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Substrate recognition and selectivity in the type IC DNA modification methylase M.*Eco*R124I

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ABSTRACT

The type I DNA modification methylase M.*Eco*R124I binds sequence specifically to DNA and protects a 25bp fragment containing its cognate recognition sequence from digestion by exonuclease III. Using modified synthetic oligonucleotide duplexes we have investigated the catalytic properties of the methylase, and have established that a specific adenine on each strand of DNA is the site of methylation. We show that the rate of methylation of each adenine is increased at least 100 fold by prior methylation at the other site. However, this is accompanied by a significant decrease in the affinity of the methylase for these substrates according to competitive gel retardation assays. In contrast, methylation of an adenine in the recognition site which is not a target for the enzyme results in only a small decrease in both DNA binding affinity and rate of methylation by the enzyme.

INTRODUCTION

Restriction and Modification (R-M) systems can be categorised as Types I, II and III [1]. Type II R-M systems have been well studied in recent years, due in large measure to the availability of a number of such enzymes from overexpressing strains [2]. The restriction (endonuclease) and modification (methyltransferase) activities of Type II R-M systems are confined to separate enzymes, which typically exist as monomers (in the case of the methylase) or homodimers (in the case of the endonuclease). The more complex Type I R-M systems, although the first to be discovered, have only recently been expressed in sufficient quantities for detailed *in vitro* characterisation [3,4]. Type I R-M systems consist of complex multisubunit enzymes which can exhibit both restriction endonuclease and modification methylase activity. Restriction endonuclease activity results in cleavage of unmodified DNA some distance from the recognition site; methyltransferase activity is confined to the methylation of individual adenines on opposite strands within the enzyme recognition sequence. The normal biological substrate for modification is hemi-methylated DNA, in which only one strand of the recognition sequence is methylated following replication of the bacterial genome [5].

Type I R-M systems have been classified into three families (IA, IB and IC) using a variety of genetic and biochemical criteria [6,7,8]. All type I systems consist of three types of subunit HsdS, HsdM and HsdR [9,7,10]. Numerous genetic experiments have shown that the HsdS protein is the determinant of DNA specificity [11,12]. Heteroduplex analysis and comparison of the DNA sequences of the *hsdS* genes for a number of type I enzymes have demonstrated that the HsdS proteins consists of two variable domains, and 2–3 regions that are highly conserved within a given family [13]. Domain swapping experiments in which the variable regions from members of the same family have been swapped have established that the specificity of DNA binding is conferred by the two variable domains of HsdS, the N-terminal variable region being responsible for recognition of the 5′ (trinucleotide) half site and the C-terminal region being responsible for binding the 3′ half site in the bipartite DNA recognition sequence [13,14].

For restriction endonuclease activity all three subunits are needed, together with the requirement for ATP, AdoMet and Mg^{2+}. In the absence of HsdR an enzyme complex can still be formed from the HsdS and HsdM subunits alone [15,16]. This complex maintains methylation activity and in the presence of AdoMet and Mg^{2+} will modify the target adenines in the recognition sequence. The modification enzyme from several type I systems has been isolated. In the only two cases where the overexpressed methyltransferase has been studied in detail (*Eco*R124I and *Eco*KI), the active methylase has been shown to be a trimeric enzyme with a subunit stoichiometry of M_2S [3,4]. For both enzymes, the binding affinity for its cognate DNA recognition sequence is at least $10^8\ M^{-1}$ [3,17].

DNA recognition by type I methylases is of interest for two reasons. Firstly the sequences the enzymes recognise are unusual in that they are bipartite, consisting of two asymmetric half sites separated by 6–8 non-specific bases. For example *Eco*R124I recognises the sequence 5′-GAANNNNNNRTCG-3′. Secondly the methylation state of the recognition site can have a profound effect on the subsequent rate of modification, but this depends on the enzyme. In the case of the type IA methylase *Eco*KI, unmethylated sites are modified very slowly, whereas if the site is hemimethylated the second adenine is rapidly modified [4,15]. The type IB enzyme *Eco*AI modifies both hemimethylated and

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non-modified sites rapidly [7,15]. Neither enzyme shows any catalytic activity if the recognition site is fully methylated.

Here, we are concerned with the modification methyltransferase from the type IC R-M system *Eco*R124I and its ability to discriminate between the various methylation states of its target DNA sequence. We report an investigation into the DNA binding and catalytic activity of the enzyme with a variety of synthetic oligonucleotide duplexes containing methyl groups at each of the adenines within the enzyme recognition sequence.

MATERIALS AND METHODS

Protein purification

*Eco*R124I methylase protein was overexpressed in *E.coli* JM109(DE3) from plasmid pJS4M [18] as described earlier. The multisubunit enzyme was purified to homogeneity from crude cell extracts by ion exchange and heparin chromatography following published procedures [3].

