

Sequence-specific DNA Binding by *EcoKI*, a Type IA DNA Restriction Enzyme

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The type I DNA restriction and modification enzymes of prokaryotes are multimeric enzymes that cleave unmethylated, foreign DNA in a complex process involving recognition of the methylation status of a DNA target sequence, extensive translocation of DNA in both directions towards the enzyme bound at the target sequence, ATP hydrolysis, which is believed to drive the translocation possibly *via* a helicase mechanism, and eventual endonucleolytic cleavage of the DNA. We have examined the DNA binding affinity and exonuclease III footprint of the *EcoKI* type IA restriction enzyme on oligonucleotide duplexes that either contain or lack the target sequence. The influence of the cofactors, *S*-adenosyl methionine and ATP, on binding to DNA of different methylation states has been assessed. *EcoKI* in the absence of ATP, with or without *S*-adenosyl methionine, binds tightly even to DNA lacking the target site and the exonuclease footprint is large, approximately 45 base-pairs. The protection is weaker on DNA lacking the target site. Partially assembled *EcoKI* lacking one or both of the subunits essential for DNA cleavage, is unable to bind tightly to DNA lacking the target site but can bind tightly to the recognition site. The addition of ATP to *EcoKI*, in the presence of AdoMet, allows tight binding only to the target site and the footprint shrinks to 30 base-pairs, almost identical to that of the modification enzyme which makes up the core of *EcoKI*. The same effect occurs when *S*-adenosyl homocysteine or sinefungin are substituted for *S*-adenosyl methionine, and ADP or ATP γ S are substituted for ATP. It is proposed that the DNA binding surface of *EcoKI* comprises three regions: a "core" region which recognises the target sequence and which is present on the modification enzyme, and a region on each DNA cleavage subunit. The cleavage subunits make tight contacts to any DNA molecule in the absence of cofactors, but this contact is weakened in the presence of cofactors to allow the protein conformational changes required for DNA translocation when a target site is recognised by the core modification enzyme. This weakening of the interaction between the DNA cleavage subunits and the DNA could allow more access of exonuclease III to the DNA and account for the shorter footprint.

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Introduction

Type I restriction/modification systems are large multimeric enzymes whose modular nature pro-

Abbreviations used: AdoMet, *S*-adenosyl methionine; AdoHcy, *S*-adenosyl homocysteine; bp, base pair(s); hsd, host specificity for DNA; K_d , dissociation constant; PVDF, polyvinylidene difluoride.

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vides an opportunity for dissecting DNA-protein interactions, especially in terms of cofactor effects. *EcoKI* is a type IA restriction/modification system (for reviews see Modrich, 1979; Yuan, 1981; Wilson & Murray, 1991; Bickle & Kruger, 1993; Bickle, 1993; King & Murray, 1994; Barcus & Murray, 1995) comprising three polypeptide subunits HsdR, M and S, for restriction, modification and specificity. *EcoKI* requires *S*-adenosyl methionine (AdoMet), Mg²⁺ and ATP (Meselson & Yuan, 1968). The oligomeric enzyme recognises an asymmetric, bipartite

sequence, 5'-AAC(N)₆GTGC-3' (Kan *et al.*, 1979), and can act as either a methyltransferase or endonuclease depending upon the methylation state of the DNA substrate. The targets for methylation are adenine bases on opposite strands of the DNA (Burckhardt *et al.*, 1981b) and S-adenosyl methionine acts as both cofactor and methyl donor in the methylation reaction. ATP induces a conformational change in the enzyme that triggers the relevant response to the DNA methylation state (Bickle *et al.*, 1978; Burckhardt *et al.*, 1981b). If both adenine bases of the target sequence are methylated there is no reaction, while hemimethylated DNA is the preferred substrate for methylation (Vovis *et al.*, 1974; Burckhardt *et al.*, 1981a; Suri *et al.*, 1984). If neither adenine is methylated, the DNA is cleaved at variable positions up to several thousand base-pairs away from the recognition sequence following ATP hydrolysis and DNA translocation, while the enzyme complex remains bound at the recognition site (Yuan *et al.*, 1980; Endlich & Linn, 1985; Studier & Bandyopadhyay, 1988; Dreier *et al.*, 1996; Janscak *et al.*, 1996; Szczelcun *et al.*, 1996, 1997). DNA cleavage is proposed to follow stalling of the enzyme either by collision with a second translocating EcoKI complex (Studier & Bandyopadhyay, 1988; Dreier *et al.*, 1996) or due to topological constraints as the result of supercoiling of the DNA (Studier & Bandyopadhyay, 1988; Szczelcun *et al.*, 1996).

While all three subunits are necessary to form the restriction endonuclease, which has the stoichiometry R₂M₂S₁ (Dryden *et al.*, 1997), only M and S subunits are necessary to form the methyltransferase (M₂S₁) (Dryden *et al.*, 1993). A complex equilibrium exists between different subunit stoichiometries which has been proposed to be important for the regulation of type I systems *in vivo* (Dryden *et al.*, 1997).

DNA binding studies for the EcoKI methyltransferase showed that differences in binding affinity contribute to the distinction between specific and non-specific DNA sequences (Powell *et al.*, 1993, 1998). However, the methylation state of the recognition sequence had no effect on the binding affinity, suggesting that the preference for a hemimethylated rather than an unmethylated DNA substrate is effected mainly at the level of catalysis. Similarly for the Type IC EcoR124I methylase an increase in k_{cat} rather than a decrease in K_m was suggested to be the reason for the faster methylation of hemimethylated DNA; in fact the binding was tighter to unmethylated DNA for this enzyme (Taylor *et al.*, 1993). Methylation interference (Powell & Murray, 1995) and protein-DNA crosslinking results (Chen *et al.*, 1995) have provided evidence for interactions of the EcoKI methyltransferase with the major groove of its recognition sequence. The methylation interference was influenced by the methylation state of the DNA and the cofactor AdoMet. Similar methylation interference patterns have been observed for EcoR124I, but no cofactor effect was observed (G. G. Kneale & D. R. Mernagh, personal communication).

Here we present an analysis of the DNA binding characteristics of EcoKI by gel retardation and footprinting, and describe the effects of the cofactors ATP and AdoMet, and various cofactor analogues upon binding to DNA molecules of different methylation states. On the basis of our results we present a model of the arrangement of the protein subunits when bound to DNA.

Results

Measurement of DNA binding affinity by gel retardation

The gel retardation technique has proved useful for the characterisation of DNA binding by the EcoKI methyltransferase (Powell *et al.*, 1993). The apparent dissociation constant for binding to an EcoKI target sequence was 9 nM in the absence of AdoMet and 2 nM in its presence. The K_d did not depend on the target methylation state. The dissociation constants were 90 nM and 43 nM for binding to DNA without the target sequence in the absence or presence of AdoMet, respectively.

Gel retardation of 0.1 nM unmethylated DNA by 0–10 nM EcoKI nuclease is shown in Figure 1. An intense retarded band due to the complex between the nuclease and the DNA was observed (Figure 1(a)–(e)) except when both cofactors and non-specific DNA were present (Figure 1(f)). Tight binding was seen for both unmethylated specific and non-specific DNA with an apparent K_d between 2 and 5 nM, for the nuclease in the absence of both cofactors or plus AdoMet but minus ATP (Figure 1(a), (b), (d), (e)). In the presence of ATP and AdoMet, however, nuclease binding remained tight for the EcoKI-specific DNA (Figure 1(c)), but was abolished, over the concentration range examined for non-specific DNA (Figure 1(f)). This indicates that the R subunits allowed tight binding of EcoKI to any stretch of DNA when no cofactors were present, in contrast to the discrimination shown by the methyltransferase in the same situation.

