

The DNA Binding Characteristics of the Trimeric *Eco*KI Methyltransferase and its Partially Assembled Dimeric Form Determined by Fluorescence Polarisation and DNA Footprinting

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The type I DNA restriction and modification systems of enteric bacteria display several enzymatic activities due to their oligomeric structure. Partially assembled forms of the *Eco*KI enzyme from *E. coli* K12 can display specific DNA binding properties and modification methyltransferase activity. The heterodimer of one specificity (S) subunit and one modification (M) subunit can only bind DNA whereas the addition of a second modification subunit to form M_2S_1 also confers methyltransferase activity. We have examined the DNA binding specificity of M_1S_1 and M_2S_1 using the change in fluorescence anisotropy which occurs on binding of a DNA probe labelled with a hexachlorofluorescein fluorophore. The dimer has much weaker affinity for the *Eco*KI target sequence than the trimer and slightly less ability to discriminate against other DNA sequences. Binding of both proteins is strongly dependent on salt concentration. The fluorescence results compare favourably with those obtained with the gel retardation method. DNA footprinting using exonucleaseIII and DNaseI, and methylation interference show no asymmetry, with both DNA strands being protected by the dimer and the trimer. This indicates that the dimer is a mixture of the two possible forms, M_1S_1 and S_1M_1 . The dimer has a footprint on the DNA substrate of the same length as the trimer implying that the modification subunits are located on either side of the DNA helical axis rather than lying along the helical axis.

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Keywords: DNA binding; DNA footprinting; DNA methyltransferase; fluorescence anisotropy; hexachlorofluorescein

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Introduction

Bacterial DNA restriction and modification systems restrict the propagation of foreign DNA. This is performed using a restriction endonuclease which makes a double strand break in foreign DNA which lacks methylation on a specific, target DNA sequence. A modification methyltransferase maintains the modification of the same target sequence within the host's DNA (Modrich, 1979; Yuan, 1981; Wilson & Murray, 1991; Bickle & Kruger, 1993; King & Murray, 1994).

Abbreviations used: AdoMet, S-adenosyl methionine; bp, base-pair; DNase I, deoxyribonuclease I; hsd, host specificity for DNA; K_d , dissociation constant; TBE, tris-borate-EDTA; UV, ultra-violet.

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The methyltransferases of type I DNA restriction and modification systems comprise one DNA sequence recognition S subunit, and two modification M subunits that each bind one molecule of the cofactor and methyl group donor, S-adenosyl methionine (Taylor *et al.*, 1992, 1993; Dryden *et al.*, 1993). Simple structural models for type I enzymes based on biochemical, crystallographic and sequence data have been proposed (Burckhardt *et al.*, 1981; Kneale, 1994; Dryden *et al.*, 1995; Figure 1).

The M subunits methylate the N6 position of a specific adenine nucleotide on each strand of the DNA recognition for the enzyme. The target sequence for the type I *Eco*KI system for *Escherichia coli* K12 is AAC (N₆) GTGC and methylation occurs at either the underlined A or the A, on the complementary strand, opposite the underlined T.

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The M subunits methylate the N6 position of a specific adenine nucleotide on each strand of the DNA recognition for the enzyme. The target sequence for the type I *Eco*KI system for *Escherichia coli* K12 is AAC (N₆) GTGC and methylation occurs at either the underlined A or the A, on the complementary strand, opposite the underlined T.

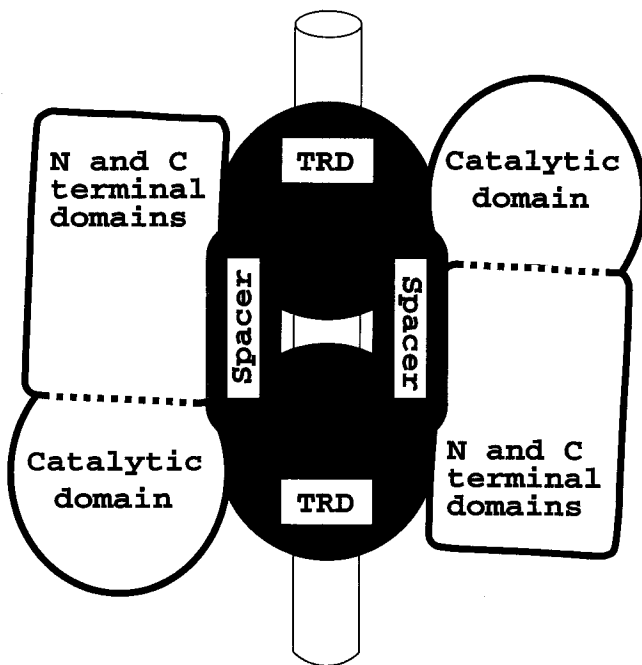


Figure 1. Model of a type I methyltransferase bound to DNA (Dryden *et al.*, 1995). The S subunit (shaded) lies along the helical axis of the DNA (cylinder). The approximately 2-fold symmetrical S subunit is comprised of two target recognition domains (TRDs) each of which recognises one part of the bipartite target sequence. The TRDs are joined by short amino acid spacers, one of which is postulated to contain the N and C termini of the subunit (Kneale, 1994). In this model, the M subunits (bold outline) are placed on either side of the DNA helical axis and flank the S subunit. A catalytic domain in the M subunit is associated with the TRDs and the location of the N and C-terminal regions of the M subunits is postulated from genetic (Kelleher *et al.*, 1991) and biochemical (Cooper & Dryden, 1994) results.

N_6 represents a non-specific hexameric sequence. EcoKI shows an approximately 100-fold preference for methylating a target which is already contains one methylated adenine, such as is found on hemimethylated DNA produced after DNA replication, rather than unmethylated DNA which is typically found in foreign DNA (Suri *et al.*, 1984; Dryden *et al.*, 1993; Winter, 1998). The recognition of whether the target is unmethylated or hemimethylated is believed to be a function of the M subunits since mutations in the *hsdM* gene can abolish the mechanism for recognition of target site methylation without affecting the methylation reaction itself (Kelleher *et al.*, 1991). The addition of two restriction subunits to the EcoKI $M_2 S_1$ complex forms the complete bifunctional restriction endonuclease and methyltransferase enzyme with methylates hemimethylated DNA but cleaves unmethylated DNA in a complex reaction (Yuan, 1981; Studier & Bandyopadhyay, 1988; Dryden *et al.*, 1997). The initial step in the assembly of EcoKI

appears to be the formation of an inactive $M_1 S_1$ dimer (Dryden *et al.*, 1993, 1997). This $M_1 S_1$ complex can be isolated during the purification of the $M_2 S_1$ methyltransferase (Dryden *et al.*, 1993).

We have examined AdoMet and DNA binding using gel retardation and fluorescence anisotropy of hexachlorofluorescein labelled DNA. The contacts of the methyltransferase and $M_1 S_1$ on a DNA substrate have been determined using exonuclease III and DNaseI footprinting and methylation interference. The novel hexachlorofluorescein probe used for the anisotropy studies is an excellent probe of protein-DNA interactions. Our experiments show that the partially assembled dimer is a mixture of $M_1 S_1$ and $S_1 M_1$ rather than the M subunit having one preferred position on the S subunit. The M subunits are positioned on either side of the S subunit and off the DNA helical axis, confirming structural models (Kneale, 1994; Dryden *et al.*, 1995), instead of projecting along the DNA axis. Additionally, the absence of one M subunit weakens DNA binding but has little effect on DNA sequence recognition.

Results

AdoMet binding

The ability of $M_1 S_1$ to bind the cofactor AdoMet was examined in a qualitative way by gel filtration as previously performed for the methyltransferase (Willcock *et al.*, 1994). It was observed that about twice as much radioactivity passed through the column in a complex with $M_2 S_1$ as with $M_1 S_1$ or the M subunit alone (data not shown). Unbound AdoMet passed through the column more slowly. As equimolar amounts of both proteins were loaded onto the column, this result is simply explained by $M_2 S_1$ having two AdoMet binding sites and $M_1 S_1$ only one. In addition, UV cross-linking (results not shown), showed that the M subunit of $M_1 S_1$ bound to the cofactor as previously shown for $M_2 S_1$ (Willcock *et al.*, 1994). These results indicated that $M_1 S_1$ binds AdoMet with a similar affinity as the methyltransferase and therefore that subsequent observations on the DNA binding by $M_1 S_1$ were not influenced by a difference in the AdoMet binding properties of the two proteins.

Fluorescence polarisation of hexachlorofluorescein-labelled oligonucleotide duplexes

The nanosecond fluorescence decay of the 21 bp hexachlorofluorescein-labelled, EcoKI-specific DNA duplex was well fitted by a single exponential decay of $2.87(\pm 0.02)$ ns (data not shown). The attachment of the fluorescent probe to the DNA *via* a flexible six-carbon linker meant that it may have been possible for the probe to move independently of the DNA or DNA-protein complex. In the case of complete flexibility and no correlation between

the dynamics of the probe and the DNA to which it was attached, measurements of fluorescence anisotropy would be insensitive to protein binding. We have examined the correlation between motion of the duplex and motion of the fluorescent probe by varying the viscosity of the solution. Figure 2 shows the variation in $1/\text{anisotropy}$ as a function of temperature/viscosity. The variation in slopes of the data collected at different concentrations of glycerol was indicative of segmental motion between the fluorescent probe and the DNA duplex (Lakowicz, 1983; Figure 2(a)). Extrapolation to infinite viscosity where the probe would be immobile gave an average intercept of $1/r_0 = 3.068(\pm 0.324)$ and an intrinsic anisotropy value for r_0 of 0.326. Using the value of $1/r_0$, the average anisotropy value of 0.137 at 25°C for the labelled DNA duplex and the fluorescence lifetime in the Perrin equation, the rotational correlation time for the labelled DNA duplex could be calculated to be between 2.5

and 1.7 ns. This time was substantially longer than the subnanosecond value expected for motion of the probe itself, but shorter than expected if the probe was rigidly attached to the DNA. Therefore, we concluded that the label was not rigidly attached to the DNA and had some degree of movement with respect to the DNA, but that this motion was not sufficient to preclude the use of the labelled duplex for studies of protein-DNA interactions. If the same data were replotted linking points collected at the same temperature rather than the same percentage of glycerol (Figure 2(b)) it was apparent that the data collected with less than 90% glycerol and at 20°C or above formed a set of nearly parallel lines and then converged sharply towards the 90% glycerol data and r_0 below 20°C. This could imply that below 20°C, the motion of the DNA was negligible and essentially frozen, leaving only the independent motion of the probe to contribute to the anisotropy. This behaviour has been predicted for a mixture of a rapidly rotating fluorophore and a slowly rotating fluorophore (Lakowicz & Weber, 1980).

