Sequence-Specific Binding of DNA by the *Eco*RV Restriction and Modification Enzymes with Nucleic Acid and Cofactor Analogues†

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ABSTRACT: The DNA-binding properties of the *Eco*RV restriction endonuclease and modification methyltransferase with their recognition sequence (GATATC) were analyzed using the electrophoretic band-shift assay. It has previously been observed that the endonuclease does not bind specifically to GATATC sequences in the absence of the essential cofactor Mg^{2+}. To investigate any possible roles for Mg^{2+} in promoting specific DNA binding, a set of hydrolysis-resistant oligonucleotide substrates were synthesized that contained either phosphate (phosphorothioate, 3′-*S*-phosphorothiolate), sugar (4′-thiothymidine), or base (7-deaza-2′-deoxyadenosine) modifications. However, it was found that none of these were specifically bound by the endonuclease in either the absence or the presence of Mg^{2+}. In contrast, the methylase bound to GATATC sequences much more strongly than to nonspecific sites, and it was possible to observe the formation of enzyme−DNA complexes by gel retardation. Binding to GATATC sequences was increased by the addition of sinefungin, a nonreactive analogue of the essential cofactor *S*-adenosyl-L-methionine (AdoMet). Presumably this also occurs with AdoMet although methylation and turnover prevented its direct observation. In the presence of sinefungin the strongest binding was observed with hemimethylated *Eco*RV sequences (K_d = 11−13 nM), and unmethylated DNA was bound less well (K_d = 46 nM). Specific, albeit weaker binding was also seen with the dimethylated product (K_d = 143 nM). A difference in electrophoretic mobility was observed between enzyme−substrate and enzyme−product complexes suggestive of structural differences between them. The K_{app} value found for sinefungin, with the hemimethylated *Eco*RV sequence, was 10.9 mM.

Type II restriction endonucleases cut their recognition sequences with very high selectivity, and a change of just one base pair can reduce the hydrolysis rate by a factor of at least 1 million (Taylor & Halford, 1989; Lesser *et al.*, 1990; Thielking *et al.*, 1990). Endonucleases appear to divide into two classes. The first which includes *Eco*RI, *Rsr*I, and *Bam*HI binds to their cognate sequences much more tightly than others in the absence of Mg^{2+} (Halford & Johnson, 1980; Terry *et al.*, 1983; Lesser *et al.*, 1990; Aiken *et al.*, 1991; Xu & Schildkraut, 1991), and this suggests that a substantial proportion of the measured discrimination arises at the DNA-binding step. A second group that includes *Eco*RV and *Taq*I, shows little or no discrimination when Mg^{2+} is absent (Taylor *et al.*, 1991; Zebala *et al.*, 1992), and this can be accounted for in two ways. Either Mg^{2+} is essential to promote specificity in substrate binding or a large proportion of the selectivity arises at the catalytic rather than the binding step. With the best studied enzyme, *Eco*RV [for recent reviews, see Halford *et al.* (1993) and Vipond and Halford (1993)], it has been demonstrated that binding discrimination does occur providing Mg^{2+} is present. In order to prevent turnover, this has been shown by either (a) altering the protein to the inactive variant D90A (Thielking *et al.*, 1992) or (b) using Ca^{2+}, a cation that does not support hydrolysis (Vipond & Halford, 1995). However, there have been no reports of the preparation of specific complexes between the endonuclease and DNA using wild-type enzyme, in the presence of Mg^{2+}, but with a hydrolysis-resistant oligodeoxynucleotide. These experiments would obviously complement those using the D90A mutant or Ca^{2+} and might shed further light on the mechanisms the endonuclease uses to generate specificity. Such complexes may also be useful for crystallography and augment the structures already available (Winkler, 1992; Winkler *et al.*, 1993; Kostrewa & Winkler, 1995). This publication reports on such experiments using hydrolysis-resistant oligonucleotides containing a variety of phosphate, sugar, and base analogues.

The *Eco*RV methylase has not been studied to anywhere near the same depth as its partner endonuclease. This enzyme adds a CH_3 group to the first dA base in GATATC sequences (Nwosu *et al.*, 1988) using *S*-adenosylmethionine (AdoMet) as the methyl group donor. Amino acid sequence similarities with other methylases such as *Escherichia coli* and T4 *Dam* and *Dpn*II (Lauster *et al.*, 1987; Guschlbauer, 1988; Chandrasegaran & Smith, 1988), which have similar DNA targets, have been noted. A protein−DNA cocrystal structure is available for one DNA dC methylase, *Hha*I (Cheng *et al.*, 1993; Klimasauskas *et al.*, 1994), and recently a structure, in the absence of DNA, has been reported for the dA methylase *Taq*I (Labahn *et al.*, 1994). All of the DNA methyltransferases studied to date bind their recognition sequences more strongly than random DNA, and

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AdoMet, as well as being involved in catalysis, often increases the binding of target sequences and improves selectivity (Reich & Mashhoon, 1990; Bergerat & Guschlbauer, 1990; Bergerat *et al.*, 1991; Dubey & Roberts, 1992; Powell *et al.*, 1993). In this study we have evaluated the ability of the *Eco*RV methylase to specifically bind to its GATATC recognition sequence in the unmethylated, hemimethylated, and dimethylated states. In addition, the effects of AdoMet, the product of the reaction of *S*-adenosylhomocysteine (AdoHcy) and the nonmethylating AdoMet analogue sinefungin, on the specific binding of DNA have been investigated. This is the first time such studies have been carried out with the *Eco*RV methylase, and these studies allow a comparison of how the endonuclease and the methylase, which recognize the same DNA sequence but otherwise are completely different, achieve their observed high specificity.

MATERIALS AND METHODS

Protein Purification. The purification and the determination of the concentration of both the *Eco*RV endonuclease (Luke *et al.*, 1987; Newman *et al.*, 1990a) and methylase (Nwosu *et al.*, 1988) have been described. The Blue-Sepharose column previously used as the second step in the methylase purification was replaced by gel filtration on a 30 × 0.8 cm Protein-Pak 300W column (Waters-Millipore) eluting with 20 mM PIPES (pH 6.5), 1 mM EDTA, 200 mM NaCl, and 10% (v/v) glycerol at a flow rate of 1 mL min^{-1} and utilizing a Waters 650E Fast Protein purification system. The eluent was monitored at 280 nm, and fractions that contained the methylase were pooled and concentrated to 500 μL using an Amicon Centriprep-10 spun concentrator at 4 °C.

Oligonucleotide Preparation. Oligodeoxynucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer using the phosphoramidite method and with reagents from either Applied Biosystems or Cruachem (Newman *et al.*, 1990a; Connolly, 1991, 1992; Waters & Connolly, 1994). The phosphoramidites of N^6-methyldeoxyadenosine and 7-deazadeoxyadenosine were purchased from Pharmacia and Glen Research (Sterling, VA). Oligonucleotides containing a 3′-*S*-phosphorothiolate linkage were produced using a 3′-thiothymidine phosphoramidite (kindly supplied by Dr. R. Cosstick and Dr. J. Vyle, University of Liverpool, U.K.) as described (Cosstick & Vyle, 1989; Vyle *et al.*, 1992). Oligonucleotides that had a 4′-thiothymidine residue were synthesized using a 4′-thiothymidine phosphoramidite (Hancox *et al.*, 1993) that was a kind gift from Prof. R. T. Walker and Dr. L. Hancox (University of Birmingham, U.K.). Phosphorothioate-containing oligonucleotides were prepared using the sulfurizing reagent 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Iyer *et al.*, 1990a,b) as previously outlined (Grasby & Connolly, 1992). This reagent was purchased from Glen Research. Oligodeoxynucleotides were purified using DMT on separation on reverse-phase (C-18) HPLC columns (Newman *et al.*, 1990a; Connolly, 1991, 1992). This protocol gave oligonucleotides of greater than 97% purity. The separation of the phosphorothioate diastereoisomers was carried out by reverse-phase HPLC on analytical size C-18 columns using a 25 min gradient of 5−35% acetonitrile in 0.1 M morpholine acetate buffer (pH 7.0) at a flow rate of 1 mL min^{-1} and a column temperature of 55 °C. The R_p diastereomer eluted before the S_p (Connolly *et al.*, 1984; Stec *et al.*, 1984, 1985; Grasby & Connolly, 1992). All oligodeoxynucleotides were desalted by passage through disposable NAP-25 gel filtration columns (Pharmacia) eluting with water. Desalted oligonucleotides were lyophilized, redissolved in small quantities of water, and stored at −20 °C. The concentrations of these oligomers were determined as previously described (Newman *et al.*, 1990a; Connolly, 1991, 1992). Oligodeoxynucleotides were labeled at their 5′-termini using [γ-^{32}P]ATP and polynucleotide kinase (Sambrook *et al.*, 1989) and following the reaction were desalted and separated from excess ATP using a Sep-Pak C-18 cartridge (Waters-Millipore). Oligonucleotides were annealed by mixing *n* pmol of the radioactively labeled strand with 1.5 × *n* pmol of the complementary unlabeled strand to give a double-stranded DNA concentration of 0.02 pmol/mL. Samples were heated at 80 °C in a water bath for 5 min and then left to slowly cool to ambient temperature. Annealed samples were stored at −20 °C.