Identification of protein subunits present in EcoKI-DNA complexes

It was apparent from Figure 1, particularly in the presence of ATP, that two minor protein-DNA complexes as well as the major species, were present when the DNA contained the EcoKI target. These additional species were only visible with non-specific DNA when higher protein and DNA concentrations than those shown in Figure 1(d)–(f) were used (data not shown). In order to investigate the nature of the additional species, two approaches were taken.

Firstly, the DNA contained in these bands was recovered by cutting the band from the polyacrylamide gel and eluting the DNA. The length of the DNA was checked on a sequencing gel and shown to be still 45 nucleotides (data not shown). The

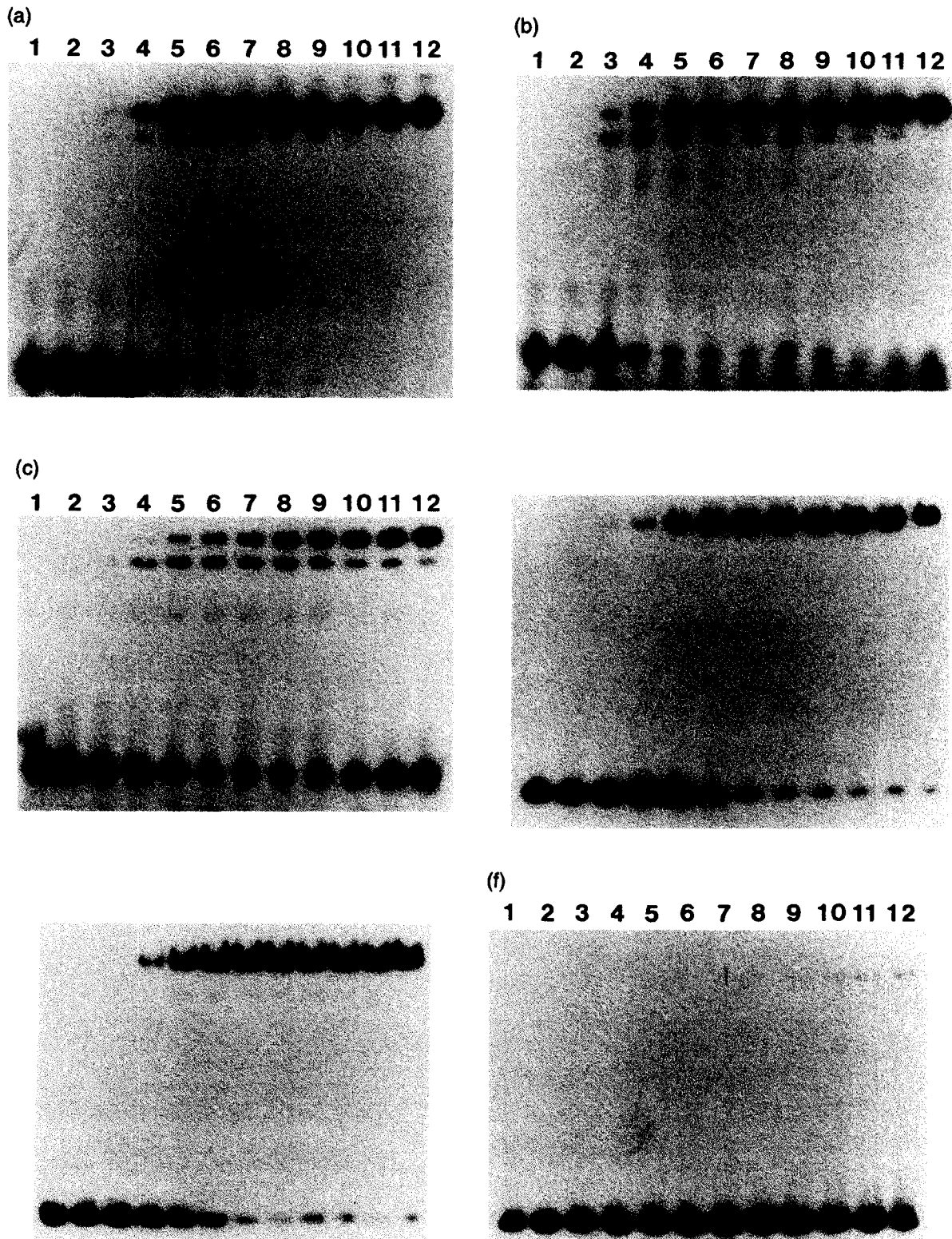


Figure 1. Measurement of *EcoKI* DNA-binding affinity by gel retardation. The concentration of the 45 bp unmethylated DNA in all experiments was 0.1 nM. In each panel, lanes 1 and 2 contain no protein while lanes 3 to 12 contain 0.5, 1, 2, 3, 4, 5, 6, 7, 8 and 10 nM *EcoKI*, respectively.

- (a) *EcoKI*-specific DNA binding in the absence of cofactors.
- (b) *EcoKI*-specific DNA binding in the presence of 100 μM AdoMet.
- (c) *EcoKI*-specific DNA binding in the presence of 100 μM AdoMet and 2 mM ATP.
- (d) Non-specific DNA binding in the absence of cofactors.
- (e) Non-specific DNA binding in the presence of 100 μM AdoMet.
- (f) Non-specific DNA binding in the presence of 100 μM AdoMet and 2 mM ATP.

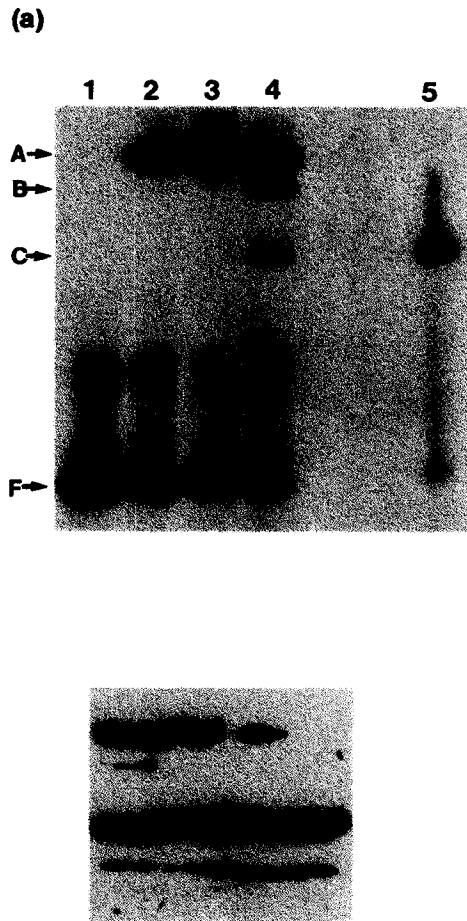


Figure 2. Identification of protein subunits in multiple complexes found by gel retardation of unmethylated DNA by *EcoKI*. (a) Binding of unmethylated DNA by *EcoKI*. Unmethylated specific 45 bp DNA (315 nM) and *EcoKI* nuclease (342 nM; lanes 2-4) or methyltransferase (342 nM; lane 5) were incubated in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 6 mM MgCl₂, 7 mM β -mercaptoethanol, 5% glycerol for 20 minutes at 22°C prior to running for two hours on a 5% non-denaturing polyacrylamide gel. AdoMet was at 100 μ M and ATP at 2 mM, if present. Lane 1: free DNA; lane 2: DNA + *EcoKI*, no cofactors; lane 3: DNA + *EcoKI* + AdoMet; lane 4: DNA + *EcoKI* + AdoMet + ATP; lane 5: DNA + methyltransferase + AdoMet + ATP. (b) Western blot of SDS-PAGE of protein eluted from DNA-protein complexes in non-denaturing gel. Protein was eluted from complexes of the type labelled A, B and C in the non-denaturing gel shown in a) and run on a 7.5% SDS-PAGE gel. This gel was blotted onto PVDF membrane and the blot developed using a chemiluminescence horse radish peroxidase system and antiserum raised against purified *EcoKI* protein. Lane 1: purified *EcoKI*; lane 2: protein eluted from complex A; lane 3: protein eluted from complex B; lane 4: protein eluted from complex C.