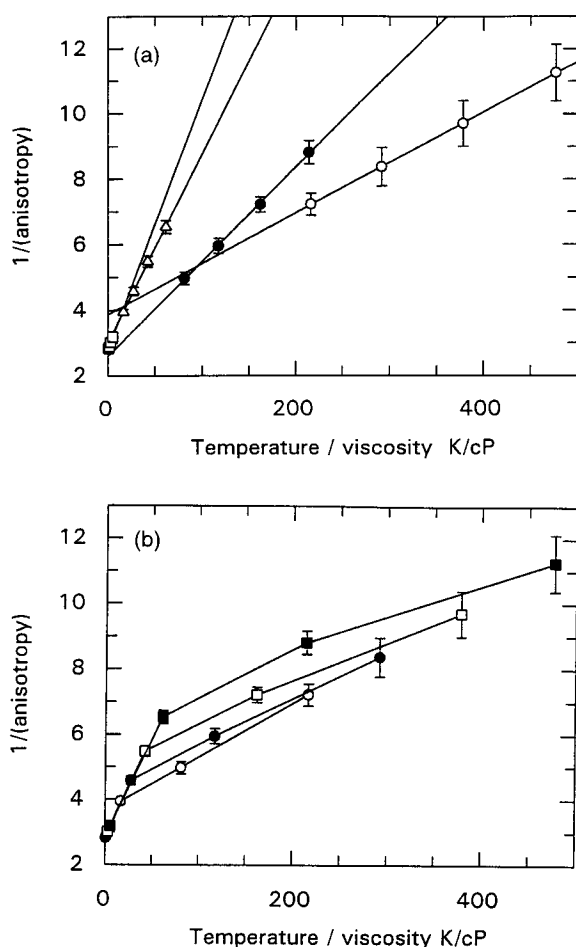


Figure 2. Variation of $1/\text{anisotropy}$ as a function of temperature/viscosity for the $1\mu\text{M}$ hexachlorofluorescein-labelled 21 bp DNA duplex. Temperatures of 10, 20, 30 and 40°C were used with glycerol added to 0, 30, 60 and 90%. Linear fits to data with 0% (○), 30% (●), 60% (△) and 90% (□) are shown in (a) and the same data are shown in (b) but with points collected at 10°C (○), 20°C (●), 30°C (□) and 40°C (■) joined together.

Fluorescence anisotropy of protein-DNA complexes

The binding of protein to hexachlorofluorescein-labelled 21 bp duplexes and also variations in NaCl concentrations caused changes of less than 10% in the fluorescence emission intensity of the fluorescent probe thereby nearly satisfying the assumption in the binding equation (Heyduk & Lee, 1990) that the quantum yield was invariant (Lakowicz, 1983; Eftink, 1997; Hill & Royer, 1997). Table 1 shows the values obtained for the K_d and maximum anisotropy change for the binding of DNA by M_1S_1 and M_2S_1 in a variety of conditions. The average anisotropy value in these experiments for the hexachlorofluorescein-labelled DNA in the absence of protein was 0.137. Figure 3 shows some typical titration results.

The presence of AdoMet enhanced the affinity of the methyltransferase for its DNA target site by four to 20-fold with the greatest enhancement observed at the higher NaCl concentrations. The affinity of the methyltransferase for DNA lacking the target site was little affected by AdoMet and was much weaker than the affinity for the target site. Addition of NaCl weakened binding to non-specific DNA so much that it was almost unobservable. The maximum anisotropy change was higher for the non-specific duplex than for the *EcoKI*-specific duplex.

The M_1S_1 protein bound six to 20-fold more weakly to specific DNA than the methyltransferase and the presence of AdoMet caused slightly less enhancement in affinity. The affinity of M_1S_1 for non-specific DNA appeared to be better than the affinity of the methyltransferase for non-specific DNA and the degree of discrimination between the two duplexes was correspondingly less for M_1S_1 . The maximum change in anisotropy was greater

Table 1. Dissociation constants and maximum anisotropy changes for the binding of methyltransferase and M_1S_1 to hexachlorofluorescein-labelled 21 bp duplex DNA

DNA duplex	NaCl conc. (M)	Methyltransferase		Methyltransferase + AdoMet	
		Dissociation constant, K_d (nM)	Max. anisotropy change, Δr	Dissociation constant, K_d (nM)	Max. anisotropy change, Δr
<i>Eco</i> KI specific	0	2.36 ± 1.18	0.050 ± 0.012	0.60 ± 0.53	0.048 ± 0.004
<i>Eco</i> KI specific	0.05	9.43 ± 14.05	0.039 ± 0.015	0.65 ± 0.40	0.039 ± 0.006
<i>Eco</i> KI specific	0.1	98.23 ± 18.23	0.040 ± 0.006	4.50 ± 1.07	0.042 ± 0.002
<i>Eco</i> KI non-specific	0	91.95 ± 10.96	0.095 ± 0.002	111.00 ± 24.75	0.092 ± 0.010
<i>Eco</i> KI non-specific	0.05	NC	NC	NC	NC
<i>Eco</i> KI non-specific	0.1	NC	NC	NC	NC

DNA duplex	NaCl conc. (M)	M_1S_1		M_1S_1 + AdoMet	
		Dissociation constant, K_d (nM)	Max. anisotropy change, Δr	Dissociation constant, K_d (nM)	Max. anisotropy change, Δr
<i>Eco</i> KI specific	0	13.97 ± 9.99	0.059 ± 0.007	14.67 ± 6.37	0.059 ± 0.015
<i>Eco</i> KI specific	0.05	170.10 ± 29.41	0.042 ± 0.001	19.35 ± 9.26	0.035 ± 0.001
<i>Eco</i> KI specific	0.1	NC	NC	80.05 ± 7.14	0.024 ± 0.001
<i>Eco</i> KI non-specific	0	63.30 ± 1.41	0.128 ± 0.002	27.80 ± 2.40	0.127 ± 0.012
<i>Eco</i> KI non-specific	0.05	NC	NC	NC	NC
<i>Eco</i> KI non-specific	0.1	NC	NC	NC	NC

NC, not calculated due to poor binding over protein concentration range examined

for complexes with the non-specific DNA and this change was larger than that observed for the methyltransferase.

DNA binding observed by gel retardation

Gel retardation using the labelled 45 bp duplex containing an unmethylated *Eco*KI site clearly showed a retarded complex containing M_1S_1 and DNA when AdoMet was present in the binding solution. This complex was hardly visible when AdoMet was absent, even though a reduction in the amount of free DNA was observable (gel not shown). At high concentrations of M_1S_1 , two faint bands of protein-DNA complex could be discerned suggesting that more than one molecule of M_1S_1 could bind to the duplex. The weakness of these bands suggested that the M_1S_1 -DNA complex was not stable during electrophoresis. A smear of radioactivity could be observed on the gel above the free DNA representing DNA which had dissociated from the protein during electrophoresis. The non-equilibrium nature of the gel retardation experiment in which free DNA is separated from bound DNA also distorts the results if one examines the amount of bound DNA in the titration, so binding affinities were derived from the amount of free DNA. The average amount of free DNA as a function of increasing concentration of M_1S_1 from four different gels is shown in Figure 4. Analysis of these data allowed the calculation of apparent K_d of 47.1(±10.5) nM without AdoMet and 9.4(±1.0) nM with AdoMet. The methyltransferase in the absence and presence of AdoMet gave K_d values of 9.0(±4.1) and 1.6(±0.7) nM respectively (Powell *et al.*, 1993). AdoMet caused a similar enhancement in the degree of discrimination by

M_1S_1 and M_2S_1 between DNA containing an *Eco*KI target and DNA without a target. The K_d values determined by gel retardation were of the same order of magnitude as those determined by fluorescence anisotropy. The absolute differences were most probably due to the non-equilibrium nature of the gel retardation experiment.

The rates of migration in the gel of the DNA complexes with either of the two proteins were very similar. However, an extended electrophoresis time allowed separation of the M_2S_1 -DNA and M_1S_1 -DNA complexes. To identify these species a gel retardation experiment was performed with the protein and DNA concentrations both at 300 nM in the presence or absence of AdoMet (Figure 5). Free DNA was run off the bottom to improve resolution of the complexes. This gel revealed that in the absence of AdoMet, the methyltransferase gave a minor fast-running band and a major slow-running species. For M_1S_1 , practically all of the DNA was in a slow-running complex with a migration rate identical to the slow-running methyltransferase-DNA complex. The presence of AdoMet converted all of the methyltransferase-DNA complexes into the faster moving form and had no effect on the slower M_1S_1 -DNA complex. This suggested that, in the absence of AdoMet, the methyltransferase had dissociated into M_1S_1 prior to binding the DNA. The methyltransferase-DNA complex ran faster than the M_1S_1 -DNA complex because it carried a greater negative charge. AdoMet stabilised the methyltransferase and reduced the K_d for this dissociation, at least in the presence of DNA. This dissociation would be expected at low concentrations from the equilibrium between methyltransferase and M_1S_1 (Dryden *et al.*, 1997). This methyltransferase dissociation effect was not observed in the ani-