Preparation of oligonucleotide duplexes

Oligonucleotides were either purchased HPLC-purified from Oswel DNA services (University of Edinburgh) or synthesised on a Cruachem PS250 DNA synthesiser and purified on Nensorb-prep columns (Dupont). N6-methyl deoxyadenosine cyanoethyl phosporamidite (Glen Research) was used to incorporate N6-methyl adenine at specific sites within oligonucleotides during the synthesis. The molar extinction coefficient of each oligonucleotide was determined by digestion to completion with snake venom phosphodiesterase and summing the contributions from individual nucleotides. This value was adjusted to give the corrected value for the intact oligonucleotide, taking account of the hyperchromicity observed following digestion [19].

In total, five oligonucleotides were prepared: the unmodifed A-strand, two variants on this sequence bearing methyl groups at adenine 9 or 10, the unmodified B-strand, and a single variant of this sequence in which adenine 13 was methylated (Fig. 1). All pairwise combinations of complementary oligonucleotides were mixed in equimolar proportions, heated to 80°C and allowed to cool to RT to generate 30 b.p. double stranded DNA fragments containing the *Eco*R124I recognition sequence. Six synthetic duplexes were prepared in total: one unmodified (AB), three methylated on one strand (A9B, A10B and AB13) and two that are methylated on both strands (A9B13, A10B13).

^{32}P end labelling of oligonucleotides

Preparation of labelled duplex DNA for gel retardation experiments. Approximately 10μg of duplex was labelled for use in a particular set of gel retardation experiments. Duplex DNA was phosphorylated using T4 polynucleotide kinase as described previously [20]. Labelled nucleic acid was purified from unincorporated material using 'Nuctrap' columns (Stratagene), precipitated with 2.5 volumes of absolute ethanol and washed with 80% ethanol. The pellets were resuspended in 400μl of dH_2O. The concentration of DNA was determined from its absorbance at 260nm (using E_{260}= 396,000 $M^{-1}cm^{-1}$) and the specific activity determined by Cerenkov counting in a liquid scintillation counter.

Preparation of uniquely labelled duplexes for exonuclease III footprinting. Single stranded oligonucleotides were end-labelled and purified using the same method as for double stranded DNA. Material eluted from the Nuctrap column was precipitated with ethanol and redissolved in 50μl of 10mM Tris−HCl (pH 8.2), 1mM EDTA. A slight molar excess of the unlabelled complementary strand was added to the mixture, heated to 80°C for 10 minutes and allowed to cool slowly to room temperature. This annealing mixture was made up to 10% glycerol and applied to a 12% polyacrylamide native TAE gel (40mM Tris-acetate pH 7.4, 1mM EDTA). Gels were electrophoresed in TAE buffer at 15W constant power until the bromophenol blue dye was two thirds down the gel. After electrophoresis plates were prised apart and the gel exposed to X-ray film for 3 minutes. The X-ray film was developed and used as a stencil to cut out a gel slice containing the labelled duplex DNA. The gel slice was crushed by centrifugation in a microfuge tube for 5 minutes and the DNA eluted by shaking the crushed gel slice in 300μl of an elution buffer (0.5M ammonium acetate, 1mM EDTA, 10mM $MgCl_2$, 0.1% SDS) at 37°C for 16 hours. Eluted DNA was precipitated with absolute ethanol, washed in 80% ethanol and redissolved in 400μl of dH_2O. The concentration of labelled duplex was determined from the absorbance at 260nm and the specific activity determined from counting the Cerenkov radiation in a liquid scintillation counter.

Exonuclease III footprinting

A 5μl sample (4pmoles) of uniquely end labelled duplex prepared as described above was mixed with 1μl (5pmoles) of methylase protein in a buffer containing 50mM Tris−HCl (pH 8.2), 5mM $MgCl_2$, 1mM DTT in a total volume of 10μl. The oligonucleotide-protein complex was digested with Exonuclease III (6400 units/ml) at room temperature for 30 minutes. A control sample of oligonucleotide duplex with no methylase present was also digested under the same conditions. The reactions were stopped by the addition of 10μl of 95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol and heated to 95°C for 5 minutes. Samples (5μl) of digestion products were loaded onto denaturing 7M urea/24% polyacrylamide TBE gels (90mM Tris-borate pH 8.2, 2.5mM EDTA) which were run at 60W constant power for 3 hours. A Maxam and Gilbert sequencing reaction of the duplex was prepared and co-electrophoresed to allow the fragment protected from exonuclease III digestion to be identified. After electrophoresis, gels were dried under vacuum and autoradiographed.