45 bp duplex has been shown to support ATP hydrolysis (Davies *et al.*, 1998; Webb, 1998), but not to be cleaved by the nuclease (see below).

Secondly, in order to identify the *EcoKI* subunits included in each of the complexes A, B and C in

Figure 2(a), the complexes were excised from the gel and the protein was extracted. The extracted protein was run on 7.5% SDS-PAGE and detected by Western blotting using antibodies raised against *EcoKI* (Figure 2(b)). Complex C, which migrated in the gel retardation experiment at the same rate as a methyltransferase-DNA complex, was indeed found to lack the R subunit. Complexes A and B both contain R, M and S subunits, but complex B contains significantly less R subunit than A. This is consistent with A being R₂M₂S₁-DNA and B being R₁M₂S₁-DNA. Therefore it appears that when *EcoKI* nuclease binds unmethylated DNA, a small amount of the enzyme dissociates losing first one R subunit and then the other. This dissociation is particularly apparent in the presence of ATP.

***In vitro* reconstitution of nuclease by titration of the methyltransferase with R subunit**

When increasing amounts of R subunit were added to the methyltransferase and the resultant protein was tested for DNA-binding in the presence or absence of cofactors, the three complexes observed in Figures 1 and 2 appeared sequentially on the DNA-binding gel (Figure 3). First methyltransferase-DNA alone, then R₁M₂S₁-DNA after addition of a small amount of R subunit, then R₂M₂S₁-DNA at higher R concentrations, until at R:methyltransferase ratios of 5:1 and 10:1, only the R₂M₂S₁-DNA was seen. In the absence of cofactors, extended electrophoresis of the methyltransferase-DNA complex revealed a doublet of methyltransferase-DNA and M₁S₁-DNA complexes as observed previously (Powell *et al.*, 1998). In the presence of cofactors, only M₂S₁-DNA is detected even if the gel is subjected to extended electrophoresis.

Cofactors had no effect on the concentration-dependent appearance of species containing the R subunit. Quantification of the amounts of each complex present at each R:methyltransferase ratio indicated that when [R] = [methyltransferase], in the absence of cofactors, 17.4% of the methyltransferase was uncomplexed with R, 50.4% had one R bound and 32.2% had two R bound. In the presence of cofactors the figures were 37.0%, 42.8% and 20.1%, respectively. These values were close to the 1:2:1 ratio expected if there was no substantial difference in binding affinity for the interaction of each R subunit with the methyltransferase. This contrasts with the situation for *EcoR124I* where the second R subunit is bound more weakly by the methyltransferase than the first (Mernagh *et al.*, 1998; Janscak *et al.*, 1998).

Exonuclease III footprinting of *EcoKI*-DNA complexes

Exonuclease III footprinting was used to determine the region of DNA protected by the nuclease under various conditions. The 45 bp duplexes used included those with the recognition sequence in the unmethylated, hemimethylated

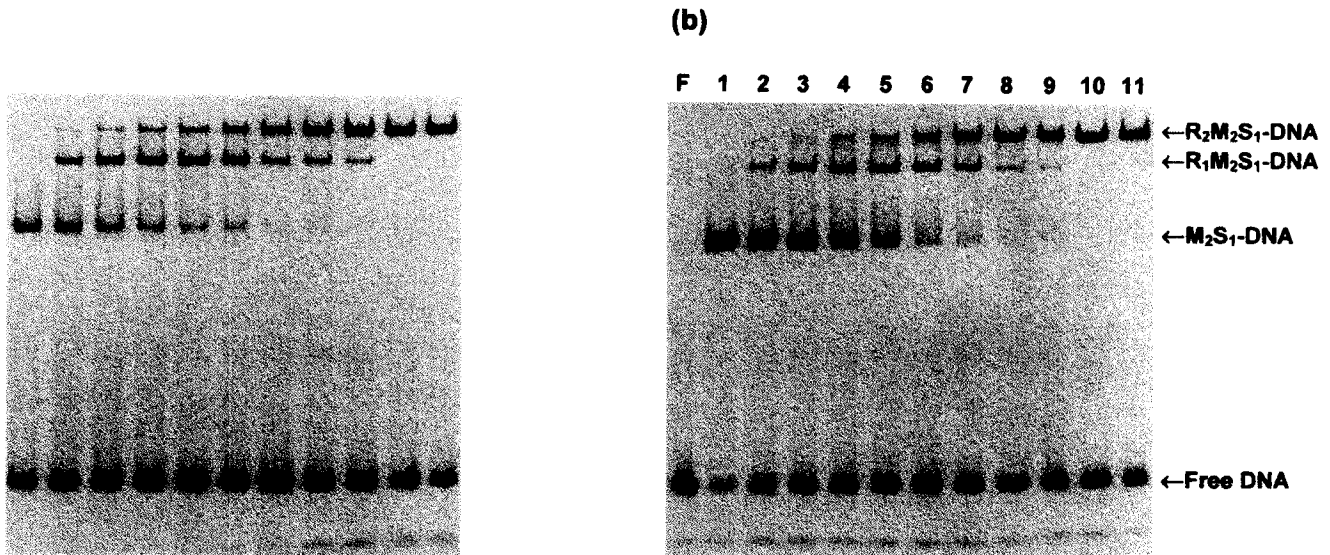


Figure 3. Reconstitution of *EcoKI* *in vitro*: titration of methyltransferase with R subunit and DNA binding by products. R subunit and methyltransferase (300 nM) were mixed in varying ratios and incubated for ten minutes on ice. (a) in the absence of cofactors and (b) in the presence of 100 μ M AdoMet and 2 mM ATP. Then unmethylated specific 45 bp DNA was added and incubated for ten minutes at 22°C prior to loading onto a 5% polyacrylamide non-denaturing gel and running for two hours at 40 mA. Lane F: DNA alone. Lanes 1-11: methyltransferase (300 nM), DNA (300 nM), and increasing amounts of R subunit to give R:methyltransferase ratios of 0:1, 0.25:1, 0.5:1, 0.75:1, 1.0:1, 1.25:1, 1.5:1, 1.75:1, 2.0:1, 5:1 and 10:1. The protein-DNA complex is identified in the labels on the right of each diagram. The asterisk in (a) against M_2S_1 -DNA is to indicate that in the absence of cofactors this species can be resolved into a mixture of M_2S_1 -DNA and M_1S_1 -DNA if the gel is run for longer.

and fully modified states (all referred to here as specific 45 bp duplexes) and also duplexes which lacked the recognition sequence (non-specific 45 bp duplexes) as described previously (Powell *et al.*, 1993). Unmethylated specific 75 bp duplexes were also used. The conditions chosen gave complete digestion of the free DNA during the time course.

