Specific DNA Recognition by *Eco*RV Restriction Endonuclease Induced by Calcium Ions†

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ABSTRACT: In the presence of Mg^{2+}, the *Eco*RV restriction endonuclease cleaves DNA specifically at its recognition sequence, but in the absence of divalent metal ions, it binds DNA without any specificity: gel-shift experiments had revealed multiple *Eco*RV−DNA complexes, due to the binding of one, two, three, or more molecules of protein per molecule of DNA, with the same equilibrium constant for each association. In this study, the binding of *Eco*RV to DNA was measured by gel shift in the presence of Ca^{2+}, an ion that perturbs the Mg^{2+}-dependent activity of *Eco*RV but that fails to support DNA cleavage. With Ca^{2+}, and at a lower concentration of *Eco*RV protein than that required for binding in the absence of divalent metal ions, a single complex was observed with DNA containing the *Eco*RV recognition site. This complex was not formed with DNA that had been methylated at the *Eco*RV site nor with an isogenic DNA lacking the *Eco*RV recognition site. The single complex thus is due to the specific binding of *Eco*RV to its recognition site on the DNA. From gel shifts with a permuted set of DNA fragments, the degree of DNA bending by *Eco*RV at its recognition site was estimated to be 53° ± 4°. This angle is similar to that seen in the crystal structure of the cognate DNA−protein complex. Calcium ions thus appear to mimic the role of Mg^{2+} in generating a specific protein−metal−DNA complex, but in contrast to Mg^{2+}, Ca^{2+} gives a stable ternary complex in which the DNA-bound nuclease cannot cleave the DNA.

In the presence of Mg^{2+}, type II restriction endonucleases cleave DNA at their respective recognition sequence with extremely high specificities (Roberts & Halford, 1993). DNA sequences that differ from the recognition site by just 1 bp[1] typically are cleaved over a million times more slowly than the cognate site (Taylor & Halford, 1989; Lesser et al., 1990; Thielking et al., 1990). In the absence of divalent metal ions, restriction enzymes can bind to DNA to form stable DNA−protein complexes, but these complexes lack nuclease activity (Roberts & Halford, 1993). Under these conditions, some restriction enzymes, such as *Eco*RI, *Rsr*I, *Bam*HI, and *Fok*I, bind their recognition sites much more strongly than any other sequence (Halford & Johnson, 1980; Terry et al., 1983; Aiken et al., 1991; Xu & Schildkraut, 1991; Waugh & Sauer, 1993). However, a number of other restriction enzymes, including *Eco*RV, *Taq*I, *Cfr*9I, and *Bcg*I, bind equally well to DNA containing or lacking their recognition sites (Taylor et al., 1991; Zebala et al., 1992; Siksnys & Pleckaityte, 1993; Kong et al., 1994).

For *Eco*RV, the apparent discrepancy between DNA binding without sequence specificity and DNA cleavage with extreme specificity can be explained by the different affinities of the *Eco*RV−DNA complexes for Mg^{2+} ions, depending on whether the protein is bound to cognate or noncognate DNA (Halford et al., 1993; Vipond & Halford, 1993). At the cognate site for *Eco*RV, GATATC (Schildkraut et al., 1984), the kinetics of DNA cleavage, as a function of the concentration of $MgCl_2$, demonstrates that the *Eco*RV protein has a high affinity for Mg^{2+} ions. In contrast, the kinetics for the reaction of *Eco*RV at a noncognate site, GTTATC, indicates a very low affinity for Mg^{2+} (Taylor & Halford, 1989). The different affinities for Mg^{2+} can be correlated to the crystal structures of the *Eco*RV enzyme bound to either specific or nonspecific DNA (Winkler et al., 1993). The DNA in the specific complex has a 55° kink in its helical axis, while the DNA in the nonspecific complex retains a B-like structure. The distortion of the specific DNA inserts the phosphodiester group at the scissile bond into the active site of the enzyme, where it forms part of a binding site for Mg^{2+}, while the lack of distortion in the nonspecific DNA leaves it too far away from the active site to coordinate Mg^{2+} (Winkler et al., 1993; Kostrewa & Winkler, 1995).