Binding Studies. The binding of the *Eco*RV endonuclease to oligodeoxynucleotides was performed in 20 μL volumes of 50 mM Hepes (pH 7.0) containing 130 mM NaCl, 5 mM EDTA, and 1 mg of acetylated BSA (Sigma). The amounts of the endonuclease and ^{32}P-labeled annealed oligonucleotides varied as detailed in the Results section. Where appropriate, the EDTA was replaced by 5−40 mM Mg^{2+} and the concentration of the NaCl reduced to maintain a constant ionic strength. The mixtures were incubated at 20 °C for 30 min and were analyzed by the gel-shift method using nondenaturing gel electrophoresis [12 × 15 × 0.5 cm gels, 10.8% polyacrylamide run in TBE buffer (89 mM Tris−borate, pH 8.2, 1 mM EDTA) at 40 W for 90 min]. When binding in the presence of Mg^{2+} was studied, the EDTA in TBE buffer was replaced with 5 mM $MgCl_2$. Following electrophoresis the gels were dried and the bands corresponding to free and protein-bound DNA detected by autoradiography. The binding of the *Eco*RV methylase to oligodeoxynucleotides was carried out in 20 μL volumes of 50 mM Hepes (pH 7.5) containing 10 mM EDTA, 100 mM NaCl, 5 mM DTT, and 1 mg of acetylated BSA. The amounts of the methylase and ^{32}P-labeled oligonucleotide varied as detailed in the Results section. Where appropriate, AdoMet, AdoHcy, or sinefungin was included in the binding mixtures at the concentrations given in the Results section. AdoMet, AdoHcy, and sinefungin were purchased from Sigma. The gel-shift method and analysis of the bands by autoradiography were as given for the endonuclease. For the determination of K_d values the intensities of each band (and hence the proportion of nucleic acid in each) were evaluated by scanning of the developed X-ray films using a Joyce-Lobel ChromoScan fitted with a 530 nm filter at an aperture width of 1.0 mm. The percent DNA bound was plotted against the methylase concentration and the binding isotherm fitted using Fig. P. Usually the enzyme concentration used is much larger than the level of DNA, and in these cases the K_d is simply the amount of protein needed to complex 50% of the DNA. However, we obtained the most satisfactory and reproducible gel shifts at DNA levels of 5 nM and methylase concentrations between 10 and 200 nM (unmethylated sequences), 5 and 80 nM (hemimethylated sequences), and 50−300 nM (dimethylated sequences). In these instances, where the enzyme levels are not in great excess over the DNA, the K_d can be estimated using K_d = [methylase]$_{1/2}$ − $^1/_2$[DNA], where [methylase]$_{1/2}$ is the

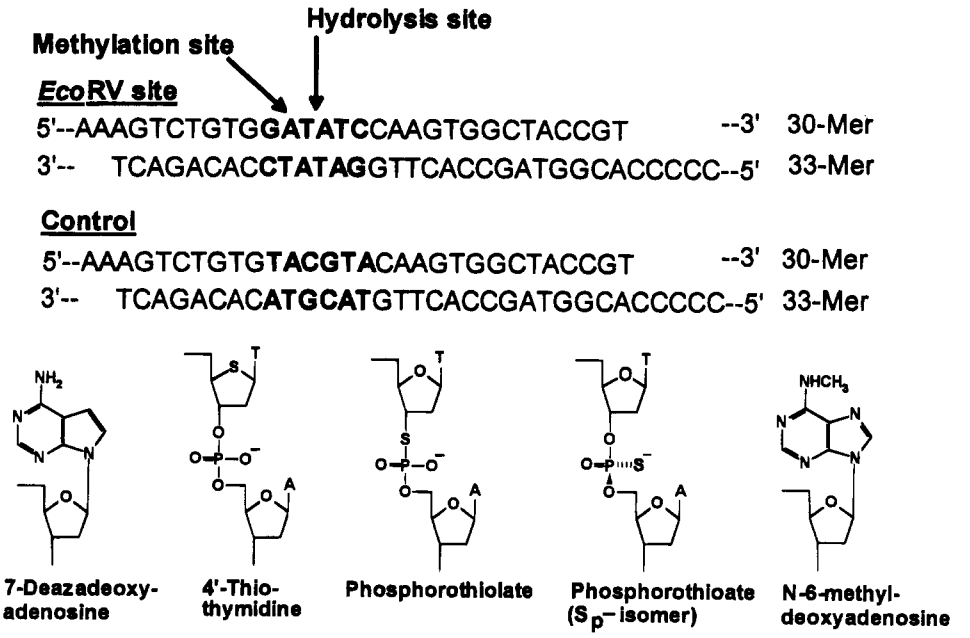

FIGURE 1: Oligodeoxynucleotides used in this study. The parent oligonucleotide consists of a 30- and 33-mer which can be hybridized to give a duplex with an *Eco*RV site (GATATC, bold). The positions of hydrolysis by the endonuclease and CH_3 addition by the methylase are indicated. In the control the GATATC site is replaced by TACGTA. The hydrolysis-resistant oligonucleotides used with the endonuclease have (1) a 7-deazadeoxyadenosine (at the first dA in GATATC), (2) a 4′-thiothymidine (at the first T in GATATC), and (3) a phosphorothiolate or a phosphorothioate at the scissile bond. With the methylase an N^6-methyldeoxyadenosine was used at the first dA in GATATC.

enzyme concentration at half-maximal binding and [DNA] is the input DNA concentration (Klig *et al.*, 1987).

Removal of Tightly Bound AdoMet from the Methylase and Investigation of Inhibition of Methylation by Sinefungin. When required, tightly bound AdoMet was removed from the methylase by incubating equimolar amounts (usually 100 nmol) of the protein and GACGATATCGTC in 200 μL of 50 mM Hepes (pH 7.5), 100 mM NaCl, 10 mM EDTA, and 5 mM DTT for 30 min at room temperature. The resulting methylase preparation was used without further treatment in gel-shift experiments. To determine whether sinefungin prevented CH_3 group addition to oligonucleotides (due to the copurified AdoMet in the methylase preparations), appropriate samples of the above methylase−oligonucleotide−sinefungin mixtures were treated as follows after the 30 min incubation. $MgCl_2$ (to a final concentration of 25 mM) was added, followed by 5 mg of the *Eco*RV endonuclease. The mixture was incubated for a further 1 h at 37 °C and then analyzed by denaturing polyacrylamide gel electrophoresis (Newman *et al.*, 1990b) with detection of oligonucleotides by autoradiography.