Gel retardation assays

In a typical experiment, end-labelled duplexes of known concentration were incubated with methylase in the presence of various concentrations of competitor. Either sonicated *E.coli* chromosomal DNA (average length 700bp) or non-labelled modified oligonucleotide duplexes were added at known concentration ratios (w/w) and used to compete off the labelled duplex. Samples were mixed in a binding buffer of 10% glycerol, 50mM Tris−HCl (pH 8.2), 5mM $MgCl_2$, 1mM DTT and incubated at 4°C for 15 minutes and then loaded onto a 6% polyacrylamide native TAE gel (40mM Tris-acetate, pH 7.4, 1mM EDTA). Gels were run at 100V at 4°C until the bromophenol blue dye was two thirds down the gel. After electrophoresis gels were dried under vacuum and autoradiographed. The intensities of the various bands were estimated by densitometry of the autoradiograph and integration of the peaks.

Competition experiments

Competition experiments using labelled and unlabelled (competitor) oligonucleotide duplexes were analysed by gel

retardation. For such experiments, one can derive an equation relating the equilibrium binding constants for the labelled fragment (K_1) and the competitor (K_2) to the concentration of components throughout the titration [23]. However, a simpler equation can be derived if one considers the concentration C_2 of competitor required to give an equal concentration of free and bound labelled duplex at a fixed concentration C_1 of labelled duplex, as long as the experiment is done (1) with a 1:1 stoichiometry (protein to labelled duplex) (2) under conditions where all the protein is bound.

The binding constants for labelled DNA (D) and competitor DNA (N) are given by

$$K_1 = [PD]/[P].[D] \quad \text{(Equation 1)}$$

$$K_2 = [PN]/[P].[N] \quad \text{(Equation 2)}$$

If the conditions above are valid, then the total concentrations of protein and labelled duplex are equal i.e. $[P_t] = [D_t]$. If all the protein is bound and is equally distributed between the two duplexes, then

$$[PD] = [PN] = [D_t]/2 \quad \text{(Equation 3)}$$

Thus combining equations (1), (2) and (3)

$$K_1 .[D] = K_2 .[N] \quad \text{(Equation 4)}$$

Since $[D] = [D_t] - [PD]$ and $[N] = [N_t] - [PN]$ it follows that

$$K_1/K_2 = [N_t] - [PN]/[D_t] - [PD] \quad \text{(Equation 5)}$$

Thus combining equations (3) and (5)

$$K_1/K_2 = ([N_t] - [D_t]/2)/([D_t] - [D_t]/2) = 2([N_t]/[D_t]) - 1$$

Reverting to our earlier nomenclature

$$K_1/K_2 = (2C_2/C_1) - 1 \quad \text{(Equation 6)}$$

Equation (6) was therefore used to estimate the ratio of specific to non-specific binding affinities from the concentration ratio C_2/C_1 required to dissociate 50% of the labelled complex. This derivation assumes that there is no free protein (as judged by the presence of 100% labelled complex in the absence of competitor) and that both duplexes posess a single site (as judged, for example, by DNA footprinting). Furthermore, the experiment must be done at a 1:1 stoichiometry of protein to labelled duplex. In the case of non-specific competitor DNA, a similar equation can be used if one allows for the presence of overlapping protein binding sites on the DNA each shifted by 1 base pair, in which case the concentration of non-specific sites becomes effectively equal to the concentration of base pairs in the competitor DNA.

Methylation assays

Determination of K_m for AdoMet. Radiolabelled $H^3[CH_3]$-S-adenosyl-L-methionine (AdoMet) was purchased from Amersham (specific activity 85Ci/mmole). A range of stock solutions of radiolabelled AdoMet was generated by mixing in various proportions high concentration non-labelled AdoMet (Sigma) with the labelled AdoMet. The new specific activity and concentration for each stock solution was determined from the UV absorption at 260nm ($E_{260} = 15{,}000\ M^{-1}\ cm^{-1}$) and from scintillation counting. To determine the initial rate for the enzyme at each AdoMet concentration 40nM methylase was incubated in 25μl of 50mM Tris−HCl (pH 8.2), 5mM $MgCl_2$, 1mM DTT at 37°C with 10μM hemi-methylated duplex A10B. In a series of experiments, the AdoMet concentration was varied between 0.54 and 212μM. At time-points of 2 and 7 minutes after starting the reaction, 10μl aliquots were withdrawn . The reaction was stopped by incubation at 65°C for 5 minutes and the sample spotted onto Whatman DE81 filter papers (2.3cm diameter circles). Unincorporated label was washed off the filters by 3 successive 2 minute washes in 20mls of 500mM sodium phosphate (pH 7.0). Filters were finally washed with 100% ethanol for 3 minutes and then allowed to dry under a lamp. Dry filters were placed into scintillation vials containing 5mls of Optiphase III scintillation fluid and the amount of incorporation determined by scintillation counting the filters in a Packard 2000CA Tri-carb liquid scintillation analyser. Cpm data taken from sets of experiments was converted to pmoles of incorporated label and initial rates calculated. These data were fitted directly to a Michaelis−Menton expression using a non-linear least squares program to obtain K_m and V_{max} for the reaction. Because of possible signal loss due to quenching by the filters the values obtained for V_{max} cannot be used to calculate an absolute value of k_{cat} for the enzyme (although it provides an upper limit for this parameter).