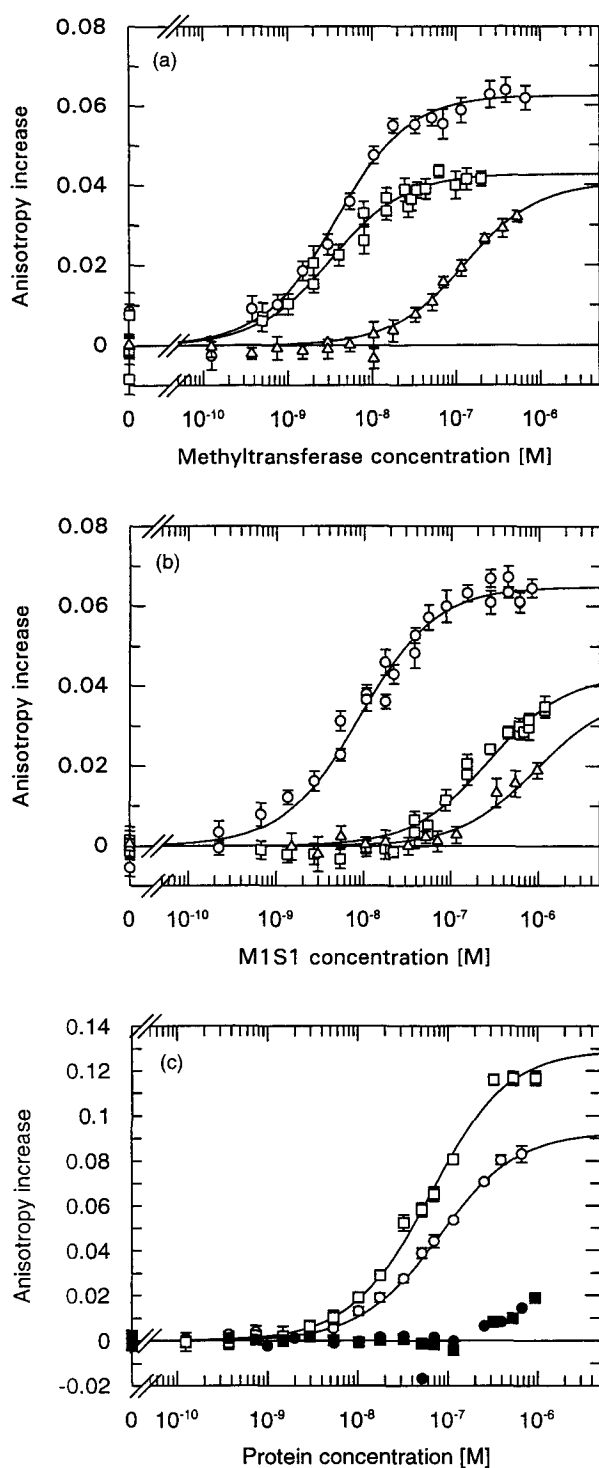


Figure 3. Representative fluorescence anisotropy titrations of 1 nM hexachlorofluorescein-labelled 21 bp duplex with M_1S_1 and M_2S_1 in the absence of AdoMet as used to derive the binding constants given in Table 1. The increase in anisotropy from the initial value in the absence of protein is shown *versus* protein concentration. Where possible, data are fitted to a single site binding equation as described in Materials and Methods. (a) Data for the binding of the methyltransferase, M_2S_1 , in the presence of 0, 0.05 or 0.1 M NaCl are shown as \circ , \square and \triangle , respectively. (b) Data for the binding of M_1S_1 in the presence of 0, 0.05 or 0.1 M NaCl are shown as \circ ,

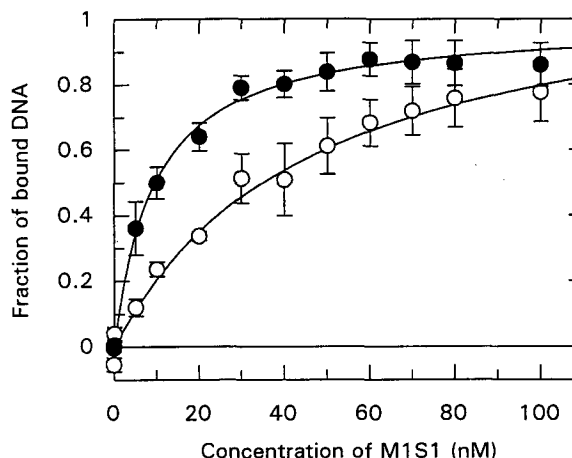


Figure 4. Quantification of unbound DNA following gel retardation of 0.1 nM EcoKI-specific 45 bp DNA duplex with M_1S_1 allows the calculation of the fraction of DNA bound by the protein in the absence (\circ) and presence (\bullet) of 100 μ M AdoMet. The K_d values are calculated to be $47.13(\pm 10.50)$ nM and $9.35(\pm 0.99)$ nM with saturation being reached at $117(\pm 16)\%$ and $99(\pm 2)\%$ of the DNA bound, respectively. In the absence of AdoMet, a protein-DNA complex was not visible except at the highest protein concentrations whereas AdoMet clearly stabilised the retarded complex.

sotropy experiments partly because the solution in the cuvette was always in equilibrium in contrast to the situation in the electrophoresis gel. In the gel, any free M subunit would migrate away from the protein-DNA complex and perturb the equilibrium. In addition, the binding of DNA stabilised the methyltransferase (see Conclusions) and diminished any dissociation effect in the anisotropy experiments. More sensitive experiments at even lower concentrations may reveal dissociation.

Exonuclease III footprinting for M_2S_1 and M_1S_1

Exonuclease III releases 5' mononucleotides by digesting each single strand of DNA in a double-stranded molecule from the 3' end. This can be used to obtain a footprint of a DNA binding site for a protein, as protein bound to its recognition site in a given DNA molecule can halt the progress of exonuclease III (Metzger & Heumann, 1994). If the DNA is labelled at the 5' end of one strand of the DNA, the protected fragment can be visualised on a urea-denaturing DNA sequencing gel.

Figure 6(a) shows the results obtained for exonuclease III footprinting of the top strand of the specific 45 bp duplex. Two major species 37 and 38 bases long were protected by the methyltransfer-

\square and \triangle , respectively. (c) Data for the binding of M_1S_1 (\square) and M_2S_1 (\circ) to non-specific DNA in the presence of 0 M NaCl (open symbols) or 50 mM NaCl (filled symbols).

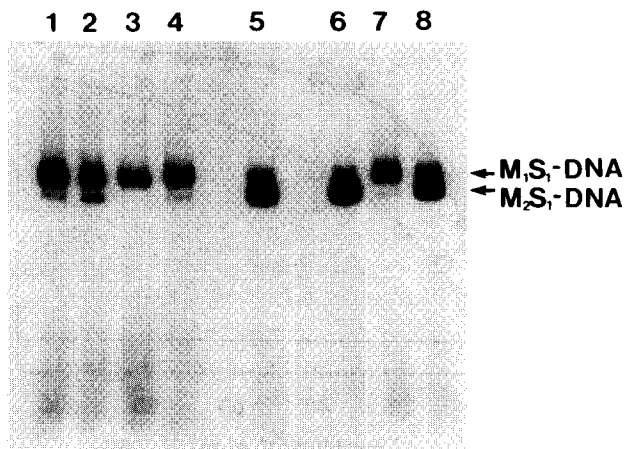


Figure 5. The effect of 100 μ M AdoMet on the migration of complexes of 300 nM M_2S_1 and 300 nM M_1S_1 with 300 nM EcoKI-specific 45 bp DNA in a gel retardation experiment. The high M_1S_1 concentration allows visualisation of the protein-DNA complex in contrast to the titrations at lower concentrations performed for Figure 4. AdoMet influences the stability of the complex between M_2S_1 and DNA. Lanes 1 and 4, mixture of methyltransferase-DNA and M_1S_1 -DNA complexes without AdoMet; lane 2, methyltransferase-DNA complex without AdoMet; lane 3, M_1S_1 -DNA complex without AdoMet. Lanes 5 to 8 are the same as lanes 1 to 4 but in the presence of 100 μ M AdoMet.

ase. The 37 base species persisted until the end of the time course indicating that the exonuclease III could remove eight bases of this strand before being stopped by the bound methyltransferase. This protected species was also seen for M_1S_1 .

Figure 6(b) shows the results for a similar experiment for the bottom strand of the 45 bp duplex, again in the presence of AdoMet. Although there was a strong protection of 34-37 bases by the methyltransferase, there was only a very weak footprint for M_1S_1 , despite gel retardation demonstrating that most of the DNA was bound by M_1S_1 . The existence of more than one protected species for the methyltransferase suggested that the exonuclease could slowly digest into the edge of the protected region. This may have been due to flexibility in the DNA-EcoKI interaction at the edge of the binding site, or to the sequence-specific exonuclease III degradation rate where the rates vary as $C \gg A \sim T \gg G$ (Ausubel *et al.*, 1992).

An alternative approach to obtaining a footprint for M_1S_1 was to perform the exonuclease III reactions as described but then use gel retardation to separate the protein-DNA complexes from unbound DNA. This method clearly showed that the methyltransferase and M_1S_1 protected the same DNA fragments (Figure 7(c)). The size of the binding site for both proteins estimated from the length of the protected species (Metzger & Heumann, 1994) was 26-30 base-pairs.

Methylation interference footprint

Methylation interference experiments for the methyltransferase (Powell & Murray, 1995) provided evidence for major groove contacts at or near the N7 position for all the guanine nucleotides in the recognition sequence for unmethylated DNA in the presence of AdoMet. In the absence of AdoMet, additional contacts in the non-specific sequence in the middle of the target sequence were observed.

Methylation interference patterns obtained in the presence or absence of AdoMet for M_1S_1 -DNA and methyltransferase-DNA complexes are shown in Figure 7. In the absence of AdoMet, interference was observed on the top strand at the two guanine bases (G27 and G29) in the tetranucleotide part of the target sequence and the guanine (G26) immediately preceding the tetranucleotide sequence for both M_1S_1 and the methyltransferase. The interference at guanine G26' was lost upon addition of AdoMet to the methyltransferase-DNA complex but not for the M_1S_1 -DNA complex. Interference was observed on the bottom strand of the DNA, for both methyltransferase and M_1S_1 , at G20', G21' and G30'. G21' and G30' are located in the tri- and tetranucleotide parts of the target and G21' is next to the trinucleotide sequence in the non-specific part of the EcoKI target (data not shown). The presence of AdoMet caused a barely perceptible loss of interference at G21' for the methyltransferase-DNA complex but not for the M_1S_1 -DNA complex.

The experiments described here for the methyltransferase were done at substantially higher protein (800 nM) and DNA (420 nM) concentrations than those used previously (Powell & Murray, 1995), in order to minimise dissociation of the methyltransferase to M_1S_1 . The possibility was considered that in the earlier study the results for reactions done in the absence of AdoMet reflected the fact that the methyltransferase dissociated under these conditions. However, the interference patterns with and without AdoMet were not observed to be dependent on protein concentration (results not shown).