Unmethylated DNA is the substrate for the *EcoKI* restriction reaction. The effects of AdoMet and ATP on the protection of unmethylated 45 bp duplex DNA by *EcoKI* were tested. Figure 4 shows the results for the top strand and Figure 5 shows the results for the bottom strand. The free DNA was completely digested under these conditions (Figures 4(a) and 5(a)). In the absence of cofactors the full length of the top strand was protected by *EcoKI* binding (Figure 4(b)). Exonuclease III footprinting of the bottom strand of the DNA in the presence of *EcoKI* alone, showed that there was slight digestion of the DNA, with 45, 44, 43 and 42mers being protected (Figure 5(b)). These results indicate a footprint of 42 to 45 bp under these conditions. The difference in the degree of protection of the two DNA strands probably reflects differences in the susceptibility of the strands to exonuclease III. In the presence of AdoMet alone, a faint 38mer fragment was seen for the top strand and a faint 37mer fragment was seen for the bottom strand, in addition to the major species (Figures 4(c) and 5(c)). In the presence of ATP alone there was no protection on either strand consistent with AdoMet being required for specific recognition of the

DNA (lower strand: Figure 5(d); results not shown for upper strand). In the presence of both cofactors, there was a clear conversion of the DNA on each strand to smaller protected species consistent with ATP triggering a conformational change in the protein. With both cofactors present, protection of 37 and 38mers for the top strand (Figure 4(d)) and of 37mers for the bottom (Figure 5(e)) suggested a protected region of \sim 30 bp. This was very similar to the 26–30 bp protection observed for the methyltransferase in the presence of AdoMet (Powell *et al.*, 1998).

As the footprint seen for *EcoKI* in the presence of ATP and AdoMet on unmethylated DNA was similar in size to that seen for the methyltransferase in the presence of AdoMet, we wished to ensure that the footprint was derived from the major $R_2M_2S_1$ -DNA complex (A) and not from the two smaller complexes, B and C seen in Figure 2. Therefore, the exonuclease III digested DNA-protein complexes were separated on a non-denaturing gel. The DNA was purified from the major complex A and run on a sequencing gel. As was expected from the relative amounts of the complexes, it was found that the major complex A gave rise to both the longer and shorter protected fragments observed in Figures 4 and 5 (data not shown, but see results for 75 bp duplex later).

Hemimethylated DNA is the substrate for methylation by *EcoKI*. Four different combinations of the position of the radio-label and methylated site can be prepared. The fragments of hemimethylated DNA protected from exonuclease III by *EcoKI* are

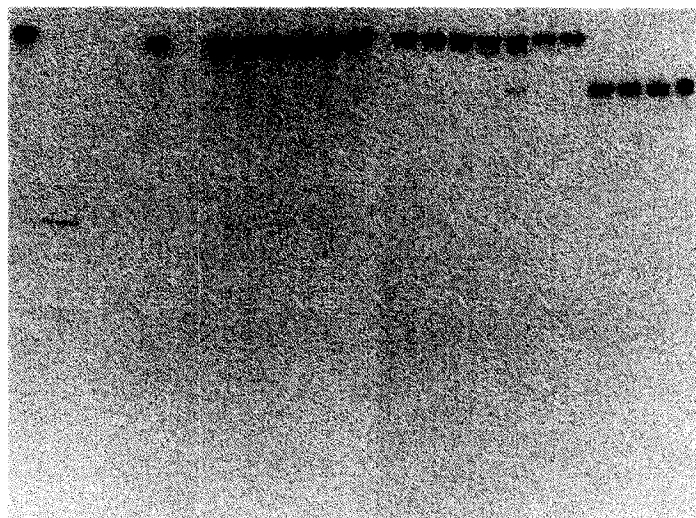


Figure 4. *Eco*KI exonuclease III footprinting for unmethylated specific DNA 45 bp duplex containing the sequence 5'AAC(N₆)GTGC3'/-3'TTG(N₆)CACG5' (top strand labelled). Protection patterns are shown for *Eco*KI (342 nM) and unmethylated DNA (315 nM) in the presence or absence of AdoMet (100 μM) or ATP (2 mM) as indicated at the top of the Figure. Each reaction set a-d shows a timecourse of exonuclease III digestion. The six lanes for reaction sets a-c show DNA after digestion for 0, 6, 10, 20, 30 and 0 minutes digestion from left to right. The first 0 minute exonuclease III reaction was for DNA incubated for 25 minutes at 22°C with *Eco*KI, while the second 0 minute exonuclease III reaction was for DNA incubated 25 minutes at 22°C followed by 25 minutes at 37°C with *Eco*KI. The five lanes in set d show DNA after digestion for 0, 6, 10, 20 and 30 minutes as in the first five lanes of a-c.

summarised in Table 1. A similar protection pattern was seen for hemimethylated DNA as for unmethylated DNA and the protected fragments were of the same size as for unmethylated DNA. Figure 6 shows the exonuclease III footprint for the hemimethylated DNA with the top strand radiolabelled and the bottom strand methylated. In the absence of cofactor (Figure 6(b)), or with AdoMet alone (Figure 6(c)), the full-length 45mer was protected. In the presence of ATP alone there was very slight protection of a 37mer (Figure 6(d)). In

the presence of AdoMet and ATP there was stronger protection of the 37mer (Figure 6(e)) although the degree of protection was lower than seen for unmethylated DNA under these conditions. This was probably because the hemimethylated DNA was becoming methylated during the exonuclease III digestion time giving fully modified DNA. The improved protection observed with AdoHcy, a product of the methylation reaction which cannot donate a methyl group, and ATP was consistent with this (Figure 6(f)).

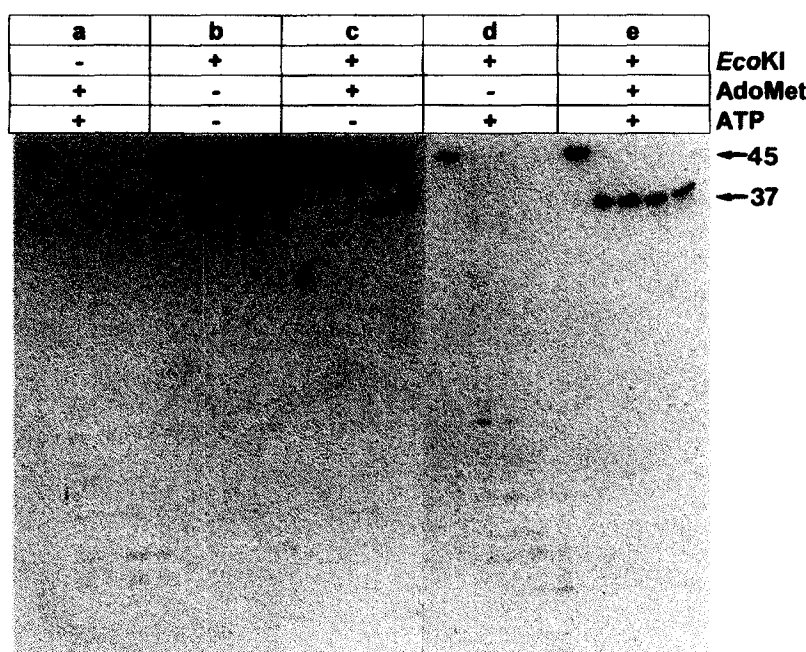


Figure 5. *Eco*KI exonuclease III footprinting for unmethylated specific DNA 45 bp duplex (bottom strand labelled). The DNA used in this footprinting experiment was as in Figure 4 except the bottom strand was labelled. Protection patterns are shown for *Eco*KI (300 nM) and unmethylated DNA (300 nM) in the presence or absence of AdoMet (100 μM) or ATP (2 mM) as indicated at the top of the Figure. The five lanes in reaction sets a-e show DNA digested for 0, 6, 10, 20 and 30 minutes, respectively. The 0 exonuclease III reactions in this experiment were for DNA incubated with *Eco*KI for ten minutes at 22°C, then 35 minutes at 37°C before ethanol precipitation.

Table 1. Fragments of 45 bp hemimethylated DNA substrates protected from exonuclease III by EcoKI.