Determination of methylation rate for modified duplex substrates. For use in a methylation assay radiolabelled AdoMet was added to a solution of non-labelled AdoMet to generate a 440μM stock solution. A typical reaction was carried out in 200μl of 50mM Tris−HCl (pH 8.2), 5mM $MgCl_2$, 1mM DTT at 37°C with 10μM of the appropriate duplex substrate and 220μM AdoMet. For hemi-methylated substrates the enzyme concentration was 100nM; for both the unmodified substrates and the fully modified sequence the enzyme concentration was increased to 2μM so that the reaction would proceed over a reasonable time-course. During the course of the reaction 25μl aliquots were withdrawn at timed intervals, the reaction stopped and the samples spotted onto Whatman DE81 filter papers. The filters were treated as for the K_m determination and the level of incorporation determined as described previously. Raw cpm data were converted to incorporated pmoles without correction for filter quenching, but correcting for enzyme concentration to give the apparent k_{cat} . Initial rates of reaction were determined by fitting data to the first order rate expression $V = V_f(1 - e^{-kt})$ using a non-linear least squares procedure.

RESULTS

Exonuclease III footprinting

Exonuclease III footprinting is a well established method for characterisation of protein binding sites on DNA [21]. ExoIII footprinting was therefore undertaken to establish directly the location and extent of the preferred binding site for the *Eco*R124I methylase on the oligonucleotide duplex AB. Duplex AB uniquely labelled on either the A or the B strand was incubated with a slight molar excess (protein:DNA = 1.1) of methylase protein and digested with exonuclease III as described in Materials and Methods. The digestion products were analysed on denaturing 24% polyacrylamide gels and are shown in Fig. 2(a,b). Comparison with the Maxam and Gilbert sequence of both strands allows the extent of methylase protection to be determined. The methylase protects about 6bp on either side of its recognition sequence giving a total protected region of 25bp (Fig. 2c). The well defined size of the DNA fragments protected from digestion indicates that the methylase binds to a unique site on the duplex.

Specific and non-specific binding

To determine the extent to which the *Eco*R124I methylase can discriminate between specific and non-specific DNA binding

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5'-CCGTGCAGAATTCGAGGTCGACGGATCCGG-3'  DUPLEX AB
3'-GGCACGTCTTAAGCTCCAGCTGCCTAGGCC-5'

          Me
          |
5'-CCGTGCAGAATTCGAGGTCGACGGATCCGG-3'  DUPLEX A9B
3'-GGCACGTCTTAAGCTCCAGCTGCCTAGGCC-5'

           Me
           |
5'-CCGTGCAGAATTCGAGGTCGACGGATCCGG-3'  DUPLEX A10B
3'-GGCACGTCTTAAGCTCCAGCTGCCTAGGCC-5'


5'-CCGTGCAGAATTCGAGGTCGACGGATCCGG-3'  DUPLEX AB13
3'-GGCACGTCTTAAGCTCCAGCTGCCTAGGCC-5'
                      |
                      Me

          Me
          |
5'-CCGTGCAGAATTCGAGGTCGACGGATCCGG-3'  DUPLEX A9B13
3'-GGCACGTCTTAAGCTCCAGCTGCCTAGGCC-5'
                      |
                      Me

           Me
           |
5'-CCGTGCAGAATTCGAGGTCGACGGATCCGG-3'  DUPLEX A10B13
3'-GGCACGTCTTAAGCTCCAGCTGCCTAGGCC-5'
                      |
                      Me
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Figure 1. Sequences of synthetic oligonucleotide duplexes used in this study. The strands are denoted A and B and are numbered 1–30 in the 5′ to 3′ direction. The positions of modifications are indicated (Me).

sites, a competition experiment was employed as this gives a direct measurement of relative binding affinities. Protein was incubated with an equimolar amount of labelled duplex AB along with varying amounts of unlabelled non-specific DNA. A gel retardation assay was used to examine the amount of specific complex formation in the presence of the non-specific DNA. The autoradiograph in Fig. 3 shows clearly that a significant fraction of methylase protein is still bound at the specific site even in the presence of a large excess of non-specific binding sites.

