The EcoRV Modification Methylase Causes Considerable Bending of DNA upon Binding to Its Recognition Sequence GATATC*

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The EcoRV methyltransferase modifies DNA by the introduction of a methyl group at the 6-NH₂ position of the first deoxyadenosine in GATATC sequences. The enzyme forms a stable and specific complex with GATATC sequences in the presence of a nonreactive analogue, such as sinefungin, of its natural cofactor S-adenosyl-L-methionine. Using circular permutation band mobility shift analysis (in which the distance between the GATATC sequence and the end of the DNA is varied) of protein-DNA-cofactor complexes we have shown the methylase induces a bend of just over 60° in the bound DNA. This was confirmed by phasing analysis, in which the spacing between the GATATC site and a poly(dA) tract is varied through a helical turn, which showed that the orientation of the induced curve was toward the major groove. There was no significant difference in the bend angle measured using unmethylated GATATC sequences and hemimethylated sequences which contain G₆-MeATATC in one strand only. These are the natural substrates for the enzyme. The EcoRV endonuclease, a very well characterized protein, served as a positive control. DNA bending by this protein has been previously determined both by crystallographic and solution methods. The two proteins bend DNA toward the major groove but the bend angle produced by the methylase, slightly greater than 60°, is a little larger than that observed with the endonuclease, which is approximately 44°.

Bending of DNA is fairly common and can be due to: 1) sequence-specific intrinsic curvature (Crothers et al., 1990; Hagerman, 1990, 1992; Trivinov, 1991), for example with poly(dA)-poly(dT), which induces curvature toward the minor groove; 2) the binding of small ligands (Feuerstein et al., 1990); and 3) the binding of proteins. One of the earliest, structurally characterized, complexes in which proteins induce bending of DNA was the nucleosome (Richmond et al., 1984). However, DNA curvature does not only occur in these macromolecular structures. A large number of proteins involved in important cellular processes such as replication, recombination, or transcription are known to produce bending of the double helix (Travers, 1990). An excellent example is the prokaryotic catabolite gene activator protein (CAP protein), which produces a 90° bend, as measured both in crystals (Schultz et al., 1991) and solution (Wu and Crothers, 1984; Kim et al., 1989; Zinkel and Crothers 1990). The importance of the bend produced by this protein has been demonstrated by its replacement with intrinsically bent DNA; this is enough to activate transcription in vitro (Bracco et al., 1989). Inducing the bending of DNA in order to activate transcription has been reported for other transcriptional proteins (Perez-Martin and Espinosa, 1991), and this seems to be a very common feature for this class of protein. The bend angles obtained using solution methods range from 30°, for Cro protein binding to O₂,₂ operator, to 112° for the GalR protein binding to O₁ operator (Kim et al., 1989).

The interactions of type II restriction enzymes with their target sites can also result in DNA bending. Crystal structures of the EcoRI (Kim et al., 1990; Rosenberg, 1991) and EcoRV (Winkler et al., 1993; Kostrewa and Winkler, 1995) endonucleases with their cognates sequences (GAATTC and GATATC, respectively) show that the bound DNA is distorted and both enzymes induce considerable bends. In both cases studies in solution have confirmed the bending of the DNA observed in the crystalline state (Thompson and Landy, 1988; Douc-Rasy et al., 1990; Stöver et al., 1993; Vipond and Halford, 1995). Bend angles of 50° and 44° have been reported for RI and RV, respectively. However, the crystal structure of the cognate DNA (ACACGTG)-PvuII complex (Cheng et al., 1994) shows no significant DNA curvature, even though the nucleosome shares structural similarities with EcoRV. Other data, obtained using only solution methods, showed that HpaI bent DNA in a very similar manner to EcoRI (Aiken et al., 1991). These two enzymes are perfect isoschizomers. SmaI and Xmal (imperfect isoschizomers that recognize the sequence CCCGGG) induce bending of 32° and 40°, respectively. Interestingly the DNA is bent in opposite directions, with SmaI toward the major groove and with Xmal in the direction of the minor groove (Withers and Dunbar, 1993).