RESULTS AND DISCUSSION

Oligonucleotide Selection. The binding of the *Eco*RV restriction endonuclease and modification methylase to DNA has been studied using the 30- and 33-mers illustrated in Figure 1. These are complementary over most of their length and can be hybridized to form a duplex which contains a single GATATC *Eco*RV site. Controls consisted of oligonucleotides in which the GATATC sequence was replaced by TACGTA. Investigation of the binding of the endonuclease to DNA in the presence of Mg^{2+} requires hydrolysis-resistant oligonucleotides, and the derivatives that have been used are shown in Figure 1. These consist of (a) two phosphate analogues in which the scissile phosphate has been replaced by a phosphorothioate of the S_p configuration or a 3′-phosphorothiolate, (b) a sugar analogue in which the 2′-deoxyribose of the T immediately 5′ to the cutting site has been replaced by a 4′-thio-2′-deoxyribose, and (c) a base analogue where the first A in the GATATC sequence has been substituted by 7-deazadeoxyadenosine. When these modifications are introduced into both strands of *Eco*RV sites, the nucleic acid becomes refractory to cleavage (Newman *et al.*, 1990a; Grasby & Connolly, 1992; Vyle *et al.*, 1992; Hancox *et al.*, 1993). The methylase is capable of adding CH_3 groups to unmethylated GATATC sequences (Nwosu *et al.*, 1988; Newman *et al.*, 1990a), but it is thought that its physiological substrate is hemimethylated DNA. Thus derivatives of the 30- and 33-mer that contain G[^{6Me}A]TATC have been prepared (Figure 1). By appropriate hybridization this enables the preparation of hemimethylated and fully methylated sequences.

All the oligodeoxynucleotides used were ≥97% pure as assessed by HPLC or nondenaturing gel electrophoresis of ^{32}P-labeled samples (not shown). The phosphorothioate diastereomers of the 30- and 33-mer could be separated by HPLC on C-18 columns using an acetonitrile gradient in a morpholine−acetate buffer (Figure 2). Purities of ≥98% with ≤2% contamination with the other isomer were achieved (not shown). Previous studies have shown that R_p isomers always elute before S_p on reverse-phase columns (Connolly *et al.*, 1984; Stec *et al.*, 1984, 1985; Grasby & Connolly, 1992), and this was confirmed here using the *Eco*RV endonuclease which cuts R_p- but not S_p-phosphorothioates (Grasby & Connolly, 1992). The phosphorothioate obtained from the early eluting HPLC peak was cut by the enzyme (when hybridized with an all-phosphate-containing complementary strand) whereas that obtained from the late peak was not (not shown).

DNA Binding by the EcoRV Endonuclease. When the binding of the *Eco*RV restriction endonuclease to the duplex formed from the unmodified 30- and 33-mer was monitored

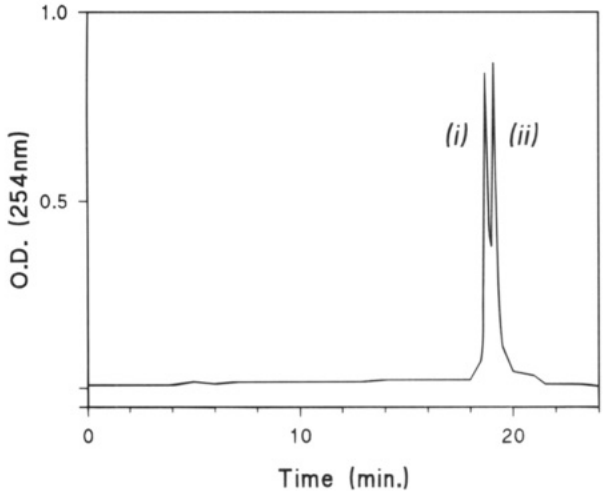

FIGURE 2: Reverse-phase HPLC separation of the two diastereomers of the 33-mer substituted with a phosphorothioate group. Peak i is the R_p and peak ii the S_p isomer. A similar separation was found with the phosphorothioates of the 30-mer.

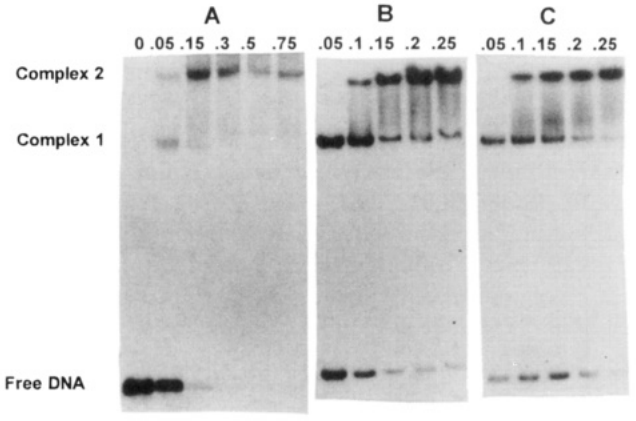

FIGURE 3: Gel shift analysis of the *Eco*RV endonuclease with oligonucleotides and Mg^{2+}: (A) GATATC-containing duplex (5 nM) without Mg^{2+}; (B) phosphorothiolate-containing duplex (5 nM) without Mg^{2+}; (C) phosphorothiolate-containing duplex (5 nM) in the presence of 5 mM $MgCl_2$. The level of the endonuclease varied as indicated by the numbers, which are concentrations in micromolar, on the top of the gels. Complexes 1 and 2 refer to the oligonucleotide duplex containing one or two molecules of the endonuclease.

by gel-shift analysis (Fried & Crothers, 1984; Garner & Revzin, 1986; Revzin, 1990), multiple bands were observed in the absence of added Mg^{2+} (Figure 3A). These correspond to the binding of one and two molecules of the endonuclease and indicate a lack of specific binding to GATATC sites. Similar ladders have been observed using plasmid DNA (Taylor *et al.*, 1991). Identical results (not shown) were observed when the gel shifts were carried out with the control, containing TACGTA rather than GATATC, confirming the lack of binding selectivity. All the hydrolysis-resistant oligonucleotides gave similar multiple bands when Mg^{2+} was omitted, and as a typical example the results found with the phosphorothiolate are shown in Figure 3B. When the binding of the phosphorothiolate was repeated, in the presence of 5 mM Mg^{2+}, multiple bands were again observed (Figure 3C). All the other inert oligonucleotides gave the same multiple bands when tested with Mg^{2+} (not shown). The identical gel-shift patterns shown for the phosphorothiolate, and the other hydrolysis-resistant species, in both the absence and presence of Mg^{2+} confirm that (a) Mg^{2+}

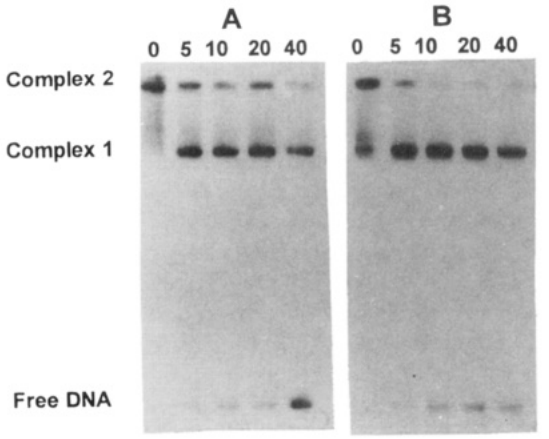

FIGURE 4: Gel-shift analysis of the *Eco*RV endonuclease (0.1 μM) with 5 nM levels of the phosphorothiolate-containing oligonucleotide (A) or 5 nM amounts of the control oligonucleotide-containing TACGTA rather than GATATC (B). The $MgCl_2$ levels (mM) were varied as indicated by the numbers on top of the gels. Complexes 1 and 2 refer to the oligonucleotide duplex containing one or two molecules of the endonuclease.