DNase I footprinting

When DNase I footprinting was carried out in solution, the methyltransferase produced a strong footprint on each strand (not shown). On the bottom strand, the region of protection was interrupted by a DNase I-sensitive region of four bases starting at the thymine which base-pairs with the adenine base normally targeted for methylation. This cleavage may have reflected distortion of the DNA in the vicinity of the adenine methylation target. No protection of either strand was seen with M_1S_1 even after adding excess protein (results not shown).

Gel retardation revealed that there was usually a significant amount of free DNA in the solutions

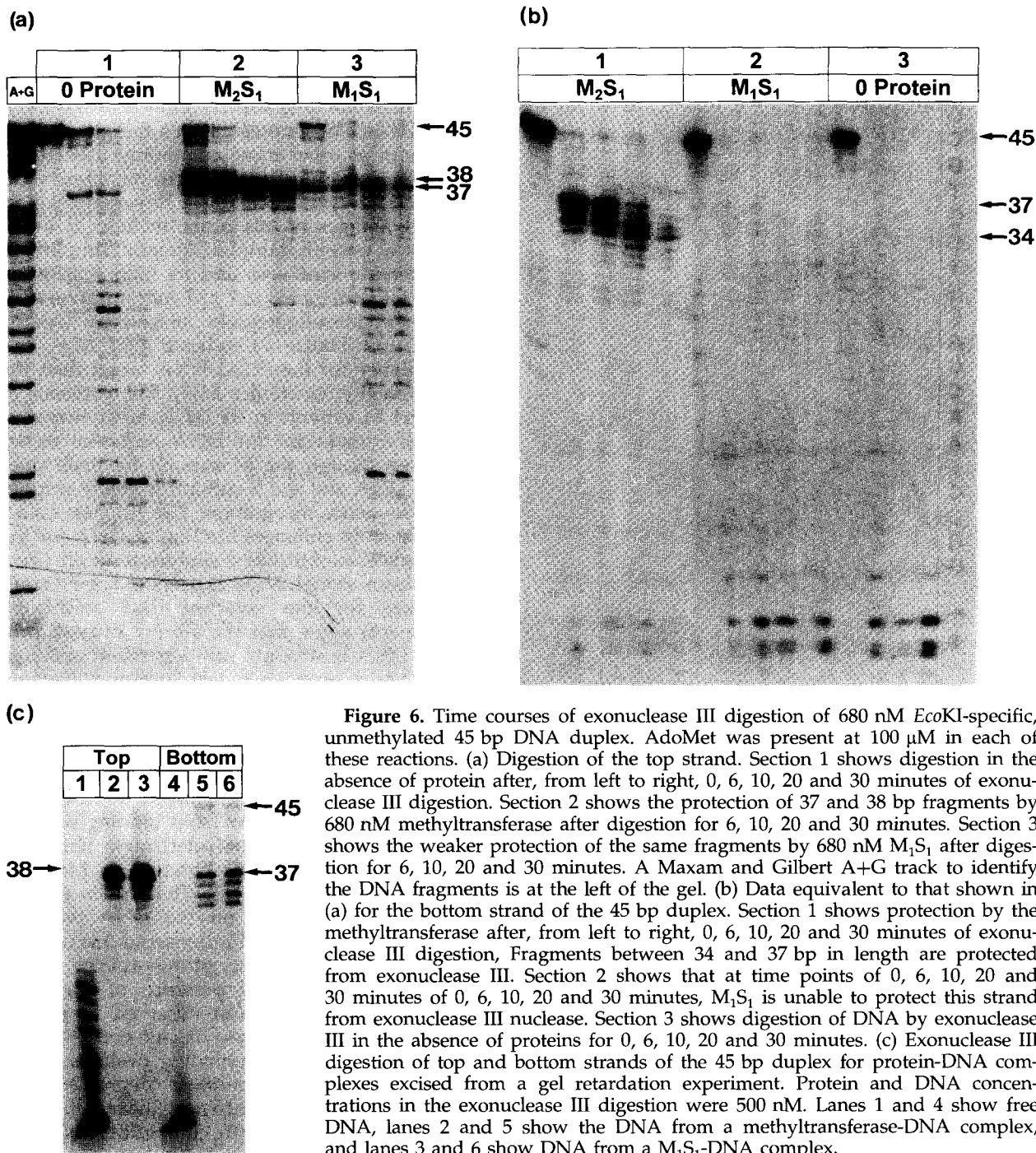


Figure 6. Time courses of exonuclease III digestion of 680 nM *EcoKI*-specific, unmethylated 45 bp DNA duplex. AdoMet was present at 100 μ M in each of these reactions. (a) Digestion of the top strand. Section 1 shows digestion in the absence of protein after, from left to right, 0, 6, 10, 20 and 30 minutes of exonuclease III digestion. Section 2 shows the protection of 37 and 38 bp fragments by 680 nM methyltransferase after digestion for 6, 10, 20 and 30 minutes. Section 3 shows the weaker protection of the same fragments by 680 nM M₁S₁ after digestion for 6, 10, 20 and 30 minutes. A Maxam and Gilbert A+G track to identify the DNA fragments is at the left of the gel. (b) Data equivalent to that shown in (a) for the bottom strand of the 45 bp duplex. Section 1 shows protection by the methyltransferase after, from left to right, 0, 6, 10, 20 and 30 minutes of exonuclease III digestion. Fragments between 34 and 37 bp in length are protected from exonuclease III. Section 2 shows that at time points of 0, 6, 10, 20 and 30 minutes of 0, 6, 10, 20 and 30 minutes, M₁S₁ is unable to protect this strand from exonuclease III nuclease. Section 3 shows digestion of DNA by exonuclease III in the absence of proteins for 0, 6, 10, 20 and 30 minutes. (c) Exonuclease III digestion of top and bottom strands of the 45 bp duplex for protein-DNA complexes excised from a gel retardation experiment. Protein and DNA concentrations in the exonuclease III digestion were 500 nM. Lanes 1 and 4 show free DNA, lanes 2 and 5 show the DNA from a methyltransferase-DNA complex, and lanes 3 and 6 show DNA from a M₁S₁-DNA complex.

containing M₁S₁ which was probably obscuring the footprint. The "in gel" footprinting method was used (Papavassiliou, 1993) to overcome this problem (Figure 8). This method gave a clear footprint for the methyltransferase on the upper strand of the 75 bp duplex of 29 bases, covering the whole of the recognition site, the spacer region and seven bases to the 5' end and nine to the 3' end of the recognition site. Hypercleavage at a guanine (G58) four bases from the edge of the footprint suggested distortion of the DNA in this region by the bound protein. The region of protection was similar with

M₁S₁ but the hypercleavage at the guanine was not as pronounced.

On the bottom strand about 31 bases were protected covering the recognition site, the spacer and ten bases to the 3' and eight to the 5' end of the recognition site. Hypercleavage at cytosine (C22') on the edge of the footprint was seen. For M₁S₁, the protected area was the same but the degree of protection was lower. The interrupted footprint for the methyltransferase-DNA complex was not observed using the "in gel" method and we suspect that the "in solution" result may be an artifact

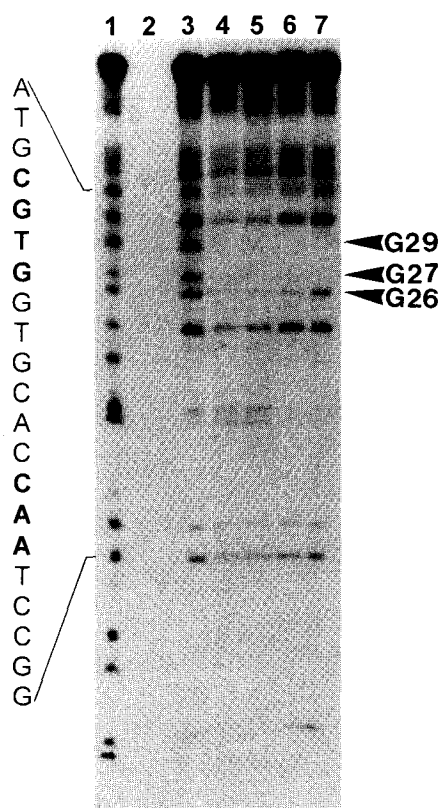


Figure 7. Methylation interference footprinting of the 45 bp *Eco*KI-specific DNA duplex. Lane 1 shows a Maxam and Gilbert A+G track and lane 2 is blank. The panel shows the footprinting on the top strand. Lane 3 is in the absence of protein, lanes 4 and 5 are in the presence of 300 nM M_1S_1 with 300 nM DNA, and lanes 6 and 7 are in the presence of 800 nM methyltransferase and 420 nM DNA. Lanes 5 and 7 contain AdoMet at 100 μ M concentration. The central sequence of the top strand is marked 5' to 3' from the bottom to the top with the *Eco*KI recognition sequence shown in bold and is numbered from 1 to 45 from the 5' end. The arrows indicate DMS-methylated guanine bases that interfere with the methyltransferase or M_1S_1 binding. G27 and G29 on the top strand are in the tetranucleotide part of the recognition sequence while G26 is in the spacer region.

as free DNA showed a similar cleavage pattern in the same region.

Conclusions

Hexachlorofluorescein when attached to the end of a DNA duplex, appears to be an excellent probe for monitoring protein-DNA interactions. The motion of the probe independent of the DNA duplex is not so large that it is insensitive to protein binding and the absence of any gross change in the fluorescence intensity simplifies the analysis of the data. The location of the hexachlorofluorescein probe at the end of the oligonucleotide, removed from the protein binding site, probably

accounts for the lack of change in the fluorescence intensity. It would be expected that this would hold for most or all DNA binding proteins. The lifetime of the excited state is of the same order of magnitude as the time scale of motion of the DNA duplex. Hexachlorofluorescein has the potential to be as useful as other more commonly used fluorescence anisotropy probes such as fluorescein (Jameson & Sawyer, 1995; Hill & Royer, 1997). Particular advantages of this probe include its availability as a phosphoramidite, greatly simplifying oligonucleotide synthesis, and its high sensitivity, allowing measurements at 1 nM concentrations.