Under the conditions of the assay we assume that duplex AB contains only one binding site for the methylase. This is reasonable, as only a single site is observed by exonuclease III footprinting of methylase bound to duplex AB. Furthermore titrations of duplex AB with methylase analysed by gel retardation show only single bound species except at high protein to DNA ratios [3]. The non-specific DNA can be considered as a series of overlapping protein binding sites, each with an equal probability of binding a protein molecule [22]. Densitometric analysis of the autoradiograph in Fig. 3 shows that a 100-fold (w/w) excess of competitor DNA is required to reduce occupancy of the labelled duplex by half. The sonicated *E.coli* DNA is long compared with the size of the methylase binding site (< 25bp) and the concentration of non-specific binding sites is essentially equal to concentration of base pairs in the competitor DNA. Thus there is a 3000-fold excess of non-specific sites at this point in the titration, leading to an estimate of the ratio of equilibrium binding constants for specific:non-specific binding of 6000 using Equation (6). This could indeed be an underestimate of the specificity ratio since the occurrence of any cognate sites in the competitor DNA sequence would lead to an overestimate of the non-specific DNA binding affinity.

Binding to methylated oligonucleotides

We have applied a similar approach to investigate the relative binding strengths of the methylase for duplexes bearing

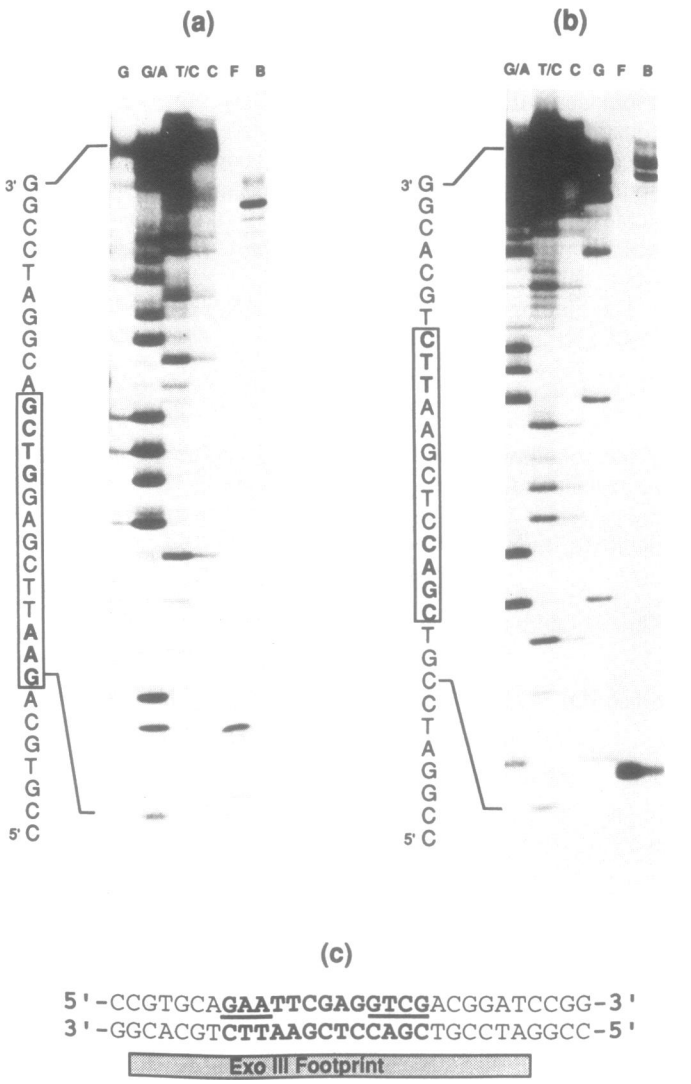

Figure 2. Exonuclease III footprint of EcoR124I methylase on the unmodified oligonucleotide duplex AB, showing protection of the A strand (**a**) and the B strand (**b**). The fragments resulting from digestion of free (F) and bound (B) duplex in each case were ran on the gel in parallel with Maxam and Gilbert sequencing reactions on the labelled strand. The region of the duplex sequence protected by the methylase is illustrated in (**c**).

methylated adenines within the recognition sequence. The competition experiments were carried out as above, but now unlabelled modified duplexes were used to compete with labelled duplex AB. Autoradiographs of the competition experiments with each of the oligonucleotide duplexes are shown in Fig. 4, the results of which are tabulated in Table 1. As a control, non-labelled duplex AB was also used as a competitor. As expected, an equimolar concentration of labelled and non-labelled duplex AB is sufficient to produce half-dissociation of the protein from the labelled duplex. Duplex A9B is only slightly less effective as a competitor, a twofold molar excess being sufficient to compete off half the protein from the labelled duplex. Duplexes A10B, AB13 and A9B13 do not differ appreciably from each other, but all show significantly weaker binding than the unmodified duplex, producing half-dissociation of the labelled complex with molar ratios from 10 to 18. Duplex A10B13 is by far the weakest competitor, a molar ratio of 75 being required