does not increase the affinity for GATATC sequences in these cases (this would have resulted in the appearance of a shifted oligonucleotide band, corresponding to a specific complex, at lower protein levels) and (b) Mg^{2+} does not engender selectivity for the *Eco*RV cognate site (this would have been manifested by the disappearance of multiple shifted bands, corresponding to nonspecific complexes, and their replacement with a single species). To determine if Mg^{2+} levels higher than 5 mM promote specific binding, panels a and b of Figure 4 show the results of 0−40 mM levels of the cation on the interaction of the endonuclease with the phosphorothiolate and TACGTA-containing oligonucleotides. The gels used only contained 5 mM levels of the metal as higher concentrations produced diffuse bands with anomalous mobilities. Increasing the Mg^{2+} concentration causes a parallel reduction in the affinity for both the phosphorothiolate and the TACGTA oligonucleotides. Thus the bands that correspond to two endonucleases per oligonucleotide decrease and the levels of free DNA rise. At 40 mM metal a single shifted band is predominantly seen for the two species, but the endonuclease still shows the same affinity for both of the oligonucleotides. It has been shown that metal ions both promote the binding of specific sequences and reduce the affinity for nonspecific ones (Thielking *et al.*, 1992; Vipond & Halford, 1995). The reductions in affinity seen here as the levels of Mg^{2+} are increased suggest that the protein sees both these sequences as noncognate DNA.

The *Eco*RV endonuclease distorts GATATC sequences on binding, and this results in the movement of the scissile phosphate toward two acidic residues (D74 and D90) to create the Mg^{2+} binding site and give efficient catalysis (Winkler, 1992; Winkler *et al.*, 1993; Taylor & Halford, 1989; Taylor *et al.*, 1991; Vermote & Halford, 1992; Vermote *et al.*, 1992). No distortion is seen for noncognate sequences, and as a consequence a low affinity for Mg^{2+} results in poor hydrolysis. New crystal structures (Kostrewa & Winkler, 1995) and solution data (Vipond & Halford, 1995; Vipond *et al.*, 1995; Baldwin *et al.*, 1995) have suggested additional bound DNA conformations and a transition state containing two metal ions. Our results with the 4′-thiothymidine-containing oligonucleotide, which nei-

ther is a substrate (Hancox *et al.*, 1993) nor forms specific complexes in the presence of Mg^{2+}, support this. A crystal structure of the endonuclease bound to a 4′-thiothymidine oligonucleotide has been solved (F. Winkler, personal communication) and shows that the DNA is in a distorted conformation that is almost identical to that observed with unmodified GATATC sequences. The sulfur atom in these derivatives is in the minor groove and is near a loop of the endonuclease which approaches this groove and interacts with several of the phosphates. Its presence appears to interfere with several water-mediated protein DNA contacts. These crystals do not contain Mg^{2+}, and crystallization in the presence of this cation or attempts to soak the metal into crystals did not give ternary complexes. This suggests that 4′-thiothymidine oligonucleotides give complexes with low Mg^{2+} affinities even though the metal ion binding site formed from the scissile phosphate and D74/D90 appears intact. These data show that the distorted conformation seen, by crystallography, with GATATC sequences and also with the 4′-thiothymidine-containing oligonucleotide, while lying along the hydrolytic pathway, is not catalytically competent and that a further conformational change is essential for hydrolysis. With the 4′-thiothymidine this is prevented by the alteration to water-mediated enzyme−DNA contacts. When crystals containing GATATC sequences are soaked with Mg^{2+}, metal ion binding takes place at the D74/D90 site but no cleavage is seen. Here it is thought that crystal packing forces stop the conformational change (Kostrewa & Winkler, 1995). The 7-deazadeoxyadenosine-, phosphorothioate-, and phosphorothiolate-containing oligonucleotides have the same properties as the 4′-thio sugar. In the absence of structural information it is difficult to conclude unambiguously why they behave as they do. However, the most likely explanation is that these analogues, which are structurally very similar to the parent, subtly perturb the protein−DNA interface and so interfere with the conformational changes needed to attain the transition state. It remains to be seen whether they bind in a distorted conformation similar to GATATC and the 4′-thio sugar. The actual effect of 7-deazadeoxyadenosine is to delete a hydrogen bond between the side chain of Asn 185 and the 7-N atom of the first dA in GATATC (Newman *et al.*, 1990b). The S_p-phosphorothioate has the oxygen that normally binds to Mg^{2+} in the enzyme−DNA complex replaced with sulfur. Given the much lower affinity that sulfur as compared to oxygen shows for this metal ion (Jaffe & Cohn, 1978; Pecoraro *et al.*, 1984), one might expect poorer binding of Mg^{2+}, and this could account for both the lack of activity and the failure to produce specific complexes. Phosphorothioate diesters are only marginally less susceptible to chemical hydrolysis than phosphate diesters (Herschlag *et al.*, 1991), and the reactivity of P−S bonds in phosphorothiolates is greater than that of P−O bonds in phosphates (Milstein & Fife, 1967; Nakamaye *et al.*, 1988). Thus in both these cases intrinsic poorer chemical reactivity cannot account for the lack of cleavage.

DNA Binding to the EcoRV Methylase. The evaluation of the binding of oligodeoxynucleotides to the methylase was complicated by the observation that the enzyme contained traces of bound AdoMet. Incubation of the methylase with GACGATATCGTC (Nwosu *et al.*, 1988; Newman *et al.*, 1990a) showed that methylation occurred, as assessed by reverse-phase HPLC, even if no AdoMet was added. Examination of the HPLC traces (not shown) revealed that

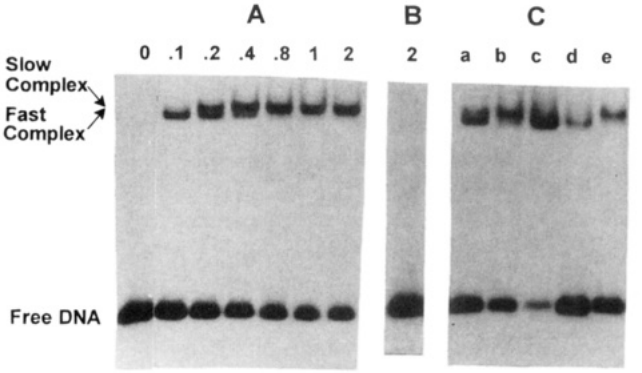

FIGURE 5: Gel shift analysis of the *Eco*RV methylase with unmethylated oligonucleotides and cofactors. (A) GATATC-containing duplex (unmethylated) (5 nM) without added cofactor at the methylase levels (μM) shown above the gel. Two just separated complexes labeled slow and fast are produced that are most obvious in the 0.2 μM lane. (B) Control oligonucleotide (5 nM) containing TACGTA rather than GATATC without added cofactor at the single methylase concentration of 2 μM. (C) GATATC-containing duplex (unmethylated) (5 nM) in the presence of 0.2 μM methylase (a), 0.4 μM methylase (b), 0.2 μM methylase plus 1.13 μM sinefungin (c), 0.2 μM methylase plus 1.13 mM AdoHcy (d), and 0.2 μM methylase plus 1.13 mM AdoMet (e). Lanes a and b give mixtures of the fast and slow complexes, lanes c and d give the fast complex, and lane e gives the slow complex.

a 10-fold excess of enzyme over GACGATATCGTC gave rise to ≈26% methylation whereas a 5-fold excess resulted in ≈12% reaction. Thus about 2.5% of the purified methylase contains an active bound AdoMet molecule. The copurification of AdoMet has been noted for other methylases (Piekarowicz & Brzezinski, 1980; Kumar *et al.*, 1992). Attempts to remove the AdoMet by dialysis or gel filtration proved unsuccessful.