Our hexachlorofluorescein anisotropy results (Table 1 and Figure 3) indicate that M_1S_1 has poorer affinity for the *Eco*KI DNA target than the complete methyltransferase M_2S_1 . The presence of the second M subunit in the M_2S_1 methyltransferase is very important for DNA sequence recognition even though the M subunit by itself does not bind DNA and the S subunit is solely responsible for sequence recognition. The AdoMet cofactor considerably enhances binding of DNA for the methyltransferase but has a slightly reduced effect for M_1S_1 even though M_1S_1 appears to have a similar affinity for the cofactor. The anisotropy measurements show that the affinity of both proteins for DNA is strongly salt-dependent and that AdoMet has a proportionately greater enhancing effect at the higher salt concentrations. The affinity for non-specific DNA is very low, particularly for the M_1S_1 protein.

The decrease in binding affinity as salt concentration increases from approximately 50 mM NaCl has been observed for many DNA binding proteins and is attributed to the polyelectrolyte nature of DNA (Record *et al.*, 1976; Lohman & Mascotti, 1992; Wong & Lohman, 1995). The protein is competing for binding sites on the DNA with an increasing concentration of cations and this reduces the apparent binding affinity of the protein.

Binding affinity in the presence of less than 50 mM NaCl has also been observed to remain constant or even to increase as salt concentration increases for certain DNA-binding proteins. These changes result in a non-linear dependence of $-\log(K_d)$ on $\log[\text{NaCl}]$. This non-linear behaviour has been attributed to both competition between different types of cations in the solution and to a change in the aggregation state of the protein at low salt concentration (Record *et al.*, 1976; Lohman & Mascotti, 1992; Wong & Lohman, 1995; Engler *et al.*, 1997). We note that our buffer A contains 10 mM MgCl_2 so that at low NaCl concentrations there will be competition between Mg^{2+} , Na^+ and the protein for the DNA substrate which may account for the non-linear behaviour of $-\log(K_d)$ with $\log[\text{salt}]$ which can be derived from the data in Table 1. Glutaraldehyde crosslinking does show a small proportion of higher order aggregates in samples of *Eco*KI (Dryden *et al.*, 1997).

The value of anisotropy at the end point of the titrations provides some information on the size of

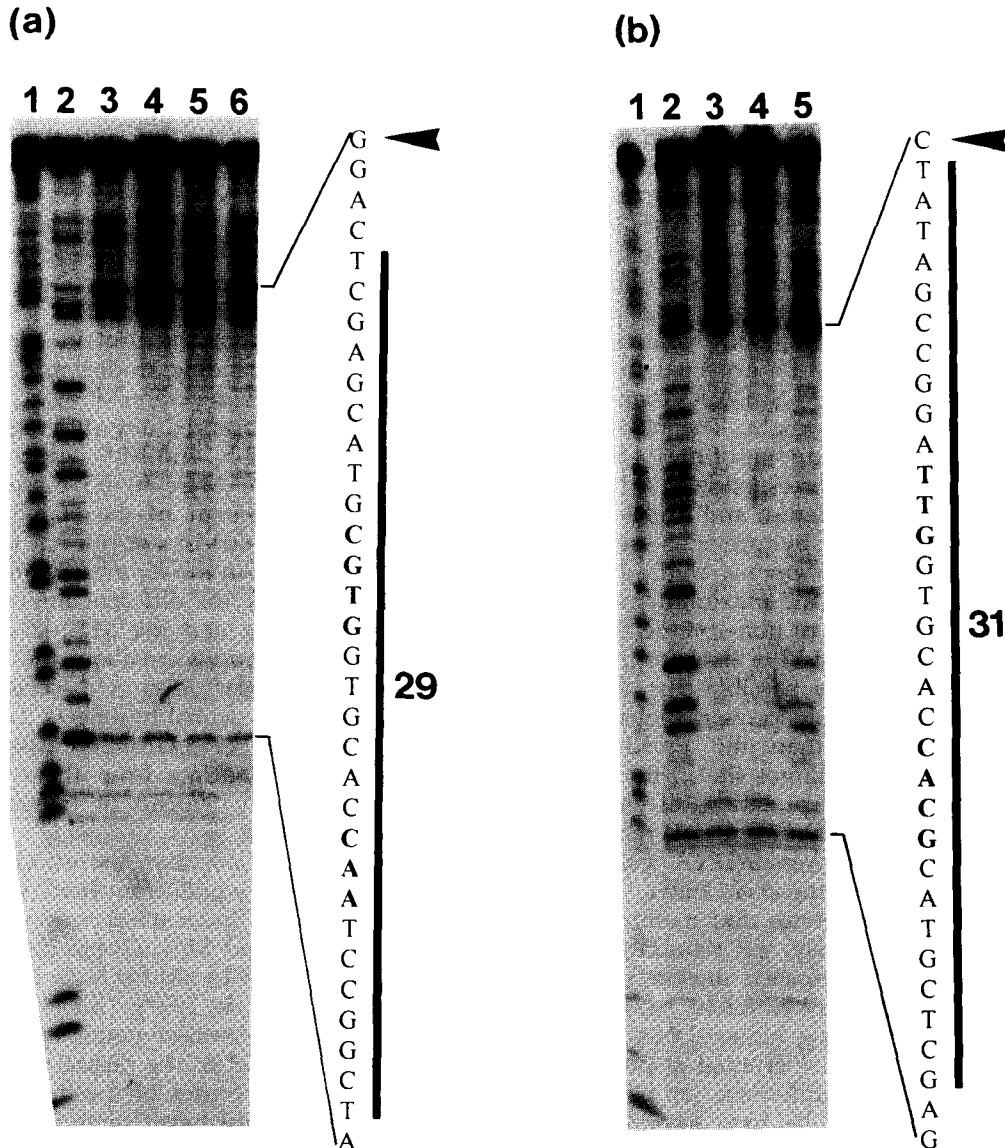


Figure 8. DNase I footprints of protein-DNA complexes as determined with the "in gel" method. 75 bp unmethylated, *EcoKI*-specific DNA and AdoMet concentrations were 180 nM and 100 μ M in all reactions, respectively. (a) Footprint on the top strand and (b) the footprint on the bottom strand. The 5' to 3' sequence A25 to G58, equivalent to A10 to G43 on the 45 bp duplex, on the top strand is marked from the bottom to the top of (a). The 3' to 5' sequence C22' to G55', equivalent to C7' to G40' on the 45 bp duplex, is marked from the top to the bottom of (b). (a) Lane 1 shows a Maxam and Gilbert A+G reaction, lane 2 has no methyltransferase, lanes 3 and 4 have 450 nM and 600 nM methyltransferase, respectively, and lanes 5 and 6 have 750 nM and 850 nM M_1S_1 , respectively. The 29 base footprint from T26 to T54 is indicated by the continuous line and DNase I hypercleavage at G58 is indicated by an arrow. The equivalent reactions are shown on the bottom strand in (b) except for the absence of a lane 6. The 31 base footprint from T23' to G53' is indicated by the continuous line and DNase I hypercleavage at C22' is indicated by an arrow.

the fluorescent molecule due to the relation between rotational diffusion and molecular size. The maximum value of Δr , the difference between the measured anisotropy of the protein-DNA complex and the free DNA, increases in the titration of *EcoKI*-specific DNA with M_1S_1 as the salt concentration is reduced. This is in contrast to the nearly constant value of Δr at the endpoints of the titrations with M_2S_1 . The increase in Δr indicates that the M_1S_1 -DNA complex is tumbling more

slowly and suggests that the size of the fluorescent complex is increasing. We interpret this as either the binding of multiple copies of M_1S_1 to the DNA or to the presence of aggregates of M_1S_1 , e.g. $(M_1S_1)_2$, which can still bind to the DNA with the same affinity as M_1S_1 . The data for binding of M_1S_1 to DNA are adequately described by the single-site binding equation even though multiple binding sites would require a more complex description. Therefore, the increase in Δr at the endpoint of the

titration may be most probably due to the formation of higher order multimers of M_1S_1 . Our data are not of high enough resolution to determine if there are multiple binding sites on the DNA with different affinities for protein or if the increase in anisotropy at the titration endpoints is due to binding of slightly aggregated forms of the proteins. The number of molecules of M_1S_1 binding to the DNA is probably of the order of two or three at low salt concentration, falling to one copy at 100 mM salt where the maximum value of Δr is lower than that of the M_2S_1 -DNA complex. The M_1S_1 -DNA complex would be expected to have a lower anisotropy than the M_2S_1 -DNA complex due to its smaller molecular weight. The nearly constant value of Δr at the endpoint of the M_2S_1 -DNA titrations most probably corresponds to a 1:1 complex between methyltransferase and EcoKI-specific DNA. This is supported by previous gel retardation experiments which demonstrated a 1:1 complex (Powell *et al.*, 1993). The maximum values of Δr are higher for binding of non-specific DNA by both methyltransferase and M_1S_1 than for the binding of specific DNA. It is possible that the non-specific duplex allows more protein molecules to crowd onto the DNA than the EcoKI-specific duplex which contains a target site in the middle of the duplex. Similar interpretations of increasing anisotropy being the result of increased mass have also been made for repressor-DNA complexes (LeTilly & Royer, 1993; Wang *et al.*, 1998).

The two different substrates and methods used to measure binding, namely, gel retardation of a 45 bp duplex and fluorescence anisotropy of a 21 bp duplex, give slightly different results with the gel retardation showing higher DNA binding affinity even though the experiment is performed in the same buffers. This difference can be attributed to several effects. It has recently been suggested that equilibration of a protein-DNA sample in the electrophoresis buffer may have occurred prior to the start of electrophoresis (Engler *et al.*, 1997), thus the gel retardation experiments are perhaps measuring DNA binding affinity in the electrophoresis buffer which is substantially different from buffer A. This suggestion has been contested by Erskine & Halford (1998) who demonstrated that fluorescence anisotropy and gel retardation gave comparable results for DNA binding by the EcoRV endonuclease. Gel retardation can also be severely affected by the kinetics of formation of the complex and electrophoresis conditions and show caging effects which artificially hold the complex together in the gel matrix (Fried, 1989; Lane *et al.*, 1992). It is clear in Figures 4 and 5 that AdoMet binding and the cage effect enhance the stability of the M_1S_1 -DNA complex in the gel. The short length of the hexachloro-fluorescein duplex cannot account for the observed difference in DNA binding affinity between gel retardation and fluorescence anisotropy methods because a 21 bp duplex can bind tightly to the methyltransferase in a gel retardation experiment

(Powell *et al.*, 1993). A major advantage of the fluorescence technique is that it is an equilibrium technique in contrast to the comparative measurements made with gel retardation.