DNA duplex		Cofactors				
Location of methyl group (DNA strand)	Location of ³² P label (DNA strand)	No cofactor	+AdoMet -ATP	-AdoMet +ATP	+AdoMet +ATP	+AdoHcy +ATP
Top	Top	45mer +++++ ^a	45mer +++++	37mer ++	37mer ++	37-38mer +++++
Bottom ^b	Top ^b	45mer +++++	45mer +++++	37mer +	37mer ++	37-38mer +++
Top	Bottom	42-45mer +++++	42-45mer +++++	-	37mer +	37-38mer ++
Bottom	Bottom	42-45mer +++++	42-45mer +++++	-	-	37-38mer ++

^a -, no protection; +, very weak protection; ++, weak protection; +++, strong protection; +++++, very strong protection.
^b Results for this substrate are illustrated in Figure 6.

Improved protection in the presence of ATP when AdoHcy was used in place of AdoMet was observed for all four hemimethylated duplexes (Table 1). For hemimethylated DNA with both the methyl group and label on the top strand, weak protection of a 37mer was seen with ATP alone, and with ATP and AdoMet. Much stronger protection was seen in the presence of ATP and AdoHcy (Table 1). When the top strand was methylated and the bottom strand labelled, no protection was seen with ATP alone, very weak protection with ATP and AdoMet, and slightly stronger protection with ATP and AdoHcy (Table 1). When the bottom strand was methylated and labelled, no protection was seen in the presence of ATP irrespective of the presence or absence of AdoMet, but there was weak protection of 37 and 38mers in the presence of AdoHcy and ATP. Differences in the exonuclease III efficiency on the DNA strands probably reflect the sequence composition of the strands.

Fully modified DNA is not a substrate for EcoKI in either the restriction or methylation reactions. In the presence of ATP, EcoKI did not protect fully modified DNA against exonuclease III, regardless of the presence of AdoMet. However, there was weak protection of a 45mer for the top strand and 42-45mers for the bottom strand in the absence of

cofactor or in the presence of AdoMet alone (data not shown).

We wished to investigate whether ATP hydrolysis was necessary to induce the change in the exonuclease III footprint seen in the presence of ATP. ADP and ATP γ S, a non-hydrolysable analogue of ATP, were tested for their ability to substitute for ATP in the protection of the upper strand of the unmethylated 45mer by EcoKI. ADP alone gave no protection, whereas ATP γ S gave faint non-specific protection (data not shown). In the presence of AdoMet, both ADP and ATP γ S were able to substitute for ATP and elicit the formation of the 37 and 38mer protected species (data not shown). Similarly AdoHcy and sinefungin were able to substitute for AdoMet (data not shown). This is in contrast to results for the methyltransferase (Powell *et al.*, 1993; Powell & Murray, 1995) where only AdoMet but not AdoHcy or sinefungin can cause an increase in binding affinity for specific DNA and a change in the methylation interference pattern with unmethylated DNA.

In the absence of the cofactors, AdoMet and ATP, EcoKI protected the full length of the top strand of the specific 45 bp duplexes. In order to check whether the protein could actually protect a larger area, a longer unmethylated 75 bp duplex DNA substrate was used. The results shown in

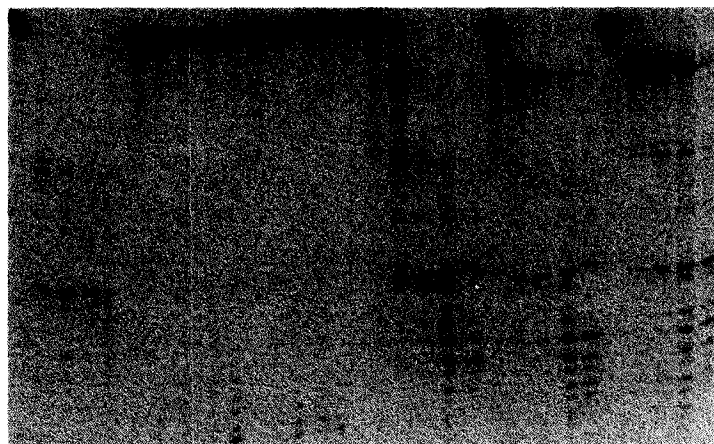


Figure 6. EcoKI exonuclease III footprinting for hemimethylated DNA 5' AAC(N₆)GTGC3'/3' TTG-(N₆)C^MACG5' (top strand labelled and bottom strand methylated). Protection patterns are shown for EcoKI (300 nM) and hemimethylated DNA (300 nM) in the presence or absence of AdoMet (100 μM), AdoHcy (100 μM), or ATP (2 mM) as indicated at the top of the Figure. Reaction sets a-f each contain 5 lanes showing DNA digested for 0, 6, 10, 20 and 30 minutes.

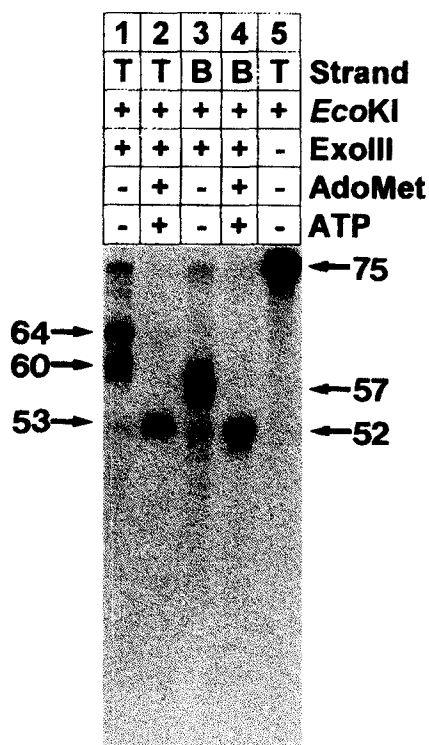


Figure 7. Exonuclease III footprint for unmethylated, specific 75 bp duplex top strand (T) and bottom strand (B), for DNA purified from complexes excised from a polyacrylamide gel. Lanes 1 to 4 show fragments of the top (T) and bottom (B) strands of the 75 bp duplex DNA protected from exonuclease III digestion in the presence or absence of the cofactors. The fragment sizes of the major protected species for the top strand are indicated to the left of the Figure, while those for the bottom strand are indicated to the right, along with the position of the full length undigested 75mer (lane 5; 75mer after incubation 30 minutes, 37°C with *EcoKI*). In the absence of cofactors the major protected species for the top strand were 60 and 64mers (lane 1) and for the bottom strand a 57mer (lane 3). In the presence of AdoMet and ATP, the major protected species for the top strand was a 53mer (lane 2) and for the bottom strand, a 52mer (lane 4).

Figure 7 are for an experiment in which, following 30 minutes exonuclease III digestion, the reactions were run on a 5% non-denaturing polyacrylamide gel and the DNA eluted from gel slices containing the major $R_2M_2S_1$ -DNA complex. In this experiment very little DNA remains to be recovered from the polyacrylamide gel in the absence of *EcoKI*. Therefore, the control experiment in the absence of *EcoKI* was performed in solution and no protected fragments were observed after 30 minutes digestion for either strand (data not shown). In the presence of *EcoKI*, ATP and AdoMet, a strong protected band was seen for both top and bottom strands of this longer substrate. For the top strand a 53mer (Figure 7; lane 2) was protected and for the bottom strand a 52mer (Figure 7; lane 4). This suggested a protected length of DNA

of 30 bp as seen for the 45 bp duplex. In the absence of cofactors there were two major protected species seen for the top strand, 60 and 64mers (Figure 7; lane 1), while the major protected species for the bottom strand was a 57mer. This suggested a protected region of 42-46 bp in the absence of cofactors. The 64 and 60mer fragments from the top strand also occurred in the absence of *EcoKI* after six and ten minute exonuclease III digestions, but the fragments did not persist after longer digestion times (data not shown). In contrast, these fragments persisted in the presence of *EcoKI* even after 30 minutes of exonuclease III digestion. The presence of these long fragments may be due to non-specific protection of the DNA. Digestion of the bottom strand in the absence of *EcoKI* did not produce any stable non-specific fragments.