There is much less structural data available for type II DNA methyltransferases. Two deoxycytidine methylases, Hhal (Cheng et al., 1993; Klimasauskas et al., 1994) and HaeII (Reinisch et al., 1995), have been crystallized as complexes with DNA. Both structures show that the target deoxycytidine base is completely flipped out of the helix, but there is little or no bending to the nucleic acid. One deoxyadenosine methylase, TaqI (Labahn et al., 1994), has also had its structure determined albeit in the absence of an oligonucleotide. We are unaware of experiments describing solution studies in which any DNA curvature, induced by complexation with a DNA methyltransferase, has been measured. In this study we investigate DNA bending induced by the EcoRV methyltransferase. This is a type II deoxycytidine methylase which adds CH₃ groups to the first deoxyadenosine in GATATC sequences (Nwosu et al., 1988). We have recently shown that this methylase binds specifically to GATATC sequences with the following order of affinity: hemimethylated DNA > unmethylated DNA > dimethylated DNA. Sinefungin, an analogue of the cofactor S-adenosyl-L-methionine (AdoMet)✝ increased binding by a factor

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1 The abbreviations used are: AdoMet, S-adenosyl-L-methionine; N-methyl-AdoMet, 2,4-diamino-N-methyl-N'-(5-deoxyadenosyl)-butanamide; PCR, polymerase chain reaction; bp, base pair(s).
of 20, and ternary enzyme/cofactor/Substrate (un-/ hemimethy-
lated DNA) migrated slightly more rapidly than the equiva-
 lent product complexes containing dimethylated DNA. The en-
 zyme copurified with traces of AdoMet and so was capable of 
 methylating small quantities of DNA without the addition of 
 this cofactor (Szczelkun and Connolly, 1995). Several of the 
 methylase-DNA contacts were determined using ethylation and 
 methylation interference analysis (Szczelkun et al., 1995). The 
 formation of a specific protein-DNA complex means that 
 solution methods, such as the circular permutation band mo-
 bility shift assay (Wu and Crothers, 1984; Liu-Johnson et al., 
 1986; Kim et al., 1989; Crothers et al., 1991; Zwieb and Adhya, 
 1994) and helical phasing analysis (Zinkel and Crothers, 1987; 
 Crothers et al., 1991; Gartenberg et al., 1990; Withers and 
 Dunbar, 1993), can be used to see if the methylase bends DNA. 
 The two approaches have been used with both unmethylated 
 and hemimethylated GATATC sites to reveal details of DNA 
 bending by the EcoRV methylase and to elucidate the means by 
 which this protein recognizes its target sequence.

**EXPERIMENTAL PROCEDURES**

Materials—The purification of the EcoRV methyltransferase has 
been described previously (Nwosu et al., 1988; Szczelkun and 
Connolly, 1995). The gel filtration column used in the second step was a Superdex 
G-75 10/30 (Pharmacia, St. Albans, UK) instead of Protein-Pak 300W 
(Waters-Millipore). The EcoRV endonuclease, purified as previously 
described (Luke et al., 1987; Newman et al., 1990), was a generous gift 
from Sarah A. Cullinane (University of Newcastle upon Tyne, UK). The 
AdoMet analogue, N-methyl-AdoMet, was kindly provided by Professor 
Michael Blackburn (University of Sheffield, UK). Restriction enzymes 
were purchased from NBL (Northumbria Biological Laboratories, 
Cramlington, UK) and Taq DNA polymerase was from Boehringer 
Mannheim (Leus, UK). Oligonucleotides primers for PCR amplifica-
tion were synthesized on an Applied Biosystems 381A DNA synthesizer 
using the phosphoramidite method (Newman et al., 1987; Newman, 1990). 
Taq DNA polymerase was from Boehringer (Mannheim, Germany) 
and was used as specified by the supplier. The concentration of each amplified DNA 
was carried out in volumes of 20 μl containing: 50 mM HEPES-NaOH, pH 7.0, 1 mM EDTA, 100 mM NaCl, 5 mM dithio-
reitol, and 1 μg of acetylated bovine serum albumin. 1.5 mM levels of 
either the AdoMet analogue N-methyl-AdoMet or 0.2 mM sinefungin, 
−100 pm amounts of each amplified DNA, and 100 mM concentrations of 
the methylase were used (Szczelkun and Connolly, 1995). The binding 
of EcoRV endonuclease was carried out in 20-μl volumes containing: 50 mM 
Tris-HCl, pH 7.0, 100 mM NaCl, 10 mM β-mercaptoethanol, 1 μg of 
amethylated bovine serum albumin, 0.1 mM EDTA, and 5 μM CaCl₂ 
(Vipond and Halford, 1995). The concentration of each amplified DNA 
was −25 pm and 10 mM levels of the enzyme was used. The free DNA and complexes were separated by either 7% or 8% of a nondenaturing poly-
acrylamide gel and visualized by autoradiography as has been previously 
reported (Szczelkun and Connolly, 1995). For the experiments with the endonuclease 5 mM Ca²⁺ was included in the gels, and the EDTA 
was omitted (Vipond and Halford, 1995).