The *Eco*RV methylase bound selectively to its target sequence, and Figure 5A shows that on incubation with the GATATC-containing oligonucleotide a single shifted band was seen by gel retardation. When the enzyme was incubated with the control oligonucleotide, no discrete shifted band was produced (Figure 5B). The negative result shown in Figure 5B was obtained in the absence of added cofactor, but the inclusion of sinefungin or AdoMet did not result in complexation of the control (not shown). Methylase binding to GATATC sequences is weak in the absence of cofactor, i.e., under the conditions of Figure 5A, and AdoMet (or an analogue of it) is required for tight binding. Thus a relatively large excess of the methylase, which will supply significant amounts of AdoMet, was required to gel shift the GATATC sequence in this experiment. The shifted band actually consists of two just-resolved species. The faster band is a complex of the methylase with either unmethylated or hemimethylated oligonucleotide. It predominates at low enzyme excesses as most of the oligonucleotide will be in these forms due to the low levels of AdoMet supplied. The slower band is an enzyme−dimethylated oligonucleotide complex and dominates at high protein levels because of the large quantities of AdoMet available. The identities of the fast and slow species were established by incubating the methylase with the GATATC oligonucleotide in the presence of excess AdoMet (which results in complete methylation) to give exclusively the slow band (Figure 5C). Repeating this experiment with AdoHcy or sinefungin gave the fast band. These compounds are often competitive inhibitors of AdoMet (Friedman, 1986; Bergerat & Guschlbauer, 1990;

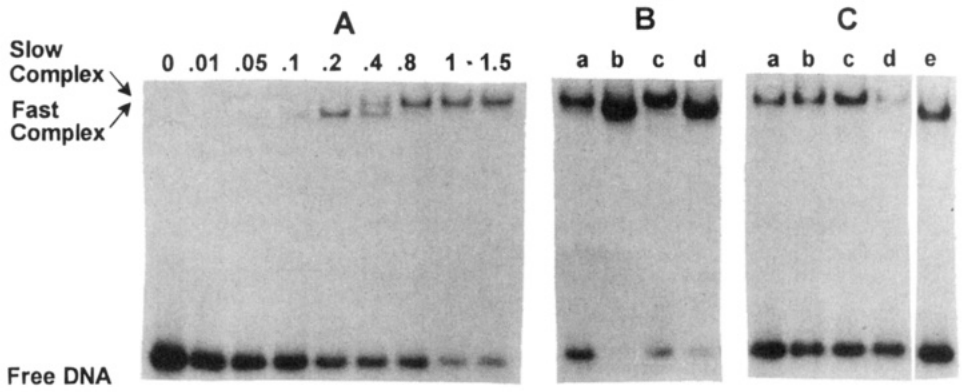

FIGURE 6: Gel shift analysis of the *Eco*RV methylase with hemi- and dimethylated oligonucleotides and cofactors. (A) Hemimethylated oligonucleotide (duplex consisted of methylated 33-mer plus unmethylated 30-mer) (5 nM) in the absence of added cofactor and the methylase concentrations (μM) shown at the top of the gels. The fast and slow complexes are best observed in the 0.4 μM lane. (B) Hemimethylated oligonucleotide (5 nM) in the presence of 0.8 μM methylase and either no addition (a), 1.13 mM sinefungin (b), 1.13 mM AdoMet (c), or 1.13 mM AdoHcy (d). The fast complex is seen in lanes b and d and the slow in lane c. (C) Dimethylated oligonucleotide (5 nM) in the presence of 0.8 μM methylase and either no addition (a), 1.13 mM sinefungin (b), 1.13 mM AdoMet (c), or 1.13 mM AdoHcy (d). The slow complex is produced in each case as illustrated by the fast complex standard (e).

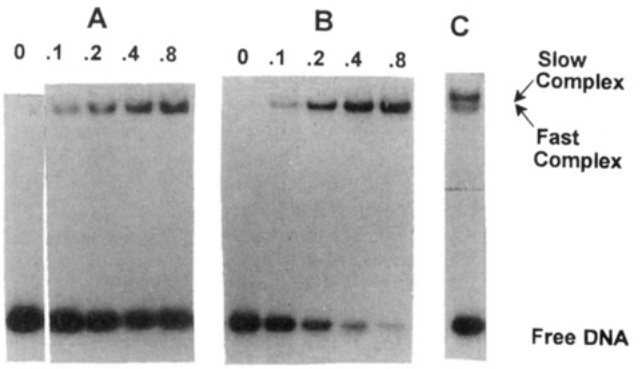

FIGURE 7: Gel shift analysis of the *Eco*RV methylase from which any tightly bound AdoMet had been removed by preincubation with GACGATATCGTC. (A) Incubation of the unmethylated GATATC-containing duplex (5 nM) without added cofactor and the indicated amounts of methylase (μM). (B) As (A) but using a hemimethylated oligonucleotide produced from the methylated 33-mer and the unmethylated 30-mer. In both cases only fast complexes are formed as shown by reference to the standards (C).

Reich & Mashoon, 1990) and presumably hinder any methylation caused by copurified AdoMet (this has been established for sinefungin as outlined below). When the band-shift assay was repeated with a hemimethylated oligonucleotide, similar results were observed. In the absence of added cofactor the fast band was initially produced but was replaced with the slow species as methylase levels increased (Figure 6A). The fast band appeared in the presence of sinefungin or AdoHcy, but the slow band was produced on incubation with AdoMet (Figure 6B). Gel shifts with the dimethylated oligonucleotide produced the slow band under all conditions (Figure 6C). Finally, gel shifts were performed with methylase in which the bound AdoMet had been converted to AdoHcy by prior incubation with GACGATATCGTC. These samples cannot add CH_3 groups to DNA, and experiments with unmethylated or hemimethylated oligonucleotides (in either the presence or absence of sinefungin) produced exclusively the fast band (Figure 7).

We have determined the K_d values for unmethylated and hemimethylated GATATC sequences, in the presence of the AdoMet cofactor sinefungin. These represent the most important complexes, i.e., ternary species containing the enzyme, a substrate DNA, and an AdoMet analogue. The low amounts of the enzyme required and the presence of the large quantities of sinefungin prevented any methylation by the copurified AdoMet. This was apparent from the study of hemimethylated oligonucleotides where only fast complexes were seen by gel retardation (any methylation would have given dimethylated oligonucleotides and hence slow bands). Furthermore, following incubation of the unmethylated GATATC sequence with the methylase and sinefungin, a sample was treated with a large excess of the *Eco*RV endonuclease. All the oligonucleotide could be cut, and as methylation prevents endonuclease catalyzed hydrolysis (Newman *et al.*, 1990a), this confirms the lack of methyl group addition. The levels of sinefungin used in these experiments (1.13 mM) are 1.5×10^5-fold larger than the maximal amount of AdoMet present (7.5 nM, arising from a 2.5% level of tightly bound AdoMet at the highest, 300 nM, level of methylase used). Thus, unless AdoMet binds extraordinarily more tightly to the methylase than sinefungin, all ternary complexes formed will contain the analogue. Very strong, preferential, binding of the AdoMet is unlikely given the total inhibition of methylation caused by sinefungin. As an example of K_d determination, the gel seen with the unmethylated oligonucleotide together with the graphical representation of these data is shown in Figure 8a,b. The K_d value found for the unmethylated duplex was 46 nM. Similar quality data (not shown) was found for the two hemimethylated species, but here binding was about four times stronger and K_d values of $\approx$12 nM were observed. All these data are summarized in Table 1. It was also possible to measure a K_d for the dimethylated product in the presence of sinefungin (data not shown), and a value of 143 nM was found (Table 1). Thus the methylase binds to product DNA more weakly than to substrate but significantly more strongly than to DNA that lacks GATATC sites. We assume that AdoMet will behave in a manner similar to that of sinefungin and potentiate DNA binding. This could not be directly observed due to competing methylation and product formation.