To ascertain that there was no cleavage or nicking of the unmethylated duplexes by *EcoKI*, extended incubations in the presence of AdoMet and ATP but without exonuclease III were carried out at 37°C. Track 1 in Figure 5(e) shows that the labelled bottom strand of the 45 bp duplex remains intact for at least 35 minutes. Labelled top strands from the 45 bp and the 75 bp duplex remained intact for at least 60 minutes (data not shown).

Non-specific 45mers showed a very small amount of generalised protection in the absence of ATP in the presence or absence of AdoMet, but no protection at all was seen when ATP was present regardless of the presence of AdoMet (Figure 8).

Discussion

The gel retardation experiments (Figures 1-3), show that the nuclease can bind very tightly to any piece of DNA in the absence of both cofactors. This binding affinity is stronger than that observed with the methyltransferase alone (Powell *et al.*, 1993, 1998) and must be due to additional interactions with DNA being provided by the presence of the R subunits. This is particularly the case for binding to non-specific DNA lacking an *EcoKI* target site where $R_2M_2S_1$ has a DNA binding affinity of approximately 2.5 nM compared to 90 nM for M_2S_1 (Powell *et al.*, 1993). The addition of the cofactor AdoMet does not change the affinity of *EcoKI* for non-specific DNA; only the addition of ATP reduces the affinity of *EcoKI* for non-specific DNA and gives the enzyme a definite preference for binding to the *EcoKI* target site. Two complexes migrating faster than the nuclease-DNA complex were observed by gel retardation when the DNA contained the *EcoKI* target site. These were identified, one contained the methyltransferase, the other $R_1M_2S_1$, an assembly intermediate in the formation of *EcoKI* (Dryden *et al.*, 1997). ~~two~~ ~~complexes~~ were not visible at these low concentrations with non-specific DNA implying that both R subunits are essential for the strong binding to non-specific DNA. Both R subunits bind to the

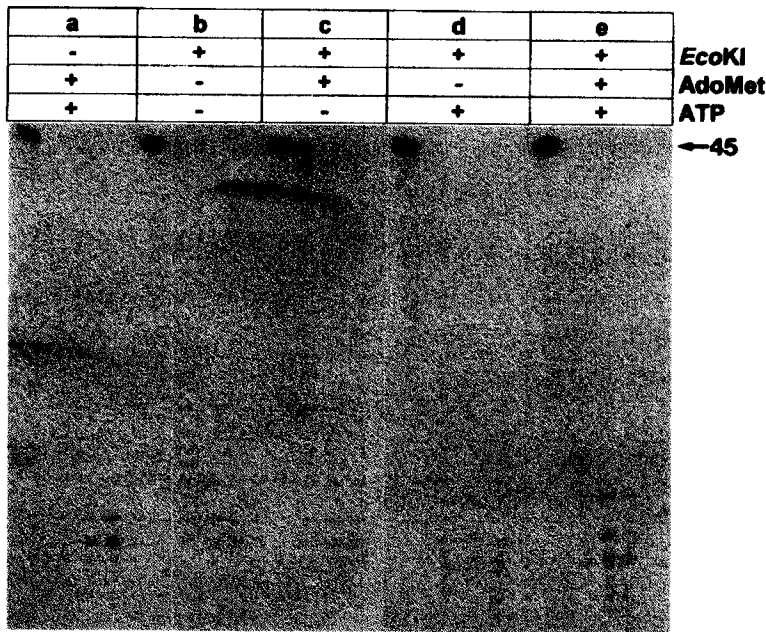


Figure 8. *EcoKI* exonuclease III footprinting for non-specific DNA 5'CCA(N₆)TGTA3'/3'GGT(N₆)ACAT5' (bottom strand labelled). Protection patterns are shown for *EcoKI* (300 nM) and non-specific DNA (300 nM) in the presence or absence of *AdoMet* (100 μM) or *ATP* (2 mM) as indicated at the top of the Figure. Each reaction set on this gel shows a time course of 0, 6, 10, 20 and 30 minutes of exonuclease III digestion.

methyltransferase with equal affinity. This is in contrast to the assembly of the *EcoR124I* type IC enzyme where one R subunit is bound more weakly than the other (Mernagh *et al.*, 1998; Janscak *et al.*, 1998) and to the *EcoAI* type IB enzyme where both R subunits appear to be weakly bound (Suri *et al.*, 1984).

The results of the exonuclease III footprinting experiments for the nuclease demonstrate the importance of both cofactors, *AdoMet* and *ATP*, in DNA recognition and are summarised in Figures 9 and 10. The cofactor analogue, *AdoHcy*, can produce the same footprint as *AdoMet*, thus the ability to methylate DNA is not essential to obtain a footprint. In the presence of *ATP*, binding is abolished unless an unmethylated or hemimethylated

EcoKI recognition sequence and *AdoMet* or *AdoHcy* are present. The fact that *ATP* hydrolysis is not essential for this recognition is clearly demonstrated by the fact that *ADP* and *ATPγS* can substitute for *ATP* in this role. Although the non-specific DNA can bind tightly to *EcoKI*, no footprint is observable implying that exonuclease III can push *EcoKI* off the DNA despite the high affinity of the nuclease for the DNA in the absence of *ATP*. It is also possible that the stability of the complex with non-specific DNA is artificially enhanced by the "caging" effects common in gel retardation experiments (Lane *et al.*, 1992).

The presence of *ATP* triggers a conformational change in *EcoKI* which results in a dramatically smaller protected region of either hemimethylated

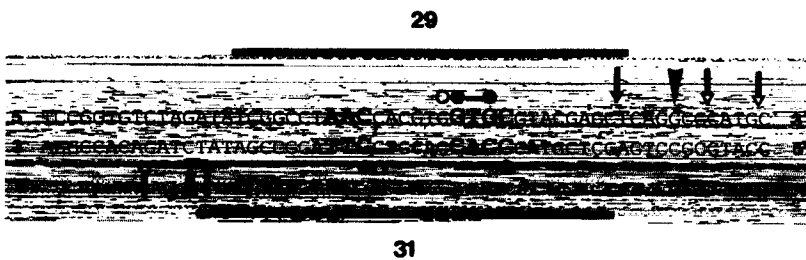


Figure 9. Summary of footprinting results for *EcoKI* nuclease and methyltransferase with unmethylated DNA. 55 bp of DNA are shown of the central portion of the 75 bp duplex. The *EcoKI* recognition sequence is shown in bold type. The circles above the guanine residues of the recognition and spacer sequences illustrate residues at which methylation interference

has been demonstrated for the methyltransferase in the absence of *AdoMet* (open and filled circles) or in the presence of *AdoMet* (filled circles only) (Powell & Murray, 1995). The continuous lines above and below the sequence represent the 29 and 31 base *DNaseI* footprints seen on the top and bottom strands, respectively of unmethylated DNA in the presence of *EcoKI* methyltransferase (Powell *et al.*, 1998). The large arrowheads show sites of *DNase I* hypercleavage at G58 on the top strand of the 75 bp duplex and C22' on the bottom strand in the presence of *EcoKI* methyltransferase (Powell *et al.*, 1998). The arrows with an open arrowhead show the limits of exonuclease III cleavage due to protection by *EcoKI* nuclease in the absence of cofactors on the top and bottom strands giving a footprint of 42-46 bp (this paper). The addition of *AdoMet* alone gives a mixture of this 42-46 bp footprint and a shorter (minor) 30 bp footprint. The arrows with a filled-in arrowhead indicate the limits of exonuclease III cleavage due to protection by *EcoKI* nuclease in the presence of *AdoMet* and *ATP* giving a protected region of 30 bp (this paper). This is similar to the exonuclease III footprint of 26-30 bp seen for the methyltransferase in the presence of *AdoMet* (Powell *et al.*, 1998).