Given the first law of thermodynamics, one consequence of the preceding mechanism is that Mg^{2+} must increase the affinity of the *Eco*RV endonuclease for its recognition site on DNA (Taylor et al., 1991). However, equilibrium binding studies on *Eco*RV cannot be carried out in the presence of Mg^{2+} because the DNA will be cleaved. One strategy to circumvent this problem has been the use of a mutant of *Eco*RV with no catalytic activity, D90A (Selent et al., 1992). In the absence of Mg^{2+}, this mutant bound DNA in the same manner as wild-type *Eco*RV, with no preference for specific over nonspecific sequences, but in the presence of Mg^{2+}, it bound preferentially to the *Eco*RV site (Thielking et al., 1992). Moreover, in the specific DNA−protein complex of D90A with Mg^{2+}, the DNA was bent by 44° (Stöver et al., 1993), an angle similar to that seen in the crystal structure with wild-type *Eco*RV (Winkler et al., 1993; Kostrewa & Winkler, 1995). However, a potential handicap to this strategy is that the mutation must destroy catalytic activity without destroying DNA-binding activity, and thus it needs

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[1] Abbreviations: bp, base pair(s); BME, β-mercaptoethanol; BSA, bovine serum albumin; CAP, catabolite-gene activator protein; EDTA, ethylenedinitrilotetraacetic acid; K_D, equilibrium dissociation constant.

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to be made at a key residue for catalysis. Consequently, the position of Mg^{2+} in the mutant protein may differ from that in the wild-type protein. This caveat is particularly acute for the D90A mutant of *Eco*RV since Asp90 is now known to be one of the residues that binds Mg^{2+} (Kostrewa & Winkler, 1995).

We describe here an alternative strategy for equilibrium binding studies on a Mg^{2+}-dependent nuclease: the use of Ca^{2+} ions in place of Mg^{2+}. Restriction enzymes generally have zero or near-zero activities with Ca^{2+} as the cofactor. For example, the activities of both *Eco*RI and *Eco*RV are least 1×10^5 times lower with Ca^{2+} than with either Mg^{2+} or Mn^{2+} (Vipond et al., 1994). However, the inability of Ca^{2+} to support DNA cleavage is not due to the exclusion of this ion from the active site of these enzymes. For *Eco*RI, Ca^{2+} inhibits the reactions with either Mg^{2+} or Mn^{2+} as cofactor, in a manner consistent with a simple competitive displacement of the active metal ion (Vipond et al., 1995). For *Eco*RV, Ca^{2+} inhibits specific DNA cleavage in the presence of Mg^{2+}, but despite giving no activity by itself, it enhances the Mn^{2+}-dependent reaction. The enhancement is probably due to the facts that *Eco*RV requires two metal ions per active site, in contrast to *Eco*RI which requires only one (Rosenberg, 1991), and that the $E-Ca^{2+}-Mn^{2+}$ complex is more active than the $E-Mn^{2+}-Mn^{2+}$ complex (Vipond et al., 1995; Baldwin et al., 1995).

EXPERIMENTAL PROCEDURES

Proteins. The *Eco*RV endonuclease was purified as described by Luke et al. (1987). The concentration of *Eco*RV is given in terms of the dimeric protein of M_r 57 000. All other enzymes were from commercial suppliers and used as recommended by the supplier.

DNA. Preparations of the 381 bp *Eco*RI–*Bam*HI fragment from pAT153 (Twigg & Sherratt, 1980) and the 389 bp *Eco*RI–*Bam*HI fragment from pAT153b (Taylor & Halford, 1989) were as described previously (Taylor et al., 1991). [The former fragment carries the *Eco*RV site from pAT153, while the latter has an 8 bp insertion that disrupts the *Eco*RV site.] Both fragments were labeled by using Klenow polymerase to fill in their 5′-extensions with [α-^{32}P]dATP and the other three dNTPs (Sambrook et al., 1989) to give flush-ended fragments with lengths as indicated. For one set of experiments, the 381 bp fragment was prepared from DNA isolated from *Escherichia coli* HB101 that had been transformed with both pAT153 and pMetB. The latter plasmid encodes the *EcoRV* modification methyltransferase (Vermote et al., 1992).