**RESULTS**

**Design of DNA Fragments for Bending Analysis**—We have 
used a number of oligonucleotides containing a single GATATC 
site to study DNA bending by the EcoRV methylase. The first 
set, illustrated in Fig. 1, are derived from the bend plasmid 
series (Zwieb and Adhya, 1994) and have been extensively 
used, for example with the EcoRV endonuclease (Vipond and 
Halford, 1995). The "permutation" of the EcoRV site along the 
DNA fragment was achieved by the PCR amplification of the 
plasmids followed by the digestion of the amplified fragments 
with the restriction enzymes shown in Fig. 1. The six 141-bp 
oligonucleotides produced have a single EcoRV site at a position 
that varies relative to the center and the ends of the molecule. The only disadvantage with these oligonucleotides is 
that the disposition of the restriction sites does not allow a 
dead-center location of the GATATC sequence. In addition the oligonucleotides, shown in Fig. 2, have been prepared and used. 
These were synthesized by the PCR amplification of a fragment 
that contains a unique GATATC sequence. Using the short 
forward and reverse pairs of primers 1–4 gives the "normal," 
i.e. unmethylated, product fragments 1–4 shown in this figure. 
Product 1 contains a centrally located EcoRV sequence and 
with products 2–4 this site moves toward the end of the oligo-

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The plasmids were transfected into Esherichia coli TGI-
compotent cells, and the resulting resolated plasmids had their 
sequences confirmed by automated DNA sequencing. These plasmids 
were then used as templates for PCR reactions with a common forward 
primer (CAGTTATGCTGCGGTG) and five different reverse primers (phasing oligonucleotide 1, AGTCAGGACGTGTTAAAA; 2, 
TCACGGCCATCC; 3, GCTCTGAGATGAAACAG; 4, AGCTGGTA-
AAAACGCGGC; 5, GTTGGAAAAACGCGGCA). This produces five 
fragments of the same length (206 bp) with a GATATC EcoRV site 
phased with the poly(dA) tract (see Fig. 3).

**Polymerase Chain Reaction—**PCR was carried out using a Technne 
Thermal Cycler from Techne (Cambridge, UK) with PCR condi-
tions of 94°C, 1 min; 55°C, 1 min; 72°C, 1 min; and 30 cycles of 94°C, 1 min; 55°C, 3 min; and 72°C, 2 min. The amplified fragments were separated in a 10% nondenaturing polyacrylamide gel and visualized by autoradiography. The required 
band was excised and eluted overnight at 37°C in Tris-EDTA buffer 
with continuous shaking and the DNA fragments separated from 
buffers and gel contaminants by ethanol precipitation (Sambrook 
et al., 1989).

