to the complete restriction enzyme it seems likely that AdoMet signals the enzyme to switch between restriction and modifica-

tion modes via a conformational change with altered base contacts.

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