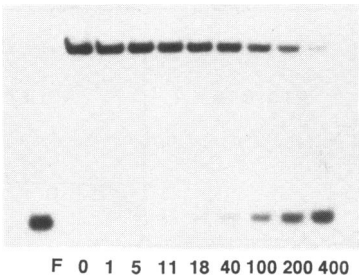

Figure 3. Gel retardation competition assay of ^{32}P-labelled duplex AB (144nM) in the presence of 144nM EcoR124I methylase, with increasing amounts of non-specific competitor DNA (E.coli). Lane 1 (F): free duplex AB in the absence of methylase; lanes 2–10 increasing ratios (w/w) of competitor DNA to labelled duplex are indicated.

Table 1. Binding selectivity of *Eco*R124I methylase to DNA substrates

Duplex	Ratio	Relative Affinity
AB	1	1.000
A9B	2	0.333
A10B	16	0.032
AB13	18	0.029
A9B13	10	0.053
A10B13	75	0.007

Ratios have been estimated from the gel retardation competition assay (Fig. 4) and refer to the concentration ratios required to produce 50% competition with the labelled duplex AB. Relative affinity of the methylase for each modified oligonucleotide substrate is expressed as the ratio of the affinity constant (K_1) for the unmodified duplex AB to that for each of the modified duplexes (K_2) according to Equation (6).

Table 2. Rates of methylation of modified oligonucleotide substrates

Duplex	Rate of Reaction
AB	$5.9\ (0.5)\times10^{-3}$
A9B	$2.9\ (0.3)\times10^{-3}$
A10B	$5.1\ (1.0)\times10^{-1}$
AB13	$10.9\ (1.0)\times10^{-1}$
A9B13	$5.6\ (1.7)\times10^{-1}$
A10B13	$5.0\ (8.0)\times10^{-6}$

The initial rate of reaction is given for each substrate, expressed in pmols of methyl groups transferred per minute. The rates have been normalised to equal concentrations of enzyme (2μM) in a 25μl reaction volume. No correction has been made for possible quenching by the filter. Figures given in parentheses refer to the standard errors derived from the least squares fitting procedure.

for half-dissociation of the labelled complex. Again the competition experiments can be used to determine the relative binding strengths of the methylase for modified and unmodified sites. Under the conditions of the assay we assume that each duplex contains only one methylase binding site i.e. non-specific binding to other regions of the duplex is negligible. The corresponding binding constant ratios estimated using Equation (6) are shown in Table 1. The strength of binding for each of the substrates fall into three distinct classes. Hemi-methylated DNA shows a 20–35 fold lower DNA binding affinity than the unmodified duplex. Fully methylated DNA (A10B13) has a 150-fold lower affinity. In contrast, DNA modified at a non-target site (A9B) is bound with almost equal affinity to unmodified DNA.

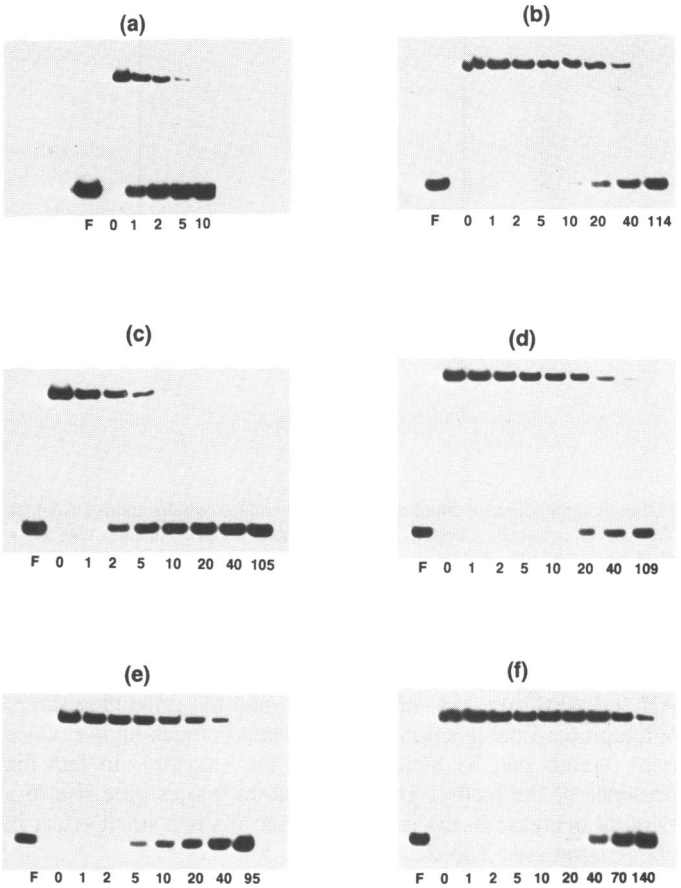

Figure 4. Competition assays of ^{32}P labelled duplex AB (144nM) in the presence of 144nM EcoR124I methylase with each of the synthetically modified oligonucleotide duplex as competitor: (a) AB, (b) A10B , (c) A9B, (d) AB13, (e) A9B13, (f) A10B13. Control tracks containing free labelled duplex AB are indicated (F). The ratio (w/w) of competitor duplex to labelled duplex is as indicated.