**Band Mobility Shift Experiments—**The interaction of the EcoRV 
methylase with DNA was carried out in volumes of 20 μl containing: 50 mM HEPES-NaOH, pH 7.0, 1 mM EDTA, 100 mM NaCl, 5 mM dithio-
reitol, and 1 μg of acetylated bovine serum albumin. 1.5 mM levels of 
either the AdoMet analogue N-methyl-AdoMet or 0.2 mM sinefungin, 
−100 pm amounts of each amplified DNA, and 100 mM concentrations of 
the methylase were used (Szczelkun and Connolly, 1995). The binding 
of EcoRV endonuclease was carried out in 20-μl volumes containing: 50 mM 
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(Vipond and Halford, 1995). The concentration of each amplified DNA 
was −25 pm and 10 mM levels of the enzyme was used. The free DNA and complexes were separated by either 7% or 8% of a nondenaturing poly-
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reported (Szczelkun and Connolly, 1995). For the experiments with the endonuclease 5 mM Ca²⁺ was included in the gels, and the EDTA 
was omitted (Vipond and Halford, 1995).

**Determination of Band Angles—**The angle of curvature induced in 
the DNA by protein binding was calculated using the empirical equa-
tion of Thompson and Landy (1988); μ/μ₀ = cos(θ/2) (where μ₀ and 
μ represent to the electrophoretic mobility for the DNA-protein com-
plexes when the binding site is at the middle and at the end of the DNA 
fragment, respectively.

**References**

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DNA Bending by the EcoRV Methylase

As mentioned in the Introduction, the EcoRV methylase copurifies with small amounts of AdoMet that can add CH₃ groups to GATATC sequences in the preincubation prior to band mobility shift analysis. This can be prevented under certain conditions, mainly a combination of the inclusion of sinefungin, a very strong competitive inhibitor of AdoMet, and the use of small quantities of the methylase (Szczelkun and Connolly, 1995). However, in this study we have selected cofactor and methylase levels that allow a degree of methylation to take place to give a mixture of un-, hemi-, and dimethylated oligonucleotides. This allows the bending of both the ternary complexes containing un/hemimethylated and dimethylated DNA to be simultaneously assessed. The phasing experiments (using the oligonucleotides shown in Fig. 3) have been carried out in the presence of 0.2 mM sinefungin, an amount sufficient to ensure the 20-fold increase in the binding of GATATC-containing sequences. However, sinefungin has recently become unavailable commercially. Therefore, the bending experiments (using the oligonucleotides shown in Figs. 1 and 2) have been performed with 1.5 mM levels of the analogue N-methyl-AdoMet. N-Methyl-AdoMet is a poorer analogue than sinefungin,² but the higher levels used ensured the 20-fold increase in affinity of cognate oligonucleotides.

Initially we evaluated any DNA bending produced by the binding of the methylase to GATATC sequences using the circular permutation band mobility shift assay (Wu and Crothers, 1984; Liu-Johnson et al., 1986; Kim et al., 1989; Crothers et al., 1991). The six fragments shown in Fig. 1 were bound to the methylase, in the presence of N-methyl-AdoMet, and the complexes formed subjected to nondenaturing gel electrophoresis. The results obtained are illustrated in Fig. 4A. This figure clearly shows that fragments containing a centrally located GATATC site give complexes with lower electrophoretic mobility than do those with the EcoRV site at the end of the DNA. Furthermore, there is a good correlation between the degree of retardation and the nearness of the GATATC site to the center of the fragment as shown in Fig. 4B, a graphical representation of the gel data. Close inspection of Fig. 4A shows that the retarded band is actually composed of two just-resolved species (this is most apparent in lane 5). However, the separation of the two bands is much less obvious here than in our earlier publication (Szczelkun and Connolly, 1995), possibly because of the much longer oligonucleotides used in this study. The two bands arise because of the presence of AdoMet that copurifies with the methylase and adds methyl groups to the first deoxyadenosine in the GATATC sequence. However, very similar results were seen if the trailing edges were used for mobility determination. This is a measure of the slower bands, which represent dimethylated oligonucleotide products. The mobility of a DNA fragment through a gel is lowered as the distance between its two ends decreases and this most commonly arises because of DNA bending. Furthermore, the more centrally located the bend the smaller the end to end distance and, in consequence, the greater the retardation on gels (Wu and Crothers, 1984; Hagerman, 1990; Crothers et al., 1991). The results shown in Figs. 4A and B are therefore consistent with the methylase bending of the DNA.