Figure 10. A sketch of *EcoKI* showing the arrangement of the three postulated DNA binding regions and the effects of cofactor binding. The DNA is shown as a rectangle with the *EcoKI* target site shaded and the relative positions of the R subunits and the methyltransferase (Mtase) which forms the core of *EcoKI* indicated. (a) Complex formed with non-specific DNA in the absence of ATP which gives rise to an extremely weak 42-46 bp exonuclease III footprint even though the affinity of *EcoKI* for non-specific DNA is high. Only the R subunits are in contact with the DNA as, in the absence of R subunits, the methyltransferase by itself has poor affinity for non-specific DNA (Powell *et al.*, 1993). AdoMet does not affect this footprint. Exonuclease III appears to be able to easily displace *EcoKI* from non-specific DNA. (b) Complex formed with specific DNA in the absence of AdoMet and ATP. Both R subunits and the methyltransferase core are in contact with the DNA giving a 42-46 bp exonuclease III footprint irrespective of the methylation state of the target site. This footprint is weak on fully methylated DNA but strong on unmethylated and hemimethylated DNA. The methyltransferase core and the R subunits all contribute to the DNA binding. The binding of AdoMet causes a shift to a 30 bp footprint for a small proportion of the protein complexed with unmethylated DNA. (c) Complex formed with specific DNA in the presence of both cofactors. Only the methyltransferase core is making a strong contact with the DNA, the R subunits having adopted a conformation which allows greater cleavage by exonuclease III to give a 30 bp footprint. This footprint occurs only with unmethylated or hemimethylated DNA. This new conformation of the R subunit may be a prerequisite for translocation on unmethylated DNA.

or unmethylated DNA (Figure 9). This change was distinct from the disassembly of the nuclease, as the larger $R_2M_2S_1$ complex excised from the gels still gave the smaller footprint under conditions

where both cofactors were present. Both M_2S_1 in the presence of AdoMet (Powell *et al.*, 1998) and $R_2M_2S_1$ in the presence of AdoMet and ATP occupy approximately the same region of the DNA, protecting about 30 bp from enzymatic probes. This is larger than the 25 bp footprint of the *EcoR124I* type IC enzyme under similar conditions (Mernagh *et al.*, 1998). In the absence of ATP, *EcoKI* covers an even larger region of the DNA. ATP induces a conformational change in the protein to give a smaller footprint and, by implication, a more compact protein structure.

Distinct steps in DNA recognition by *EcoKI* have been described, of which the early ones are common to the methylation and restriction pathways (Burckhardt *et al.*, 1981b), and also occur even if the DNA is fully methylated and is not a substrate for either reaction. The first step was described as enzyme activation where *EcoKI* binds AdoMet rapidly, and in a slow second step a conformational change was induced in the protein to give an activated form (Hadi *et al.*, 1975). This activated enzyme *EcoK** interacts with DNA regardless of the methylation state of the DNA, initially forming a non-specific complex and then a recognition complex at an *EcoKI* target site (Bickle *et al.*, 1978). ATP then allows the enzyme to discriminate between unmethylated, fully methylated and hemimethylated DNA. If the site is fully methylated, the enzyme is released from the site (Bickle *et al.*, 1978). If the site is unmethylated, ATP causes a change in the conformation of *EcoKI*, resulting in loss of enzyme-bound AdoMet and a decrease in the diameter of the protein as demonstrated by electron microscopy. It has also been shown that ATP induces the protein to trap the DNA on nitrocellulose filters, a characteristic which both initial and recognition complexes lack (Bickle *et al.*, 1978). ATP hydrolysis is not essential for the transition from "large" to "small" enzyme, as a non-hydrolysable analogue of ATP can be substituted for ATP (Bickle *et al.*, 1978). In the case of hemimethylated DNA, ATP is thought to trigger *EcoKI* to act in its modification mode, and thus methylate the DNA (Burckhardt *et al.*, 1981b). No change was apparent in the size of *EcoKI* bound to hemimethylated DNA in the presence of ATP, and filter binding complexes were not seen with hemimethylated DNA (Burckhardt *et al.*, 1981b).

Our results are broadly consistent with this scheme. The only difference would appear to be in the initial DNA binding step. Our results show that the *EcoKI* enzyme does not require either cofactor to bind to DNA. The cofactors assist the enzyme to recognise its DNA target site and cause some sort of conformational change. Evidence for AdoMet induced conformational changes in the *EcoKI* methyltransferase and the *EcoR124I* methyltransferase has been found (Powell *et al.*, 1993; Cooper & Dryden, 1994; Taylor *et al.*, 1994). AdoMet alone does not cause the large change in the exonuclease III footprint for the *EcoKI* nuclease. The large decrease in the *EcoKI* DNA footprint pro-

duced by ATP binding for both hemimethylated and unmethylated DNA may correlate with the change observed by electron microscopy for unmethylated DNA. Alternatively, the appearance of the M_2S_1 -DNA and $R_1M_2S_1$ -DNA complexes in our gel retardation experiments may be equivalent to the smaller protein-DNA complex observed by electron microscopy as it was suggested that the complexes observed by electron microscopy may have lost some protein subunits (Bickle *et al.*, 1978). There is no electron microscopy evidence for a conformational change when EcoKI binds to hemimethylated DNA. However, our footprinting results in the presence of AdoMet show that the species with the smaller footprint is transient due to methylation to fully modified DNA. If the electron microscopy was repeated in the presence of AdoHcy or sinefungin, a large change in the shape of the enzyme could be more apparent. The disappearance of the footprint on fully modified DNA in the presence of ATP agrees with the proposal that the enzyme dissociates from such a target site.

In the absence of cofactors, the EcoKI nuclease protects a much larger region of DNA than the methyltransferase. This implies that the two R subunits project out along the DNA helical axis from their contact point with the M_2S_1 centre of EcoKI. The EcoKI-DNA interface may comprise three regions; a "core" region provided by the methyltransferase and two regions on either side provided by the R subunits (Figure 10). Contact with DNA *via* two regions of EcoKI or the related EcoBI enzyme has been proposed from electron microscopic analysis of the products of the DNA cleavage reaction (Rosamund *et al.*, 1979; Yuan *et al.*, 1980). These studies suggested that EcoKI and EcoBI could translocate DNA in only one direction at a time, however, further analysis of the products of digestion of T7 DNA as a function of time clearly show that DNA translocation occurs from both sides of the EcoKI target site at the same time (Studier & Bandyopadhyay, 1988). Therefore we believe that extending the two-point contact model to a three-point contact model is reasonable, as was also concluded for the EcoR124I enzyme (Szczelkun *et al.*, 1996). The strong binding to non-specific DNA indicates that the DNA binding region on each R subunit is sufficient for tight binding and perhaps implies that the R subunits wrap around the DNA to hold the protein on to the DNA. The absence of binding to non-specific DNA by $R_1M_2S_1$ shows that both R subunits are required. However, only when EcoKI is bound to a target site and all three DNA binding regions are functioning, is binding in the absence of cofactors strong enough to prevent access of exonuclease III. The "core" DNA binding by the methyltransferase component would be absent on a non-specific substrate and DNA affinity would be due to the R subunits alone.