Kinetics of DNA methylation

Enzyme activity was monitored by the transfer of a labelled methyl group from the co-factor, S-adenosyl methionine (AdoMet), to an oligonucleotide substrate bearing the recognition sequence (Taylor *et al.*, 1992). As a prelude to kinetic measurements on the various synthetic DNA duplexes, it was necessary to investigate the dependence of initial enzyme velocity on the concentration of the AdoMet, since we have no knowledge of the K_m of the enzyme for this co-factor. Fig. 5 shows the resulting plot of initial velocity as a function of AdoMet concentration. The points were fitted to a Michaelis-Menton curve using a non-linear least squares procedure, which gave a good fit to the experimental points with a K_m of 27 ($\pm$5) μM. Subsequent experiments were therefore done at a AdoMet concentration of 220μM, well above the K_m to ensure saturation with the co-factor.

The time-course of the reaction of *Eco*R124I methylase with the cognate oligonucleotide duplex and each of the five modified duplexes is shown in Fig. 6. While the rates of methylation and the final concentration of methyl groups incorporated varies in each case, three clear classes of reaction can be identified. The substrate for which there is no detectable enzyme activity is A10B13, thus identifying the sites of enzyme methylation on each

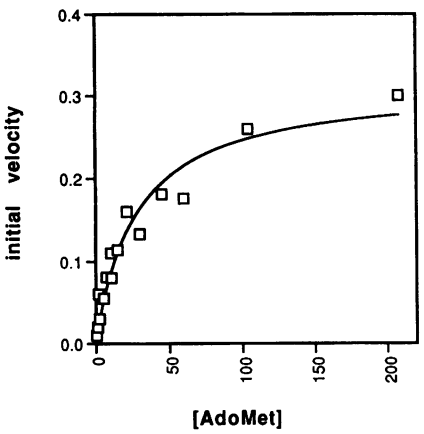

Figure 5. Dependence of initial enzyme velocity on the concentration of AdoMet. The curve represents a best fit to the Michaelis-Menten equation with K_m= 27.4μM and V_{max}= 0.31

strand. Since these two adenines are already modified chemically, they can not be substrates for the enzyme reaction. The substrates AB and A9B are very much slower than the remaining three, although the final position of equilibrium is much higher (since both strands can be methylated by the enzyme). In fact the presence of the methyl group at position 9 does give rise to a twofold decrease in the initial rate, but this is a small effect in comparison (see Table 2).

The rates of reaction for the remaining three substrates are much faster, although the final degree of incorporation is rather less. The DNA duplexes are essentially hemi-methylated in one of the two strands (A10 or B13). The prior modification of the duplex at A9 leads to an approximately twofold drop in the initial rate of reaction (compare AB13 with A9B13), as with unmethylated DNA. There is also a twofold increase in the rate of methylation by the enzyme at A10 compared to the rate of reaction at B13. However it is clear that the major difference in rates is between unmodified and hemi-methylated DNA, the latter being at least 100- fold faster than the former, regardless of the strand that is methylated.

DISCUSSION

Sites of modification

On the reasonable assumption that the target bases for the methyltransferase lie within the specific recognition sequence, there are three possible candidates for the two adenines that are methylated in the enzyme reaction. The specific recognition sequence on the B strand (5'-CGAY-'3) contains only a single adenine (position 13 in our nomenclature) and this is therefore the only possible site of methylation. On the A strand the situation is more complicated as there is a possibility of methylation at either position 9 or 10. Previous attempts to positively identify which of these two adenines is methylated by M.*Eco*R124I have been unsuccessful [24]. The results of the methylation assay allow an unambiguous assignment of this site of modification. Firstly, the presence of an additional methylated adenine at A9 does not affect the ability of the enzyme to methylate the duplex (compare A9B with AB, or A9B13 with AB13 in Fig. 6), thus ruling out the possibility that A9 is a site for methylation by the enzyme. Conversely, the duplex which is methylated at both A10 and B13