It is possible to calculate the degree of bending using the empirical equation described by Thompson and Landy (1988), in which the mobility of fragments containing the bend at the center and at the end on the molecules are compared. The fragments we have used do not contain an exactly centered GATATC site but nevertheless comparing the two most central sites (Fig. 1, fragments 3 and 4) with the two most peripheral (fragments 1 and 6) gave a bend angle of 62 ± 3° (number of determinations = 4). This angle was produced regardless of which combination of the two central and peripheral sites were used to evaluate μM and μE. An angle of 62° was found for both the faster, substrate, complexes and the slower, product, com-

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² S. Cal and B. A. Connolly, unpublished observations.
plexes. This demonstrates that the differences in mobility between enzyme/un- and hemimethylated DNA complexes and those that comprise enzyme/dimethylated DNA cannot be due to a change in DNA bending.

As a positive control we have used the binding of the very well characterized EcoRV endonuclease to the DNA fragments, in the presence of Ca²⁺. The results are also shown in Fig. 4, A and B, and quantitative analysis, exactly as above, gave a bending angle of 47 ± 3° (number of determinations = 4). The slower migration of the endonuclease-DNA complexes compared to those of the methylase-DNA are consistent with the different molecular weights of the two proteins; endonuclease 58 kDa (comprised of a dimer of subunit molecular weight 29 kDa), methylase a monomer of 32 kDa. Using the same oligonucleotides, Vipond and Halford (1995) reported a bend angle of 53° ± 4° and with a different set of oligonucleotides, Stöver et al. (1993) found an angle of 44 ± 4°. A figure of 55° has been seen by crystallography (Winkler et al., 1993; Kostrewa and Winkler, 1995). The similarity of the results we obtain with the endonuclease, to those seen by several investigators, validates the data and conclusions drawn with the relatively uncharacterized methylase.

We have confirmed that the EcoRV methylase bends DNA by using a second set of oligonucleotides, shown in Fig. 2. Once again in this set of experiments N-methyl-AdoMet was used as cofactor. One of the four 150-bp oligonucleotides (fragment 1) contains an exactly center GATATC sequence, and in the others this is moved toward the end of the DNA. Using G6-Me-FIG. 2. The production of 150-bp fragments containing either an un- or a hemimethylated EcoRV site at various distances along the oligonucleotide. These were prepared by PCR amplification of part of a DNAse I gene (shown in bold type) containing a single GATATC site (highlighted). Pairing of the short forward (→) primers (containing only normal bases) 1, 2, 3, and 4 with the reverse (←) primers 1, 2, 3, and 4, respectively, gave the fragments 1, 2, 3, and 4 which contain an unmethylated EcoRV site at the locations shown. Pairing of the longer forward (→) primers (containing a single 6-methyladenosine, shown by an asterisk (*)) 1, 2, 3, and 4 with the reverse (←) primers 1, 2, 3, and 4 gave the fragments 1, 2, 3, and 4, containing a hemimethylated EcoRV site at the positions illustrated. All primers are shown in italic type. The results found with these oligonucleotides are shown in Fig. 5.