The presence of the copurified AdoMet made experiments in the absolute absence of cofactor difficult to perform. It can be seen from Figure 5C that the tightest binding to GATATC sequences occurs in the presence of sinefungin, and significantly weaker binding is seen without added

Table 1: K_d Values Found for the *Eco*RV Methylase and the Oligonucleotides Shown in the Presence or Absence of the AdoMet Analogue Sinefungin[a]

oligonucleotide duplex	K_d (nM) +sinefungin	K_d (nM) no cofactor
GATATC/GATATC (unmethylated)	46	>800
G^{6Me}ATATC/GATATC (hemimethylated)	11	≈200
GATATC/G^{6Me}ATATC (hemimethylated)	13	not determined
G^{6Me}ATATC/G^{6Me}ATATC (dimethylated)	143	not determined
TACGTA/TACGTA (control)	≫2000	≫2000

[a] The two hemimethylated oligonucleotides have the N^6-methyldeoxyadenosine in either the 30-mer (K_d 11 nM) or the 33-mer (K_d 13 nM) strand. As mentioned in the text, the K_d values found in the absence of sinefungin have been estimated by visual inspection of the appropriate gels rather than by more accurate determination.

cofactor or with AdoHcy. To get some idea of the role the cofactor has in DNA binding, a sample of the methylase was preincubated with GACGATATCGTC (5 nM) to convert the AdoMet to AdoHcy. As shown in Figure 7, gel shifts were then carried out with 5 nM levels of unmethylated or hemimethylated DNA at increasing protein levels. We have not attempted to accurately evaluate K_d values, but a visual inspection of the gels and estimation of the lanes in which half the radioactivity appears in each band gives K_d's of >800 nM for the unmethylated and 200 nM for the hemimethylated oligonucleotides. In both cases this implies, by comparison with the K_d's given in Table 1, an approximate 20-fold increase in affinity for GATATC sequences in the presence of cofactor. Two criticisms can be made to this experiment. First, the GACGATATCGTC present might compete with the 30/33-mer duplex and compromise K_d evaluation. While GACGATATCGTC is a substrate for this enzyme, it binds very weakly and does not form a tight complex as measured by gel-shift assay (not shown). Second, the AdoHcy formed in the preincubation might result in increased binding of the oligonucleotides. However, when the gel-shift part of this experiment was repeated in the presence of extra added AdoHcy (1.13 mM), no differences in the gel patterns to those observed in Figure 7 were noted (not shown). Similarly, when the experiments shown in Figure 8 were performed with AdoHcy rather than sinefungin, weaker binding of the oligonucleotides was observed (not shown) with binding affinities reduced by a factor of 20 (not shown). Thus DNA binding is reduced approximately 20-fold if sinefungin is either omitted or replaced with AdoHcy, and so AdoHcy unlike sinefungin (and presumably AdoMet) does not increase DNA binding.

Finally, we have obtained an estimate for the binding of sinefungin to a methylase−hemimethylated DNA complex. The graphical representation of data obtained from gel-shift analysis is shown in Figure 9. These shifts do not give a true K_d as they are not carried out in the presence of saturating amounts of oligonucleotide but rather a K_{app}. The K_{app} found for sinefungin under the conditions we have used was 10.9 μM. The curve does not pass through zero probably because the methylase has an affinity, albeit reduced, for DNA even in the absence of cofactor and also due to the copurified AdoMet. Nevertheless, Figure 9 clearly shows that the presence of cofactor strengthens DNA binding and that the amounts routinely used (1.13 mM) in studies in which DNA levels were varied are saturating.

Many of the above features seen with the *Eco*RV methylase are shared with others including *Eco*RI (Reich & Mashoon, 1990; Reich *et al.*, 1991), *Eco* Dam (Bergerat & Guschlbauer, 1990), *Msp*I (Dubey & Roberts, 1992; Ford *et al.*, 1993), and *Eco*KI (Powell *et al.*, 1993) even though these are from different families (*Eco*RI, *Eco*RV, and Dam, type 2 dA methylases; *Msp*I, type 2 dC methylase; *Eco*KI, type 1 methylase). Two bands of similar mobility have been seen with Dam, *Msp*I, and *Eco*KI although the exact circumstances in which a particular band is formed (i.e., the methylation state of the DNA, whether a cognate or noncognate sequence is used, and the presence and nature of the cofactor) vary between the enzymes. These slight alterations in mobility are undoubtedly caused by particular enzyme−DNA−

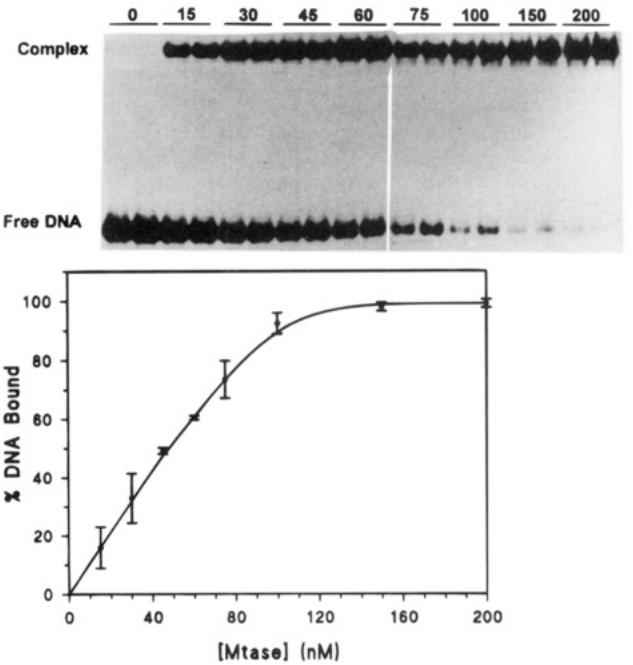

FIGURE 8: K_d determination for the binding of the *Eco*RV methylase to the unmethylated GATATC-containing oligonucleotide (at a fixed concentration of 5 nM) in the presence of 1.13 mM sinefungin. The gel shifts are shown with the concentration of the methylase (nM) varied as shown by the numbers on the top of the gel. Each individual methylase concentration was run in duplicate. In addition, the graphical representation of the data obtained from the gel experiments is given.

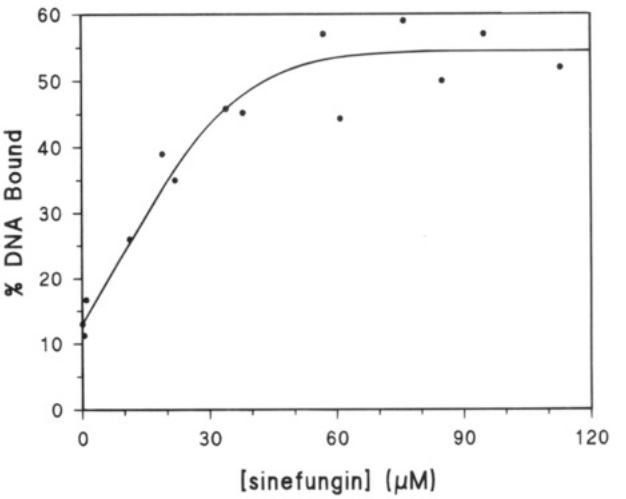

FIGURE 9: Determination of the K_{app} for sinefungin. 5 nM hemimethylated oligonucleotide (33-mer methylated, 30-mer unmethylated) and 15 nM *Eco*RV methylase were titrated with the amounts of sinefungin shown. The graphical representation of the data produced from gel-shift assays is given.