The dissociation of the M_2S_1 into the M_1S_1 at low protein concentration has been previously observed in the absence of DNA and cofactor (Dryden *et al.*, 1993, 1997). Our gel retardation experiments show that the dissociation of the methyltransferase can occur in the presence of DNA but that it is strongly reduced when cofactor is present. Given that the protein concentrations used in our DNA binding studies are of the order of the K_d for the dissociation of M_2S_1 , one might wonder why we observed any difference between DNA binding by M_2S_1 and M_1S_1 when one would expect most of the M_2S_1 to be dissociated into M_1S_1 . The enhancement of the stability of M_2S_1 when DNA and AdoMet are present can account for this observation in the K_d measurements. In the experiment to show the different migration of the M_1S_1 -DNA complex and the M_2S_1 -DNA complex where the concentrations are above the K_d for M_2S_1 dissociation, the observation of dissociation in the absence of AdoMet probably reflects the non-equilibrium nature of gel retardation, where any M subunits which dissociate from the complex can electrophorese away instead of rebinding to the M_1S_1 -DNA complex.

It is possible to calculate the effect that DNA binding has on the equilibrium between M_2S_1 and $M_1S_1 + M$ knowing dissociation constants for DNA binding by M_2S_1 and M_1S_1 (Table 1) and the dissociation constant of 15 nM for M_2S_1 in the absence of DNA (Dryden *et al.*, 1997). This is done by constructing a simple thermodynamic cycle (Figure 9) in which the product of the dissociation constants to move from one corner of the cycle to another must be the same whichever route is taken around the cycle. One arrives at a K_d of between 0.4 and 1.2 nM for the equilibrium between M_2S_1 and M_1S_1 in the presence of DNA. The exact value depends on [NaCl] and the absence of AdoMet. This represents a 12 to 37-fold stabilisation of the M_2S_1 protein over M_1S_1 when DNA containing an EcoKI target sequence is present. One can also calculate the relative stability of M_2S_1 with respect to M_1S_1 in the presence of both DNA and AdoMet by assuming that the K_d of 15 nM for the $M_2S_1 \leftrightarrow M_1S_1 + M$ equilibrium in the absence of DNA is independent of the cofactor. One arrives at a K_d of between 1.2 and 2.1 nM, depending on [NaCl], for the equilibrium between M_2S_1 and M_1S_1 . This represents a 7 to 12-fold improvement in the stability of M_2S_1 due to the presence of AdoMet and DNA. Additionally, we have some evidence from HPLC gel filtration that, in the absence of DNA, AdoMet does actually stabilise the methyltransferase with respect to M_1S_1 (D.T.F.D., unpublished results). This effect would further enhance the stability of the complex between M_2S_1 , AdoMet and DNA. The binding to non-specific DNA is so weak that no enhancement of the stab-

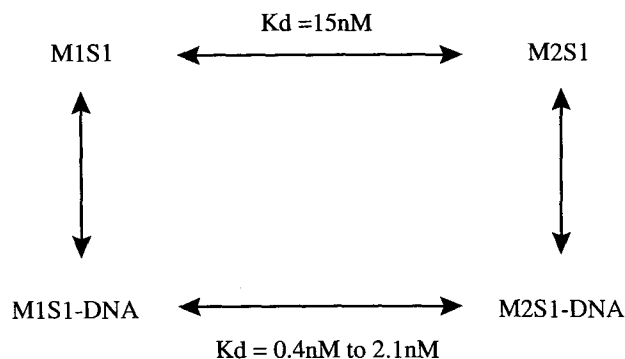


Figure 9. A diagram of the equilibria between methyltransferase, M₁S₁ and DNA as determined here and previously (Dryden *et al.*, 1997). Using the dissociation constant of 15 nM (Dryden *et al.*, 1997) for assembly of the methyltransferase, and the affinity for DNA binding by M₁S₁ and methyltransferase as given in Table 1, the dissociation constant for assembly of the methyltransferase in the presence of DNA can be calculated to be between 0.4 nM and 2.1 nM depending on the salt concentration and whether AdoMet is present. This indicates that DNA binding stabilises the methyltransferase with respect to M₁S₁.

ility of M₂S₁ occurs, indeed the slightly better binding of M₁S₁ to non-specific DNA compared to the methyltransferase implies that this form of DNA actually favours dissociation of the methyltransferase to M₁S₁. Although the M subunit does not bind DNA by itself, these calculations show that it does provide significant interactions with the DNA when the S subunit is also present.

Methylation interference results for the *Eco*KI methyltransferase and M₁S₁ show major groove contacts to all the guanine bases in the target site and to a guanine in the spacer region when AdoMet is absent (Figure 7). The S subunit is located in the major groove of the DNA (Chen *et al.*, 1995) while the M subunits are believed to make contacts with the minor groove, hence the absence of any difference between the methyltransferase and M₁S₁ in the methylation interference method, which detects major groove contacts, is expected. The addition of AdoMet removes the interference in the spacer region of the top strand for the methyltransferase but not M₁S₁. This indicates that although M₁S₁ binds AdoMet with an affinity similar to the methyltransferase, the cofactor is unable to exert the same influence at the protein-DNA interface. The exonuclease III footprint of 26-30 bp for *Eco*KI methyltransferase and M₁S₁ (Figure 6) is larger than that of *Eco*R124I methyltransferase. Exonuclease III footprinting of *Eco*R124I methyltransferase found a 25 bp footprint (Taylor *et al.*, 1993). The exonuclease III footprinting also implies that the M₁S₁ protein does not have one preferred position on the S subunit for binding the M subunit. Rather, the M subunit is probably distributed between the two possible binding sites for M on the S subunit. The DNase I footprint is similar in

extent to the exonuclease III footprint. The observation that DNase I shows the same length of footprint for methyltransferase and M₁S₁ (Figure 8) indicates that the M subunits lie on either side of the S subunit roughly parallel to the DNA helical axis as proposed previously (Burckhardt *et al.*, 1981; Kneale, 1994; Dryden *et al.*, 1995; Figure 1) rather than each M subunit being attached to only one end of the S subunit along the helical axis. In such a situation, the DNase I footprint would have been shorter for M₁S₁ when compared to the methyltransferase. DNase I footprinting of M₂S₁-DNA complexes in solution revealed some cleavage within the *Eco*KI target sequence. Although it is difficult to understand why this result should differ from that obtained with the "in gel" method and it may be artifactual, this accessibility to DNase I may correlate with hydroxyl radical footprinting of *Eco*R124I methyltransferase which revealed sites that were hypersensitive to cleavage within the target sequence (Mernagh & Kneale, 1996). Both DNase I and exonuclease III footprinting methods showed weaker footprints for the M₁S₁ complex compared to the methyltransferase, particularly on the complementary bottom strand where exonuclease III nuclease was able to completely displace M₁S₁ from the DNA. The binding sites for the two M subunits on the S subunit are not exactly equivalent because, although the M subunits are identical, the S subunit does not have two binding sites of identical amino acid sequence. Therefore, one might expect one particular M subunit to dissociate to form M₁S₁. The presence of footprints on both strands of DNA argues against this and suggests that either of the M subunits can dissociate from the methyltransferase to form M₁S₁.

It has been proposed (Burckhardt *et al.*, 1981; Kneale, 1994; Dryden *et al.*, 1995) that the S subunit lies parallel to the helical axis along one side of the DNA target and that the M subunits are attached on either side of the S subunit approximately parallel to, but displaced from, the helical axis (Figure 1). This model is supported by the results presented above and by the following observations. Comparisons with the base flipping mechanism used by C5-cytosine methyltransferases suggests that the M subunits contain a catalytic domain which moves into the minor groove at the methylation sites (Dryden *et al.*, 1995). The presence of two M subunits is essential for activity (Dryden *et al.*, 1993, 1997). Strong evidence for a large scale movement of the M subunits to wrap around the DNA target site has been found by small angle X-ray scattering of *Eco*R124I methyltransferase (Taylor *et al.*, 1994). Although the S subunits contain the determinants of sequence specificity, it has been found that the S subunit of *Eco*KI (Winter, 1998) and *Eco*R124I (Patel *et al.*, 1992) is not soluble enough to form a stable complex with DNA and that a fusion protein containing the *Eco*R124I S subunit is still unable to bind efficiently to its target (Mernagh *et al.*, 1997). The

M subunits are essential for DNA sequence recognition by type I proteins even though they contain no specificity determinants and can be exchanged between closely related type I systems (reviewed by Modrich, 1979; Yuan, 1981; Wilson & Murray, 1991; Bickle & Kruger, 1993). The influence of ionic strength and cofactor on binding by the methyltransferase and M_1S_1 , coupled with the weaker DNA binding observed for M_1S_1 and the methylation interference results, suggest that the large conformational change required to wrap around the DNA and enhance the recognition of the target sequence cannot be completely accomplished by the partially assembled M_1S_1 protein.

Methods

Protein and substrate preparation

The M subunit, M_1S_1 and M_2S_1 forms of EcoKI were purified to homogeneity and their concentrations determined by UV spectroscopy as described previously (Dryden *et al.*, 1993, 1997). Buffer A used for most experiments was 20 mM Tris-HCl, 6 mM MgCl₂, 7 mM β-mercaptoethanol (pH 8) with varying amounts of NaCl as stated. 5% (v/v) glycerol was included in gel retardation and footprinting experiments. AdoMet (New England Biolabs) was present at 100 μM in some experiments to ensure saturation of binding sites (Powell *et al.*, 1993).

Measurement of AdoMet binding

AdoMet binding was performed by mixing 6 μM protein with 0.5 μM [methyl-³H]AdoMet (Amersham) in 50 μl 20 mM Tris, 20 mM 2-(N-morpholino)ethanesulphonic acid, 10 mM MgCl₂, 200 mM NaCl, 0.1 mM EDTA, 7 mM β-mercaptoethanol (pH 6.5). The sample was passed through a 10 ml Sephadex G50 gel filtration column (Pharmacia PD10 column) and fractions collected for scintillation counting as described previously (Willcock *et al.*, 1994).