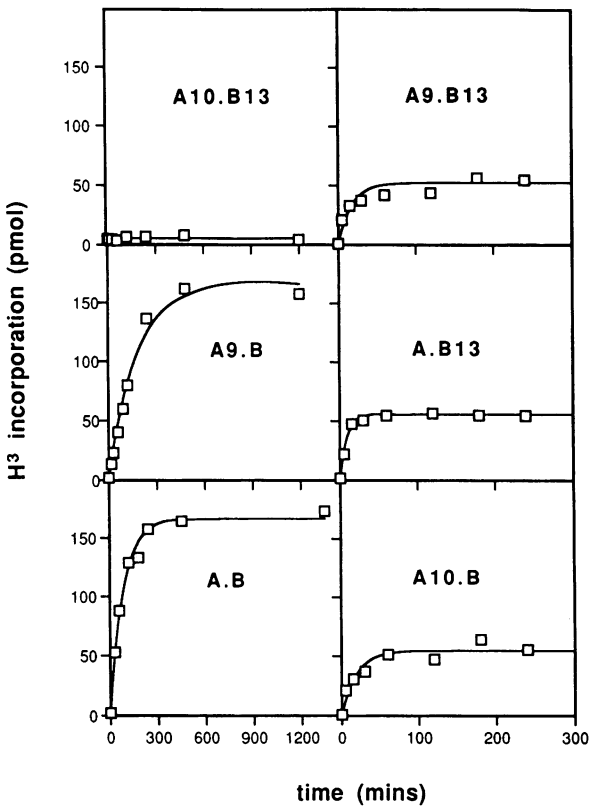

Figure 6. Time course of methylation by EcoR124I methylase for each oligonucleotide duplex. Reactions with AB, A9B and A10B13 had an enzyme concentration of 2.0 μM. Reactions with A10B, AB13 and A9B13 had an enzyme concentration of 0.1 μM. The solid curves represent the line of best fit to a first order rate equation. The rate constants for each curve correspond to the initial velocities listed in Table 2.

is no longer a substrate, since it cannot be further modified by the enzyme. Consequently A10 (i.e. the second adenine in the trinucleotide sequence GAA) must be the target site for M.*Eco*R124I.

The two adenines that are methylated by the *Eco*R124I enzyme are 8 base pairs apart, somewhat less than one turn of the helix unless the DNA is severely undertwisted. Consequently the active sites of the two methylase subunits can not line up along one side of the DNA helix, and must be less symmetrically oriented with respect to the DNA helix axis.

Substrate selectivity

*Eco*R124I methylase shows a 100−200 fold higher reaction rate for hemi-methylated DNA than for the unmodified substrate, the precise degree of enhancement depending on which of the two strands is modified (Table 2). *Eco*R124I therefore shows a substrate preference much closer to that of the type IA methylase, *Eco*KI, than to that of the IB enzyme, *Eco*AI. Our results can be compared with earlier studies on the *Eco*R124I endonuclease which although qualitative appear to show a similar preference for hemi-methylated DNA [7]. However, those studies used heteroduplex plasmid DNA rather than synthetic oligonucleotides which complicates the analysis.

The very much higher rates of reaction for hemi-methylated substrates indicates a highly cooperative interaction. Modification of either half-site profoundly affects the rate of methylation at

the other, implying a significant communication between the two active sites on the methylase. This could occur through a conformational change in the DNA or through a structural change in the methylase itself. The latter is perhaps most likely, in that the methylation state of the DNA at one site could be communicated to the other by protein-protein interactions of the sort seen in many allosteric enzymes. Indeed, such a change may be part of the mechanism whereby the endonuclease (now with HsdR present) is unable to restrict hemi-methylated DNA. Since the restriction endonuclease also has to sense the methylation state at either site, a similar conformational change could be transmitted via subunit interactions to HsdR resulting in loss of endonuclease activity, or even the dissociation of HsdR from the multisubunit enzyme.

The large increase in rate of reaction for hemi-methylated substrates is unlikely to be the result of an increase in the affinity of the methylase for the oligonucleotide substrate, since the reaction is conducted under conditions where the duplex is well in excess of the enzyme and at concentrations that are likely to be far above the K_d for the DNA substrate. Furthermore, we have direct evidence from the gel retardation competition assays that the affinity of the methylase for either hemi-methylated duplex is indeed lower than for the unmodified form. Consequently, the large increase in reaction rate for hemi-methylated DNA would seem to reflect an increase in k_{cat} rather than a decrease in K_m.

The situation is rather different for methylation at the adenine that is not susceptible to modification by the *Eco*R124I methylase (A9 in our nomenclature). Here, both the rate of reaction and the DNA binding affinity are reduced by a factor of 2–3, a small but fairly reproducible effect. In this case it seems most likely that the extra methyl group at this position gives rise to some degree of steric hindrance, suggesting an interaction between the enzyme and the major groove of DNA at this site. However the relatively small effect observed suggests that the contact here is not especially close. A parallel series of investigations is now in progress with other modified DNA sequences to probe the nature of these protein-DNA interactions throughout the DNA recognition sequence.

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