The footprint of EcoKI on unmethylated or hemimethylated DNA changes from 42-46 bp to 30 bp upon the addition of cofactors. The smaller foot-

print is very similar to the 26-30 bp found for the methyltransferase alone (Powell & Murray, 1995) and therefore the difference between the 42-46 bp footprint and the 26-30 bp footprint should be representative of the footprint of the two R subunits in EcoKI in the absence of cofactors. This difference will be equal to that contributed directly by the R subunits assuming that any steric hindrance by EcoKI which prevents exonuclease III cutting right up to the edge of the EcoKI DNA binding site is the same for the two different sizes of footprints. This allows one to calculate that the footprint of each R subunit is 6 to 10 bp on DNA. We note that this estimate lies between the step size of 5 bp for DNA unwinding by the UvrD helicase (Ali & Lohman, 1997) and the protein-DNA interface of 10 bp found in the crystal structure of the related Rep helicase when bound to DNA (Korolev *et al.*, 1997).

The smaller region of DNA protected by both the nuclease and the much smaller methyltransferase, in the presence of cofactors, could imply one of two possible structures for EcoKI. Firstly, it is possible that the R subunits do not contact the DNA at all in this complex, however, this seems very unlikely as the R subunits are necessary for the DNA translocation stage of the restriction reaction. A second possibility, suggested by our results with EcoKI and recent results with EcoR124I (Mernagh *et al.*, 1998), is that the R subunits are still in contact with the DNA positioned *via* their attachment to the S and M subunits, but that they do not make such an extensive contact with the DNA. This less extensive contact may allow the R subunits to cycle through a series of ATPase-dependent conformational states pulling the DNA past itself during translocation. Such a mechanism has been proposed for DNA and RNA helicases (Lohman & Bjornson, 1996; Bird *et al.*, 1998) to which the R subunits show sequence similarity (Gorbalenya & Koonin, 1991; Murray *et al.*, 1993; Titheradge *et al.*, 1996). This more open contact with the DNA could allow exonuclease III to move in closer to the "core" DNA binding site provided by the methyltransferase component of the nuclease and give a smaller footprint.

Materials and Methods

Proteins and cofactors

Methyltransferase (M_2S_1) was purified as described (Dryden *et al.*, 1993) but substituting the S300 Sephacryl gel filtration column with a Superdex 200 16/60 column (Pharmacia). EcoKI nuclease ($R_2M_2S_1$) was prepared using a multicopy plasmid *phsA*⁺ that expressed the *hsdR*, *M* and *S* genes from their natural promoters (O'Neill *et al.*, 1997) in *E. coli* strain NM679. Six litres of culture grown in L broth supplemented with 0.1 mg/ml ampicillin at 37°C yielded about 3 mg homogeneous nuclease. The R subunit was purified as described (Dryden *et al.*, 1997). AdoMet was obtained from New England BioLabs and all other cofactors from Sigma.

Oligonucleotides

Synthetic oligonucleotides were supplied by OSWEL DNA, University of Southampton. 45 bp duplexes were prepared by hybridization of complementary oligonucleotides, to give unmethylated, hemimethylated or fully modified specific DNA containing the EcoKI recognition sequence, or non-specific DNA, as described by Powell *et al.* (1993). The top strand of the DNA containing the EcoKI sequence was 5'-TGTCTAGATATCGGCCTAAC-CACGTGGTGCGTACGAGCTCAGGCG-3'. Nucleotides methylated at the N6-adenine position were incorporated at the underlined adenine or the adenine complementary to the underlined thymine. The top strand of the non-specific DNA sequence was 5'-TGTCTAGATATCGGCCTCCACACGTGTGTAGTACGAGCTCAGGCG-3'. The unmethylated 75 bp duplex contained a central region corresponding to the 45 bp duplex and the sequence of the top strand was 5'-CATATCCA-CATCCGGTGTCTAGATATCGGCCTAACACGCTGTGCGTACGAGCTCAGGCGCATGCCGTAGCGCGG-3'.

The oligonucleotides were end-labelled for gel retardation or footprinting on one strand only prior to hybridization to the complementary, unlabelled oligonucleotide which was included at slight molar excess. The labelling reaction used [γ -³²P]ATP (Amersham or ICN) or [γ -³³P]ATP (ICN) and T4 polynucleotide kinase (from S. Bruce and K. Murray, ICMB, Edinburgh). Unincorporated ATP was removed using G25 spun column chromatography (Sambrook *et al.*, 1989).

Gel retardation of protein-DNA complexes

Gel retardation of the unmethylated 45 bp DNA duplex was performed as described previously (Powell *et al.*, 1993).

Identification of protein subunits in DNA-protein complexes separated on 5% PAGE gels

Bands were excised from a wet 5% (w/v) polyacrylamide gel, transferred to a microcentrifuge tube and 300 μ l of SDS-PAGE sample buffer was added. The tubes were then placed on a rotating wheel at 37°C overnight to elute the protein from the gel. The sample buffer was removed from the gel slice and was loaded onto a 7.5% SDS-PAGE gel, along with prestained protein markers (Sigma) and run for three hours at 45 mA before blotting onto PVDF membrane (Millipore) for four hours at 60 V at 4°C. The efficiency of transfer could be estimated from the amount of prestained marker transferred to the membrane. The protein bands in the Western blot were visualised using primary antibody raised against EcoKI and the chemiluminescence horse radish peroxidase system (Boehringer Mannheim). The DNA in the complexes was eluted from the gel into 0.5 M ammonium acetate (pH 7.5), 1 mM EDTA, 0.1% (w/v) SDS, 10 mM MgCl₂, precipitated and analysed on 12% polyacrylamide, 7 M urea sequencing gels (Papavassiliou, 1994). This procedure confirmed that there was no degradation of the DNA in the complexes observed.

Titration of methyltransferase with R subunit

Methyltransferase (300 nM) was mixed with increasing amounts of the R subunit from 0 to 3 μ M at ratios of 0 to 10 times the amount of the methyltransferase, either

in the presence of the two cofactors AdoMet (100 μ M) and ATP (2 mM) or in their absence. After ten minutes incubation a DNA stock solution containing the same concentrations of cofactors (if present) and 5% (v/v) glycerol was added to give a final DNA concentration of 300 nM. The samples were incubated for a further ten minutes before gel retardation as described (Powell *et al.*, 1993).

Footprinting

Exonuclease III digests each single strand of DNA in a double-stranded molecule from the 3' end releasing 5' mononucleotides. 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 can halt the progress of exonuclease III. 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 sequencing gel.

Exonuclease footprinting using the 45 bp duplexes was as described by Powell *et al.* (1998). Briefly, EcoKI nuclease was incubated for ten minutes at 22°C with the appropriate DNA in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 6 mM MgCl₂, 7 mM β -mercaptoethanol, 5% glycerol before digestion with exonuclease III for 0–30 minutes at 37°C. The reactions were stopped and the DNA precipitated with ethanol. The resuspended DNA was then run on a 12% polyacrylamide (Longranger, Flowgen), 7 M urea sequencing gel for one hour at 38–42 W and 50°C. The gel was dried and exposed to film at –70°C. The concentration of EcoKI was 300 nM as was the concentration of DNA, for all of the reactions except for the footprinting reaction with unmethylated, specific 45 bp duplex DNA with the top strand labelled where EcoKI was at 342 nM and the DNA at 315 nM. Gel retardation of a sample of each reaction was used to check the DNA binding by the protein.

For the experiment with the 75 bp duplex, the method was the same except following exonuclease III digestion the samples were not immediately ethanol precipitated, but run on 5% non-denaturing gels to allow separation of complexes of the type A, B and C as illustrated in Figure 2(a). After autoradiography, DNA from the major species (A) R₂M₂S₁-DNA was eluted from gel slices, ethanol precipitated and run on a 12% polyacrylamide urea-denaturing sequencing gel. EcoKI and DNA concentrations were both at 300 nM.

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