cofactor combinations having different conformations. A variety of methods including susceptibility to proteolysis (Bergerat & Guschlbauer, 1990; Cooper & Dryden, 1994) and circular dichroism spectroscopy (Baldwin *et al.*, 1994) have indicated that DNA methylases are flexible domain proteins and that considerable changes in protein folding occur upon substrate binding. This has been confirmed by crystal structures of the *Hha*I methylase (Cheng *et al.*, 1993; Klimasauskas *et al.*, 1994) and also proposed for the *Taq*I methylase (Labahn *et al.*, 1994). With the *Eco*RV methylase the different gel mobilities of the substrate (non- or hemimethylated DNA) and product (dimethylated DNA) complexes clearly show that they are structurally distinct and that a conformational change separates them. All the methylases mentioned above bind their recognition sequences with a higher affinity than nonspecific DNA, and the K_d values found for the *Eco*RV methylase (in the lower nanomolar range) are comparable to those seen with the others. Preferential binding to the hemimethylated species, seen with *Eco*RV, was also found with *Msp*I but not the *Eco*KI methylase, and all three methylases showed significant binding to the dimethylated product. The binding selectivity (i.e., the degree to which the cognate sequence is preferred to others) varies considerably with the three methylases. A major problem is comparability of the noncognate sequences used. We have converted GATATC to TACGTA (i.e., changed all the bases in the recognition sequence), and a similar approach was used with *Eco*KI [change of AAC(N)6GTGC to CCA(N)6TGTA]. With *Msp*I the noncognate DNA was only altered in a single base relative to the true recognition site (CAGG rather than CCGG). We were unable to observe binding of *Eco*RV to the noncognate sequence selected. With *Eco*K a selectivity of ≈10 was seen in the absence of AdoMet increasing to ≈20 when cofactor was present. *Msp*I gave respective figures of about 5 and 100. Experiments with the Dam and *Eco*RI methylases, which are the most closely related to *Eco*RV, failed to detect binding to nonspecific sequences. The effects that AdoMet, its product AdoHcy, and analogues such as sinefungin have on the binding of DNA to methylases are somewhat variable. With the *Dam* methylase AdoMet, but not sinefungin or AdoHcy, strengthened the binding of specific GATC sequences, but for the *Msp*I and *Eco*RI methylases both sinefungin and AdoHcy strengthened the binding of cognate DNA sequences. In one case, the *Hga*I-2 methylase, AdoMet has only a small effect on specific DNA binding (Baldwin *et al.*, 1994). Thus it can be generally concluded that AdoMet appears to increase binding to the target sequence and often improves the binding discrimination between cognate and other sequences for DNA methylases, and this is often mimicked by sinefungin.

CONCLUSION

The discrimination between DNA sequences is the key feature of DNA recognition and has been extensively investigated with restriction endonucleases and modification methylases. Endonucleases are capable of cutting their recognition sites with selectivity factors of over 1 million (Taylor & Halford, 1989; Lesser *et al.*, 1990; Thielking *et al.*, 1990). Less information is available for the modification methylases (largely because the assays required to determine catalytic selectivity using plasmid DNA are far more cumbersome than for the endonucleases), but it is clear that they do have high binding selectivity and presumably also a catalytic preference for their cognate sequences (Bergerat & Guschlbauer, 1990; Reich & Mashoon, 1990; Dubey & Roberts, 1992; Powell *et al.*, 1993). The interactions of enzymes with nucleic acids are complicated by the requirement to carry out a chemical transformation of the target sequence. This means that enzymes cannot achieve specificity simply by increasing their affinity for cognate DNA sequences as very tight binding would compromise catalysis. The K_d values found for the methylase ($\approx 10^{-8}$ M for hemimethylated DNA) are much higher than those seen for most repressor proteins, and in general, enzymes have higher K_d values than repressors (Dubey & Roberts, 1992). Both endonucleases and methylases require cofactors (Mg^{2+} and AdoMet), and these are involved not only in chemical catalysis but in potentiating enzyme−DNA interactions and often in improving selectivity. As enzymes, endonucleases and methylases are designed to achieve maximum discrimination in catalysis rather than substrate binding, and this means that cognate DNA should be most highly favored over other sequences in the transition state. Thus at least three important complexes, enzyme−DNA, enzyme−cofactor−DNA, and the transition state, can be envisaged. All are likely to have slightly different structures, and the observed overall discrimination will be partitioned between them. It is important to study all three states in order to build up a full picture of how the enzymes function. Complicating these experiments is the requirement to prevent turnover which is fast relative to gel retardation techniques.

The *Eco*RV endonuclease does not form specific complexes with GATATC sequences when Mg^{2+} is absent (Taylor *et al.*, 1991), but it is thought that this does take place when the metal is present. This cannot be directly observed due to turnover but has been seen using an inactive mutant (Thielking *et al.*, 1992) or Ca^{2+} (Vipond & Halford, 1995). However, we have been unable to trick the enzyme into forming specific ternary complexes using a variety of hydrolysis-resistant oligonucleotides containing base, sugar, and phosphate analogues, despite the analogues being structurally very similar to the parent. This agrees with the idea that restriction enzymes have evolved to hydrolyze their recognition sequences with high fidelity as catalysis away from these sites would be lethal due to an absence of protection by host methylation. We think it is likely that all our oligonucleotide analogues are distorted on binding to give a structure similar to that reported for GATATC-containing sequences (Winkler *et al.*, 1993) as has been shown crystallographically for the 4′-thiothymidine derivative. However, this conformation is not catalytically active, and a further conformational change is needed, possibly to generate a two metal ion species, for hydrolysis to take place. It is this change that is exquisitely sensitive to oligonucleotide structure and demands an absolutely correct protein−DNA interface. The attainment of this catalytically active conformation requires the GATATC base sequence (the 7-deazadeoxyadenosine-containing oligonucleotide is not a substrate) but additionally the correct sugars and phosphates (the oligonucleotides containing sugar and phosphate analogues show no activity). This emphasizes that direct readout, i.e., interactions to the bases, and indirect readout, i.e., interactions with the sugar phosphate backbone, are both equally vital in generating high specificity. Furthermore, water-mediated

interactions (disrupted by the 4′-thiothymidine oligonucleotide) also play a critical role.

With the methylase it was very straightforward to produce specific complexes, of high affinity, with GATATC sequences providing an analogue of AdoMet was present. This opens the way for the footprinting experiments, described in the accompanying paper, which confirm location to the target sequence. The *Eco*RV methylase is similar to many other methylases in requiring AdoMet or an analogue of it for maximum binding to cognate sequences and also in showing tightest binding to its physiological substrate hemimethylated DNA. However, the methylase will still bind specifically, albeit more weakly, to GATATC sequences without its cofactor. In this respect the two *Eco*RV enzymes are subtly different. With the endonuclease the Mg^{2+} cofactor seems to be absolutely essential for specific DNA binding. In the case of the methylase the AdoMet cofactor only improves the selectivity for target sequences.

The results found in this publication (and the accompanying one) and similar ones for a number of other endonucleases and methylases (referred to above) have been useful in mapping protein−DNA interfaces and studying the interactions between the two macromolecules. However, they mainly deal with ground-state enzyme−DNA complexes either with or without the reaction cofactor. There is a pressing need to develop good transition-state models for these two classes of enzymes which might be diagnosable by very tight binding (K_d values more typical for repressor−DNA rather than enzyme−DNA complexes). This is difficult, and as yet no good transition-state analogues are available for either DNA methylation or DNA hydrolysis reactions. However, if they could be produced, their study should reveal many of the factors responsible for specificity in catalytic reactions on DNA.

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REFERENCES

Aiken, C. R., Fisher, E. W., & Gumport, R. I. (1991) *J. Biol. Chem. 266*, 19063−19069.

Baldwin, G. S., Kelly, S. M., Price, N. C., Wilson, G. W., Connolly, B. A., Artymiuk, P. J., & Hornby, D. P. (1994) *J. Mol. Biol. 235*, 545−553.

Baldwin, G. S., Vipond, I. B., & Halford, S. E. (1995) *Biochemistry 34*, 705−714.

Bergerat, A., & Guschlbauer, W. (1990) *Nucleic Acids Res. 18*, 4369−4375.

Bergerat, A., Guschlbauer, W., & Fazakerley, G. V. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6394−6397.

Chandrasegaran, S., & Smith, H. O. (1988) *Structure and Expression, Volume 1: From Proteins to Ribosomes* (Sarma, R. H., & Sarma, M. H., Eds.) pp 149−156, Adenine Press, New York.