An additional set of DNA fragments, all of 141 bp, were prepared by digesting a derivative[2] of pBend3 (Zwieb & Adhya, 1994) with either *Eco*RI–*Sal*I, *Bgl*II, *Spe*I, *Xho*I, *Dra*I, *Sma*I, *Stu*I, *Bgl*I, *Nru*I, *Ssp*I, *Bam*HI, or *Xba*I–*Hin*dIII. The fragments were purified by electrophoresis through acrylamide and labeled by using first calf intestinal alkaline phosphatase and then polynucleotide kinase with [γ-^{32}P]ATP (Sambrook et al., 1989). [pBend3 contains two copies of a polylinker sequence in tandem repeat either side of unique *Xba*I and *Sal*I sites (Zwieb & Adhya, 1994). The polylinker has an *Eco*RV site so that the fragments all contain one *Eco*RV site but at different positions along the DNA. For example, the 141 bp fragments produced by *Dra*I, *Bam*HI, and *Sma*I have the *Eco*RV site centered 6, 89, and 129 bp, respectively, from the left-hand end.]

Gel-Shift Assays. Binding reactions (10 μL) contained the ^{32}P-labeled DNA fragment (final concentration about 10 pM) and *Eco*RV endonuclease (final concentration in the range 0–100 nM, diluted to the required concentration in binding buffer containing 1 mM spermine; Halford & Goodall, 1988). The reactions were done in either EDTA-binding buffer (50 mM Tris·HCl, 100 mM NaCl, 10 mM BME, 100 μg/mL BSA, and 0.1 mM EDTA (pH 7.5); Taylor et al., 1991) or Ca^{2+}-binding buffer (the same supplemented with 5 mM $CaCl_2$). After 15 min at room temperature, loading buffer (5 μL) was added to each sample, and the samples were applied immediately to a 6% polyacrylamide gel (37.5:1 acrylamide/bis) running at 3 V/cm, after which the voltage across the gel was increased to 11 V/cm (Taylor et al., 1994). The loading buffer was the same as the buffer used for the binding reaction, with either EDTA or $CaCl_2$, augmented in both cases with 40% (v/v) glycerol and 0.01% (w/v) bromophenol blue. For reactions in EDTA-binding buffer, the polyacrylamide gel was prepared and run in 0.089 M Tris base, 0.089 M boric acid, and 2 mM EDTA. The same gel buffer was used for reactions with the Ca^{2+}-binding buffer, except that the EDTA was replaced with 5 mM $CaCl_2$. The gels were prerun for 2 h at 11 V/cm before the samples were loaded. After electrophoresis, the gels were fixed in 1:1:8 (v/v) acetic acid/methanol/water and dried. The radioactivity on the gel was measured with a 400B PhosphorImager and analyzed by ImageQuant software (both from Molecular Dynamics, Sunnyvale, CA).

For some reactions noted in the following, the concentration of $CaCl_2$ in the Ca^{2+}-binding buffer was varied from 5 mM. In these cases, the same alteration to the concentration of $CaCl_2$ was also made to both the loading buffer and the electrophoresis buffer.

RESULTS

DNA Binding by EcoRV with Ca^{2+}. The effect of Ca^{2+} ions on the binding of the *Eco*RV restriction endonuclease to DNA was examined by the gel-shift method (Fried & Crothers, 1981). Two DNA fragments were used: a 381 bp fragment containing one *Eco*RV site and a 389 bp fragment lacking an *Eco*RV site. The only difference between the two fragments is that the latter contains an 8 bp insertion at the middle of the *Eco*RV site. Hence, if the *Eco*RV endonuclease were to bind specifically to its recognition site in the presence of Ca^{2+} ions, and if the resultant complex had no DNA cleavage activity, then a titration of the 381-mer with increasing concentrations of *Eco*RV should yield initially just one stable DNA–protein complex, with perhaps additional complexes being formed at higher protein concentrations due to nonspecific binding elsewhere on the DNA. The 389 bp fragment cannot produce a specific complex, but it should generate the same nonspecific complexes as the 381-mer.

[2] The derivative of pBend3 used here (K. Gaston, personal communication) carried a 20 bp *Xba*I–*Sal*I insert with the same sequence as the P5 + 1 binding site for the transcription factor YY1 (Shi et al., 1991). This allowed for a positive control on the same set of DNA fragments: the reduction in electrophoretic mobility caused by the binding of YY1 was greatest when the P5 + 1 site was located at the center of the fragment, and it was smallest when the site was at the end of the fragment (data not shown; K. Gaston, unpublished).