Preparation of DNA oligonucleotides

Oligonucleotides with and without the EcoKI target sequence were synthesized using standard phosphoramidite chemistry. Oligonucleotides containing the EcoKI target sequence 5'-AAC (N₆) GTGC-3' or the non-specific sequence 5'-CCA (N₆) TGTA-3' were referred to as "top" strands and the complementary sequences as "bottom" strands. Oligonucleotides labelled with hexachlorofluorescein at their 5'-ends were prepared using a hexachlorofluorescein phosphoramidite (Applied Biosystems). The manufacturer's instructions were followed during the synthesis and ammonia deblocking. All oligonucleotides were purified by reversed-phase high performance liquid chromatography on a C18 column and their concentrations determined using their known base composition and UV spectroscopy. Duplex DNA was prepared by mixing one equivalent of labelled oligonucleotide with 1.1 equivalents of the complementary strand in buffer A. The solution was heated to 100°C and allowed to slowly cool to room temperature.

Synthetic, 21 bp unmethylated DNA oligonucleotide duplexes containing a hexachlorofluorescein label on the top strand were prepared as described above and used to compare specific and non-specific binding to M_1S_1

and M_2S_1 via observation of the change in fluorescence anisotropy of the label which occurs upon protein binding. The DNA sequence of the top strand was 5'-hexachlorofluorescein-GCCTAACCACGTGGTGCCTAC-3' for the oligonucleotide containing an EcoKI target (in bold) and 5'-hexachlorofluorescein-GCCTCCACACGTG-TGTAGTAC-3' for a non-specific DNA target. The 21 bp fluorescent duplex containing the EcoKI target has the same sequence as the central portion of the 45 bp duplex used in gel retardation (Powell *et al.*, 1993). These duplexes were used for measurements of fluorescence anisotropy as a function of added protein concentration. The duplex containing the EcoKI target was also used for fluorescence lifetime experiments and the measurement of anisotropy as a function of temperature/viscosity.

Synthetic 45 bp DNA duplexes containing the EcoKI recognition site in an unmethylated state were prepared by hybridisation after labelling the 5' end of the strand of interest using polynucleotide kinase and [γ -³²P]ATP (Amersham). These oligonucleotides and their use to measure DNA binding by EcoRI by gel retardation have been described (Powell *et al.*, 1993). The top strand had the sequence 5'-TGTCTAGATATCGGCCTAACCACGTGGTGCCTACGAGCTCAGGCG-3'. This duplex was used for gel retardation, exonuclease III footprinting and methylation interference.

The 75 base, top strand oligonucleotide (OSWEL DNA) had the sequence 5'-CATATCCACATCCGGTGTCTAGATATCGGCCTAACCACGTGGTGCCTACGAGCTCAGGCGCATGCCGTAGCGCGG-3', which was hybridised with its complementary strand after labelling with [γ -³²P]ATP. The central sequence of the unmethylated duplex is the same as the 45 bp duplex described above. The 75 bp duplex was used for DNaseI footprinting.

Properties of oligonucleotide duplexes labelled with hexachlorofluorescein

A measurement of the fluorescent lifetime of 0.5 μM hexachlorofluorescein-labelled 21 bp EcoKI-specific duplex in buffer A supplemented with 100 mM NaCl was made with an Edinburgh Instruments FL900 time-resolved fluorimeter. Excitation and emission wavelengths were 530 nm and 570 nm, respectively with 18 nm bandwidths. The excitation pathlength was 2 mm, emission pathlength was 10 mm and the temperature was 25°C. An estimate of the degree of correlation between probe motion and motion of this hexachlorofluorescein-labelled duplex was made by varying the viscosity of the 1 μM solution in buffer A supplemented with 100 mM NaCl by adding glycerol and measuring the anisotropy as a function of temperature/viscosity. The viscosity of the solutions was determined from tables of viscosity of glycerol/water mixtures (Segur, 1953) and assuming that the buffer viscosity was the same as that of water. A Perkin Elmer LS50B fluorimeter was used for these measurements. Excitation was at 530 nm with 10 nm bandwidth, emission was measured through a 570 nm cut-off filter (HV Skan Ltd. Solihull, UK) and the sample was in a 3 mm × 3 mm cuvette. Analysis of the fluorescence lifetime, and measurement of the anisotropy of the duplex as a function of temperature/viscosity using the Perrin equation (Lakowicz, 1983) gave a measure of the motion of the hexachlorofluorescein label with respect to the DNA duplex. The Perrin equation is $1/r = r_0/(1 + \tau/\phi)$ where r is the measured anisotropy, r_0 is the intrinsic anisotropy of the

fluorophore in the absence of any rotational motion, τ is the fluorescence lifetime and ϕ is the rotational correlation time which equals $\eta V/kT$ assuming spherical symmetry. η is the viscosity (cP), V is the molecular volume, k is Boltzmann's constant and T is the temperature (K).

Measurement of DNA binding with fluorescent oligonucleotides

Anisotropy measurements were performed at 25°C on 400 μ l samples in a SLM-Aminco 8100 fluorimeter for DNA concentrations of 1 nM. The excitation was at 530 nm and emission was detected through a 570 nm cut-off filter. The excitation pathlength was 10 mm, the emission pathlength 2 mm and excitation slitwidth was 8 nm. Small amounts of proteins were added to the DNA solution in the cuvette using a microlitre syringe and gently stirred. The cuvette was not removed from the instrument for these additions. Three to six measurements of anisotropy were made and averaged, and each protein titration repeated at least in duplicate. Each titration took approximately 45 minutes to complete. Slight differences in the anisotropy of the free DNA between different experiments were most probably due to small differences in the exact position of the cuvette in the sample chamber of the fluorimeter. Data were fitted to a single-site binding equation which accounted for significant concentrations of complex when the DNA concentration was similar to the K_d (Heyduk & Lee, 1990). The single site binding equation was also applicable to the case of multiple binding sites of equal affinity. The change in anisotropy reflected the increase in size and change of shape of the DNA duplex upon protein binding (Lakowicz, 1983; Eftink, 1997; Jameson & Sawyer, 1995; Hill & Royer, 1997).

Analysis of DNA binding by gel retardation and footprinting

Gel retardation was performed as described previously (Powell *et al.*, 1993) using the 45 bp duplex containing the EcoKI target site.

The optimum conditions for exonuclease III footprinting were established by Metzger & Heumann (1994). Methyltransferase or M_1S_1 (850 nM) in buffer A plus 100 mM NaCl, 5% glycerol and 350 nM DNA were incubated with 100 μ M AdoMet in a total volume of 60 μ l for ten minutes on ice. The 45 bp duplex labelled at the 5' end of one strand was then added to the mixture. After the addition of DNA, 40 μ l of each sample was transferred to a separate tube and the samples were incubated for a further ten minutes at 22°C to allow protein-DNA complex formation. After this time, ten units of exonuclease III (Boehringer Mannheim) were added to the 40 μ l sample. For the zero time point, 8 μ l was removed from the 20 μ l in the original tube and added to 8 μ l of 0.6 M sodium acetate, 40 mM EDTA, 100 μ g/ml calf thymus DNA (pH 5.2) to stop digestion. For the other time points, 8 μ l was removed from the tube containing the 40 μ l sample to which the exonuclease III had been added. 40 μ l of ethanol was added to these 8 μ l samples and the samples stored at -20°C for one hour before spinning in a microcentrifuge to precipitate the DNA. After ethanol precipitation, the pellets were washed with 70% ethanol, air-dried and resuspended in 10 μ l 90% formamide, 2 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol. 2-4 μ l of these samples were run on 12% polyacrylamide (Longranger, Flowgen), 7 M

urea, 0.6 \times TBE sequencing gels for one hour at 45-50°C and ~38 W. The gels were dried without fixing for two hours at 80°C and then exposed to X-ray film. The amount of protein-DNA complex formation in each reaction was tested using gel retardation on a duplicate sample.

Methylation interference was performed as described previously (Powell & Murray, 1995) except higher protein and DNA concentrations were used. This was necessary for the methyltransferase reactions in order to prevent dissociation to M_1S_1 and hence a low yield of methyltransferase-DNA complexes on the preparative bandshift gels. Electrophoresis time of the preparative bandshift gels was extended to allow separation of mtase-DNA and M_1S_1 -DNA complexes.

The DNase I footprinting method used, in solution, was adapted from Leblanc & Moss (1994). The solution used to stop DNase I digestion was as described by Leblanc & Moss (1994) except 100 μ g/ml calf thymus DNA was substituted for 40 μ g/ml tRNA. Binding reactions (20 μ l) contained 0 or 600 nM protein, 100 μ M AdoMet, 200 nM unmethylated 75 bp EcoKI-specific duplex DNA in 20 mM Tris-HCl, 100 mM NaCl, 2.5 mM CaCl₂, 5 mM MgCl₂, 5% glycerol (pH 8) buffer. The DNase I reaction was started by the reaction of 0.01 or 0.025 Kunitz units of DNase I in a volume of 1 μ l. The two minute digestion at 22°C was stopped by adding 20 μ l stop solution and 100 μ l ethanol. The precipitated DNA was then processed as described for the exonuclease III reactions. The amount of protein-DNA complex formed in each reaction was tested using gel retardation. The "in gel" DNase I footprinting method was adapted from Papavassiliou (1993). A gel retardation experiment was performed to separate free from bound DNA. The bands corresponding to a protein-DNA complex were excised after autoradiography of the wet 5% polyacrylamide gel, and the gel slices subjected to DNase I digestion by first diffusing the enzyme into the gel slices (one hour, 22°C), followed by activation by the addition of a mixture of MgCl₂ and CaCl₂. The gels used were 2 mm thick and the slices were 5 mm \times 2 mm \times 3 mm (~30 μ l). The DNase I solution contained 25 mM Tris-HCl (pH 7.8), 62.5 μ g/ml DNase I, 62.5 μ g bovine serum albumin, 0.625 mM dithiothreitol and 0.3% polyethylene glycol 20,000 (BDH). DNA eluted from the gel slice was prepared for loading onto the 12% polyacrylamide sequencing gel in the same way as the exonuclease III footprinting samples. Scintillation counting of 1 μ l spots of each sample was used to ensure equal counts were loaded for each lane of the gel. A+G reactions were performed as described (Papavassiliou, 1994).