Cheng, X., Kumar, S., Posfai, J., Pflugrath, J., & Roberts, R. J. (1993) *Cell 74*, 299−307.

Connolly, B. A. (1991) in *Oligonucleotide Analogues—a Practical Approach* (Eckstein, F., Ed.) pp 155−183, IRL Press, Oxford, U.K.

Connolly, B. A. (1992) in *Methods in Enzymology—DNA structure* (Lilley, D., & Dahlberg, J., Eds.) Vol. 211, pp 36−53, Academic Press, Orlando, FL.

Connolly, B. A., Potter, B. V. L., Eckstein, F., Pingoud, A., & Grotjahn, L. (1984) *Biochemistry 23*, 3443−3453.

Cooper, L. P., & Dryden, J. M. B. (1994) *J. Mol. Biol. 236*, 1011−1021.

Cosstick, R., & Vyle, J. S. (1989) *Tetrahedron Lett. 30*, 4693−4696.

Dubey, A., & Roberts, R. J. (1992) *Nucleic Acids Res. 20*, 3167−3173.

Ford, K., Taylor, C., Connolly, B. A., & Hornby, D. P. (1993) *J. Mol. Biol. 230*, 779−786.

Fried, M. G., & Crothers, D. M. (1984) *J. Mol. Biol. 172*, 241−262.

Friedman, S. (1986) *Nucleic Acids Res. 14*, 4543−4556.

Garner, M., & Revzin, A. (1986) *Trends Biol. Sci. 11*, 395−396.

Grasby, J. A., & Connolly, B. A. (1992) *Biochemistry 31*, 7855−7861.

Guschlbauer, W. (1988) *Gene 75*, 211−214.

Halford, S. E., & Johnson, N. P. (1980) *Biochem J. 191*, 593−604.

Halford, S. E., Taylor, J. D., Vermote, C. L., & Vipond, I. B. (1993) in *Nucleic Acids and Molecular Biology* (Eckstein, F., & Lilley, D. M. J., Eds.) Vol. 7, pp 47−69, Springer-Verlag, Berlin.

Hancox, E. L., Connolly, B. A., & Walker, R. T. (1993) *Nucleic Acids Res. 21*, 3485−3491.

Herschlag, D., Piccirilli, J. A., & Cech, T. R. (1991) *Biochemistry 30*, 4844−4854.

Iyer, R. P., Egan, W., Regan, J., & Beaucage, S. L. (1990a) *J. Am. Chem. Soc. 112*, 1253−1254.

Iyer, R. P., Phillips, L. R., Egan, W., Regan, J., & Beaucage, S. L. (1990b) *J. Org. Chem. 55*, 4693−4699.

Jaffe, E. K., & Cohn, M. J. (1978) *J. Biol. Chem. 253*, 4823−4825.

Klig, L. S., Crawford, I. P., & Yanofsky, C. (1987) *Nucleic Acids Res. 15*, 5339−5351.

Klimasauskas, S., Kumar, S., Roberts, R. J., & Cheng, X. (1994) *Cell 76*, 357−369.

Kostrewa, D., & Winkler, F. K. (1995) *Biochemistry 34*, 683−696.

Kumar, S., Cheng, X., Pflugrath, J. W., & Roberts, R. J. (1992) *Biochemistry 31*, 8648−8653.

Labahn, J., Granzin, J., Schluckebier, G., Robinson, D. P., Jack, W. E., Schildkraut, I., & Saenger, W. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 10957−10961.

Lauster, R., Kriebardis, A., & Guschlbauer, W. (1987) *FEBS Lett. 220*, 167−176.

Lesser, D. R., Kurpiewski, M. R., & Jen Jacobson, L. (1990) *Science 250*, 776−786.

Luke, P. A., McCallum, S. A., & Halford, S. E. (1987) in *Gene Amplification and Analysis: Restriction Endonucleases and Methylases* (Chirikjian, J. G., Ed.) Vol. 5, pp 185−207, Elsevier, New York.

Milstein, S., & Fife, T. H. (1967) *J. Am. Chem. Soc. 89*, 5820−5826.

Nakamaye, K. L., Gish, G., & Eckstein, F. (1988) *Nucleic Acids Res. 16*, 9947−9958.

Newman, P. C., Nwosu, V. U., Williams, D. M., Cosstick, R., Seela, F., & Connolly, B. A. (1990a) *Biochemistry 29*, 9891−9901.

Newman, P. C., Williams, D. M., Cosstick, R., Seela, F., & Connolly, B. A. (1990b) *Biochemistry 29*, 9902−9910.

Nwosu, V. U., Connolly, B. A., Halford, S. E., & Garnett, J. (1988) *Nucleic Acids Res. 16*, 3705−3720.

Pecoraro, V. L., Hermes, J. D., & Cleland, W. W. (1984) *Biochemistry 23*, 5262−5271.

Piekarowicz, A., & Brzezinski, R. (1980) *J. Mol. Biol. 144*, 415−429.

Powell, L. M., Dryden, D. T. F., Pain, R. H., & Murray, N. E. (1993) *J. Mol. Biol. 234*, 60−71.

Reich, N. O., & Mashhoon, N. (1990) *J. Biol. Chem. 265*, 8966−8970.

Reich, N. O., Maegley, K. A., Shoemaker, D. D., & Everett, E. (1991) *Biochemistry 30*, 2940−2946.
Revzin, A. (1990) *J. Chem. Educ. 67*, 749−753.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular cloning: a laboratory manual*, 2nd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Stec, W., Zon, G., Egan, V., & Stec, B. (1984) *J. Am. Chem. Soc. 106*, 6077−6079.
Stec, W., Zon, G., & Uznanski, B., (1985) *J. Chromatogr. 326*, 263−280.
Taylor, J. D., & Halford, S. E. (1989) *Biochemistry 28*, 6198−6207.
Taylor, J. D., Badcoe, I. G., Clarke, A. R., & Halford, S. E. (1991) *Biochemistry 30*, 8743−8753.
Taylor, S. M., & Jones, P. A. (1979) *Cell 17*, 771−779.
Terry, B. J., Jack, W. E., Rubin, R. A., & Modrich, P. (1983) *J. Biol. Chem. 258*, 9820−9825.
Thielking, V., Alves, J., Fliess, A., Maass, G., & Pingoud, A. (1990) *Biochemistry 29*, 4682−4691.
Thiekling, V., Selent, U., Köhler, E., Landgraf, A., Wolfes, H., Alves, J., & Pingoud, A. (1992) *Biochemistry 31*, 3727−3732.
Vermote, C. L. M., & Halford, S. E. (1992) *Biochemistry 31*, 6082−6089.
Vermote, C. L. M., Vipond, I. B., & Halford, S. E. (1992) *Biochemistry 31*, 6089−6097.
Vipond, I. B., & Halford, S. E. (1993) *Mol. Microbiol. 9*, 225−231.
Vipond, I. B., & Halford, S. E. (1995) *Biochemistry 34*, 697−704.
Vipond, I. B., Baldwin, G. S., & Halford, S. E. (1995) *Biochemistry 34*, 697−704.
Vyle, J. S., Connolly, B. A., Kemp, D., & Cosstick, R. (1992) *Biochemistry 31*, 3012−3018.
Waters, T. R., & Connolly, B. A. (1994) *Biochemistry 33*, 1812−1819.
Winkler, F. K. (1992) *Curr. Opin. Struct. Biol. 2*, 93−99.
Winkler, F. K., Banner, D. W., Oefner, C., Tsernoglou, D. J., Brown, R. S., Heathman, S. P., Bryan, R. K., Martin, P. D., Petratos, K., & Wilson, K. S. (1993) *EMBO J. 12*, 1781−1795.
Xu, S.-Y., & Schildkraut, I. (1991) *J. Biol. Chem. 266*, 4425−4429.
Zebala, J. A., Choi, J., & Barany, F. (1992) *J. Biol. Chem. 267*, 8097−8105.

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