FIG. 3. The DNA fragments used for phasing analysis. These were prepared by inserting an EcoRV site between the EcoRI and Xml sites of the five phasing plasmids described by Withers and Dunbar (1993). This gives the five plasmids shown, each of which contains a GATATC site at a defined distance from a poly(dA) tract. These distances, measured from the center of the EcoRV site (between the thymidine and deoxyadenosine bases) to the middle deoxyadenosine of the poly(dA) tract are given. The five plasmids were then amplified by PCR to give the 206-bp fragments illustrated which were used to confirm DNA bending by the EcoRV methylase and gave the results shown in Fig. 6.
over 60°. It is interesting that the hemimethylated oligonucleotide, and dimethylated) bend DNA to the same extent, just methylase-nucleic acid complexes (unmethylated, hemimethylated, and dimethylated) DNA) was poor, and the leading edges have been used to evaluate fragment mobility. By analogy with the above experiment we assume that the bands seen in Fig. 5, using the unmethylated oligonucleotides, are actually a mixture of un-, hemi-, and dimethylated species. Once again we observe that the more central the location of the EcoRV site, and hence any bend induced on methylase binding, the more retarded the complex runs on gels (Fig. 5, A and B). Quantitation using the Thompson-Landy equation and fragments 1 (center) and 4 (end) gives a bend angle of 60 ± 3° (four determinations) in excellent agreement with the values produced above. When samples of these unmethylated fragments were reslated, following a normal pre-gel shift incubation with the methylase, they could be gel-shifted by the endonuclease (not shown). This confirms that at least some of the DNA remains fully unmethylated, because hemimethylated species are not gel-shifted by the endonuclease (see later). The EcoRV endonuclease was again used as a positive control, and the data for the unmethylated oligonucleotides shown in Fig. 5, A and B, give a bend angle of 46 ± 3°. Almost identical results were seen when gel shifts were carried out with the methylase and hemimethylated oligonucleotides (Fig. 5, A and B), and the bend angle found was 61 ± 3°. In this instance the material in the shifted band will consist of hemi- and dimethylated DNA, and unmethylated material cannot be present. These experiments confirm the bending angle determined above but also show that methylase/un- and hemimethylated DNA complexes have the nucleic acid bent to the same degree. Thus all three possible methylase-nucleic acid complexes (unmethylated, hemimethylated, and dimethylated) DNA) were not gel-shifted by the endonuclease (not shown). Under the conditions used, i.e. in the presence of Ca²⁺, an analogue of the normal cofactor Mg²⁺, the endonuclease binds specifically to GATATC sequences and rejects all others (Vipond and Halford, 1995). The function of methylation is to protect the host DNA from endonuclease action, and the lack of binding of hemimethylated (and presumably dimethylated) DNA (in the presence of cations, presumably facilitates this.

Confirmation of DNA Bending and Determination of Its Direction by Phasing Analysis—In the above discussion we have assumed that the retardation of centrally placed GATATC sequences on binding to the methylase is due to DNA bending. However, it is well known that DNA conformational effects other than bending can give rise to the gel mobilities of the sort observed above (Zinkel and Crothers, 1987; Gartenberg et al., 1990; Crothers et al., 1991). In order to discriminate between DNA bending and alternative conformational features and to determine the bend direction we have used phasing analysis. For this purpose, the data for the unmethylated fragments shown in Fig. 3 were used. These contain a GATATC site phased (i.e. separated over a helical turn) with an intrinsically curved poly(dA) tract. The results seen with these fragments and both the methylase and endonuclease (which once again acted as a positive control) are shown in Fig. 6. In these experiments sinefungin, rather than N-methyl-AdoMet was used as cofactor. From the gels (Fig. 6A) and their associated graphical representations (Fig. 6B) it can be seen that, for both enzymes, the mobility of the DNA-enzyme complex is dependent on the spacing between the
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**DISCUSSION**

When the EcoRV methylase binds to its GATATC target site, the DNA is bent toward the major groove by just over 60°. The degree of bending is identical, irrespective of whether the GATATC site is un-, hemi-, or dimethylated. The hemimethylated species is the true physiological substrate for the enzyme although the protein is also capable of de novo CH$_3$ group addition to unmethylated sequences. Dimethylated oligonucleotides represent enzyme-product complexes. The different gel mobility of the enzyme-substrate and enzyme-product complexes, observed here and previously (Szczelkun and Connolly, 1995), are most probably due to a protein conformational change, given that the bending of the DNA does not alter. The parallel, positive control experiments, carried out with EcoRV endonuclease, gave a bend angle of about 47°, in excellent agreement with earlier crystallographic (Winkler et al., 1993; Kostrewa and Winkler, 1995) and solution studies (Stöver et al., 1993; Vi Gonz and Halford, 1995). It is also apparent that the two proteins, which recognize the same GATATC sequence, both bend DNA toward the major groove, although the methylase induces more curvature than does the endonuclease.