Acknowledgements

We thank Professor Noreen Murray (Edinburgh) and our colleagues in her laboratory for advice and encouragement and particularly Mr Laurie Cooper (Edinburgh) for preparing the protein samples. We also thank Dr Jeremy Lakey (Newcastle) for extensive use of his SLM-Aminco Fluorimeter and Dr John Gilchrist (Edinburgh Instruments) for providing access to the time-resolved fluorimeter. This work was supported by grants from the Biotechnology and Biological Research Council, the Medical Research Council and The Royal Society. D.D. thanks the Royal Society for a University Research Fellowship.

References

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1992). *Short Protocols in Molecular Biology*, 2nd edit., J. Wiley & Sons, New York.
- Bickle, T. A. & Kruger, D. H. (1993). Biology of DNA restriction. *Microbiol. Rev.* **57**, 434–450.
- Burckhardt, J., Weisemann, J., Hamilton, D. L. & Yuan, R. (1981). Complexes formed between the restriction endonuclease EcoKI can be UV-crosslinked to a bromodeoxyuridine-substituted DNA target sequence. *J. Mol. Biol.* **153**, 425–440.
- Chen, A., Powell, L. M., Dryden, D. T. F., Murray, N. E. & Brown, T. (1995). Tyrosine 27 of the specificity polypeptide of EcoKI can be UV-crosslinked to a bromodeoxyuridine-substituted DNA target sequence. *Nucl. Acids Res.* **23**, 1177–1183.
- Cooper, L. P. & Dryden, D. T. F. (1994). The domains of a type I DNA methyltransferase. Interactions and role in recognition of DNA methylation. *J. Mol. Biol.* **236**, 1101–1021.
- Dryden, D. T. F., Cooper, L. P. & Murray, N. E. (1993). Purification and characterisation of the methyltransferase from the type I restriction and modification system of *Escherichia coli* K12. *J. Biol. Chem.* **268**, 13228–13236.
- Dryden, D. T. F., Sturrock, S. S. & Winter, M. (1995). Structural modelling of a type I DNA methyltransferase. *Nature Struct. Biol.* **2**, 632–635.
- Dryden, D. T. F., Cooper, L. P., Thorpe, P. H. & Byron, O. (1997). The *in vitro* assembly of the EcoKI type I DNA restriction/modification enzyme and its *in vivo* implications. *Biochemistry*, **36**, 1065–1076.
- Eftink, M. R. (1997). Fluorescence methods for studying equilibrium macromolecule-ligand interactions. *Methods Enzymol.* **278**, 221–257.
- Engler, L. E., Welch, K. K. & Jen-Jacobson, L. (1997). Specific binding of EcoKV endonuclease to its recognition site GATATC. *J. Mol. Biol.* **269**, 82–101.
- Erskine, S. G. & Halford, S. E. (1998). Reactions of the EcoRV restriction endonuclease with fluorescent oligodeoxynucleotides: identical equilibrium constants for binding to specific and non-specific DNA. *J. Mol. Biol.* **275**, 759–772.
- Fried, M. G. (1989). Measurement of protein-DNA interaction parameters by electrophoresis mobility shift assay. *Electrophoresis*, **10**, 366–376.
- Heyduk, T. & Lee, J. C. (1990). Application of fluorescence energy transfer and polarisation to monitor *Escherichia coli* cAMP receptor protein and lac promoter interaction. *Proc. Natl Acad. Sci. USA*, **87**, 1744–1748.
- Hill, J. J. & Royer, C. A. (1997). Fluorescence approaches to study of protein-nucleic acid complexation. *Methods Enzymol.* **278**, 390–416.
- Jameson, D. M. & Sawyer, W. H. (1995). Fluorescence anisotropy applied to biomolecular interactions. *Methods Enzymol.* **246**, 283–300.
- Kelleher, J. E., Daniel, A. S. & Murray, N. E. (1991). Mutations that confer *de novo* activity upon a maintenance methyltransferase. *J. Mol. Biol.* **221**, 431–440.
- King, G. & Murray, N. E. (1994). Restriction enzymes in cells, not eppendorfs. *Trends Microbiol.* **2**, 465–469.
- Kneale, G. G. (1994). A symmetrical model for the domain structure of type I DNA methyltransferases. *J. Mol. Biol.* **243**, 1–5.
- Lakowicz, J. R. (1983). *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.
- Lakowicz, J. R. & Weber, G. (1980). Nanosecond segmental mobilities of tryptophan residues in proteins observed by lifetime-resolved fluorescence anisotropies. *Biophys. J.* **32**, 591–601.
- Lane, D., Prentki, P. & Chandler, M. (1992). Use of gel retardation to analyse protein-nucleic acid interactions. *Microbiol. Rev.* **56**, 509–528.
- Leblanc, B. & Moss, T. (1994). DNaseI footprinting. In *DNA-protein Interactions. Principles and Protocols* (Kneale, G. G., ed.), pp. 1–10, Humana Press, New Jersey.
- LeTilly, V. & Royer, C. A. (1993). Fluorescence anisotropy assays implicate protein-protein interactions in regulating trp repressor DNA-binding. *Biochemistry*, **32**, 7753–7758.
- Lohman, T. M. & Mascotti, D. P. (1992). Thermodynamics of ligand-nucleic acid interactions. *Methods Enzymol.* **212**, 400–424.
- Mernagh, D. R. & Kneale, G. G. (1996). High resolution footprinting of a type I methyltransferase reveals a large structural distortion within the DNA recognition site. *Nucl. Acids Res.* **24**, 4853–4858.
- Mernagh, D. R., Reynolds, L. A. & Kneale, G. G. (1997). DNA binding and subunit interactions in the type I methyltransferase M.EcoR124I. *Nucl. Acids Res.* **25**, 987–991.
- Metzger, W. & Heumann, H. (1994). Footprinting with exonuclease III. In *Methods in Molecular Biology, DNA-Protein Interactions: Principles and Protocols* (Kneale, G. G., ed.), vol. 30, pp. 11–20, Humana Press Inc., Totowa, NJ.
- Modrich, P. (1979). Structures and mechanisms of DNA restriction and modification enzymes. *Quart. Rev. Biophys.* **12**, 316–369.
- Papavassiliou, A. G. (1993). *In gel* DNaseI protection analysis of electrophoretically fractionated DNA-binding reactions. *Methods Mol. Cell. Biol.* **4**, 95–104.
- Papavassiliou, A. G. (1994). 1,10-Phenanthroline-copper ion nuclease footprinting of DNA-protein complexes *in situ* following mobility-shift electrophoresis assays. In *Methods in Molecular Biology, DNA-Protein Interactions, Principles and protocols* (Kneale, G. G., ed.), vol. 30, pp. 43–78, Humana Press, New Jersey.
- Patel, J., Taylor, I., Dutta, C., Kneale, G. & Firman, K. (1992). High level expression of the subunits and intact DNA methyltransferase of the type I restriction and modification system EcoR124I. *Gene*, **112**, 21–27.
- Powell, L. M. & Murray, N. E. (1995). S-Adenosyl methionine alters the DNA contacts of the EcoKI methyltransferase. *Nucl. Acids Res.* **23**, 967–974.
- Powell, L. M., Dryden, D. T. F., Willcock, D. F., Pain, R. H. & Murray, N. E. (1993). DNA recognition by the EcoKI methyltransferase: the influence of DNA methylation and the cofactor S-adenosyl-L-methionine. *J. Mol. Biol.* **234**, 60–71.
- Record, M. T., Jr., Lohman, T. M. & De Haseth, P. (1976). Ion effects on ligand-nucleic acid interactions. *J. Mol. Biol.* **107**, 145–158.
- Segur, J. B. (1953). Physical properties of glycerol and its solutions. In *Glycerol* (Miner, C. S. & Dalton, N. N., eds), vol. 117, pp. 238–334, Reinhold Publishing Corp. New York.
- Studier, F. W. & Bandyopadhyay, P. K. (1988). Model for how type I restriction enzymes select cleavage sites in DNA. *Proc. Natl Acad. Sci. USA*, **85**, 4677–4681.

- Suri, B., Nagaraja, V. & Bickle, T. A. (1984). Bacterial DNA modification. *Curr. Top. Microbiol, Immunol.* **108**, 1–9.
- Taylor, I. A., Patel, J., Firman, K. & Kneale, G. G. (1992). Purification and biochemical characterisation of the Eco124 type I modification methylase. *Nucl. Acids Res.* **20**, 179–186.
- Taylor, I., Watts, D. & Kneale, G. (1993). Substrate recognition and selectivity in the type IC DNA modification methylase M.EcoR124I. *Nucl. Acids Res.* **21**, 4929–4935.
- Taylor, I. A., Davis, K. G., Watts, D. & Kneale, G. G. (1994). DNA binding induces a major structural transition in a type I methyltransferase. *EMBO J.* **13**, 5772–5778.
- Wang, K., Rodgers, M. E., Toptygin, D., Munsen, V. A. & Brand, L. (1998). Fluorescence study of the multiple ligand binding equilibria of the galactose repressor. *Biochemistry*, **37**, 41–50.
- Willcock, D. F., Dryden, D. T. F. & Murray, N. E. (1994). A mutational analysis of the two motifs common to adenine methyltransferase. *EMBO J.* **13**, 3902–3908.
- Wilson, G. G. & Murray, N. E. (1991). Restriction and modification systems. *Annu. Rev. Genet.* **25**, 585–627.
- Winter, M. (1988). Investigation of *de novo* methylation activity in mutants of the EcoKI methyltransferase. Ph.D thesis, University of Edinburgh.
- Wong, I. & Lohman, T. M. (1995). Linkage of protein assembly to protein-DNA binding. *Methods Enzymol.* **259**, 95–127.
- Yuan, R. (1981). Structure and mechanism of multifunctional restriction endonucleases. *Annu. Rev. Biochem.* **50**, 285–315.

Edited by J. Karn

(Received 15 May 1998; received in revised form 11 August 1998; accepted 13 August 1998)