It has been extensively reported that the binding to DNA of a great number of proteins introduces curvature into the double helix. This is often observed with repressor/activator proteins that must bind specifically to particular DNA sequences (Wu and Crothers, 1984; Kim et al., 1989; Schultz et al., 1991; Aggarwal et al., 1988; Kerrola and Curran 1991a, 1991b) or enzymes, especially restriction endonucleases, that must catalyze a chemical transformation with high fidelity at their target sites (Thompson and Landy, 1988; Dou-Rasy et al., 1989; Rosenberg, 1991; Aiken et al., 1991; Stöver et al., 1993; Winkler et al., 1993; Vi Gonz and Halford, 1995; Kostrewa and Winkler, 1995; Withers and Dunbar, 1993). In all of these cases the bending of the DNA serves to increase the specificity of the proteins and to aid in the selection of target sequences and in the discrimination against noncognate ones. Proteins achieve selectivity for particular DNA sequences by a variety of mechanisms that include: 1) direct readout, where proteins recognize the bases (Seeman et al., 1976) and 2) indirect readout, where a particular, nontypical, DNA structure is recognized (Otwinowski et al., 1988; Matthews, 1988; Brennan and Matthews, 1989). It is known that many DNA sequences have intrinsic curvature or other distortions, and it is thought that this can be used in the recognition process (Heitman, 1992). Thus it has been suggested that the GAATTC EcoRI recognition sequence is intrinsically distorted and that this is enhanced on binding to the EcoRI restriction endonuclease (Thomas et al., 1989). A variant on this theme is the concept of a DNA sequence that is easy to bend or distort. Here a bendable cognate sequence can easily adapt to a complementary protein binding site, whereas stiff noncognate sequences cannot. This seems to occur with DNase I, where rigid poly(dA)poly(dT) tracts are poorly cut, whereas more flexible sequences are able to adopt the bound, bent, conformation and so are better substrates (Drew and Travers, 1984; Suck et al., 1988; Weston et al., 1992). It should be recognized that bending of DNA also serves to correctly line up the protein and nucleic acid partners and so enhance protein-DNA interaction. Thus direct and indirect readout are intimately linked. Formation of interactions between the bases and the protein may produce DNA curvature, but equally the ability to adopt a bent conformation may enhance favorable contacts between the two macromolecules. Finally, although DNA bending is common with proteins that act on specific DNA sequences, it is not universal. Thus certain repressors, e.g. the λ repressor (Jordan and Pabo, 1988), the 434 Cro protein (Wolberger et al., 1988) and the PvuII restric-

**Fig. 6.** Gel retardation of DNA fragments containing a GATATC site at varying separations from a poly(dA) tract. A, autoradiographs of the gels obtained. The lanes 1-5 correspond to the fragments 1-5 shown in Fig. 3. Two bands are once again visible for the methylase and the endonuclease, the maximum electrophoretic mobility, corresponding to the least flexibility, of which are consistent with the results seen in the preceding experiments. B, Gel mobility of the enzyme-substrate and enzyme-product complexes. The different gel mobility of the enzyme-substrate and enzyme-product complexes, observed here and previously (Szczelkun and Connolly, 1995), are most probably due to a protein conformational change, given that the bending of the DNA does not alter. The parallel, positive control experiments, carried out with EcoRV endonuclease, gave a bend angle of about 47°, in excellent agreement with earlier crystallographic (Winkler et al., 1993; Kostrewa and Winkler, 1995) and solution studies (Stöver et al., 1993; Vi Gonz and Halford, 1995). It is also apparent that the two proteins, which recognize the same GATATC sequence, both bend DNA toward the major groove, although the methylase induces more curvature than does the endonuclease.

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