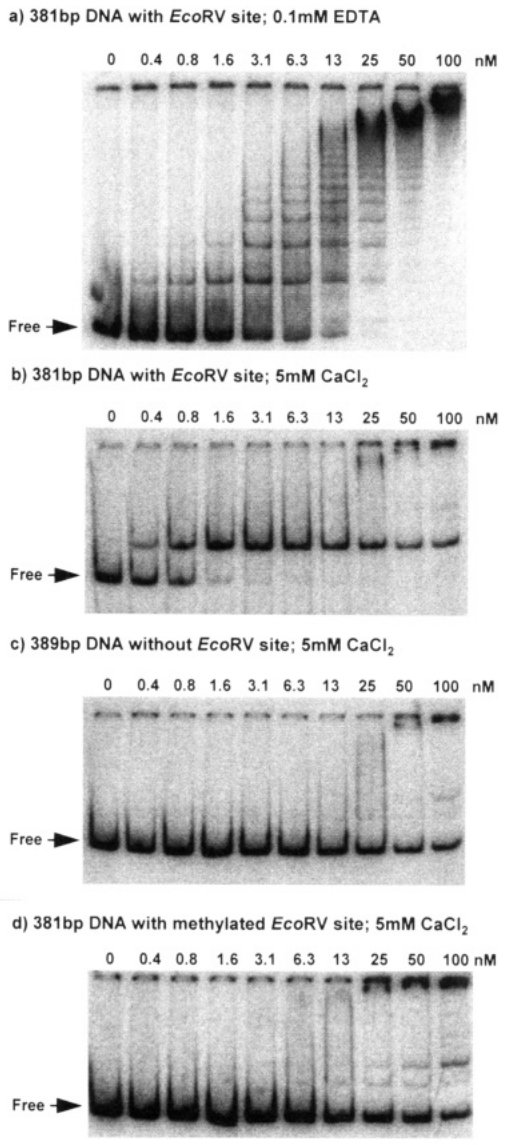

FIGURE 1: Binding of *Eco*RV to specific and nonspecific DNA, with or without Ca^{2+} ions. The binding reactions contained ^{32}P-labeled DNA at about 10 pM and *Eco*RV endonuclease at the concentrations (nM) noted above each lane of the gels (the lanes marked 0 had no enzyme). The DNA was the 381 bp fragment with an unmodified *Eco*RV site (panels a and b), the isogenic 389 bp fragment lacking the *Eco*RV site (panel c), or the 381 bp fragment methylated at the *Eco*RV recognition site (panel d). The reactions were done in either EDTA-binding buffer (a) or Ca^{2+}-binding buffer with 5 mM $CaCl_2$ (b–d). After electrophoresis through polyacrylamide in the presence of either EDTA (for a) or 5 mM $CaCl_2$ (for b–d), the gels were analyzed on a Phosphorimager and the phosphorescence records are shown here. An arrow on the left side of each gel marks the position of free DNA.

To act as a control for the effects of Ca^{2+}, we repeated the gel-shift experiments that had been done previously to measure the binding of *Eco*RV to DNA in the absence of divalent metal ions (Taylor et al., 1991). Increasing concentrations of the *Eco*RV endonuclease were added to the specific 381 bp fragment, and the free DNA was then separated by electrophoresis from the DNA complexed with protein. This revealed multiple forms of the DNA with progressively retarded electrophoretic mobilities (Figure 1a). The multiple forms of the DNA correspond to complexes with 1, 2, 3, ..., n molecules of *Eco*RV bound per molecule of DNA, where n is the maximum that can fit onto the DNA (Taylor et al., 1991). When bound to DNA, the *Eco*RV protein covers about 15 bp, in agreement with the crystal structures of *Eco*RV–DNA complexes (Winkler et al., 1993; Kostrewa & Winkler, 1995). A DNA molecule of 381 bp contains 367 potential sites for a ligand that occludes 15 bp (from $N - l + 1$, where N and l are the lengths of the DNA and the ligand, respectively; McGhee & von Hippel, 1974), but the overlaps between the sites mean that a maximum of 25 sites can be occupied at any one time (from $n = N/l$). Gel-shift experiments on the binding of *Eco*RV to the nonspecific 389 bp fragment revealed the same series of complexes in the same yield at each concentration of protein tested (data not shown). In the absence of divalent metal ions, the *Eco*RV endonuclease thus binds to DNA without any detectable preference for its recognition site over any other site on the DNA. This confirms numerous studies on the binding of *Eco*RV to DNA under similar conditions, carried out previously with a variety of DNA fragments from 14 to 400 bp (Taylor et al., 1991; Selent et al., 1992; Thielking et al., 1992; Vermote et al., 1992; J. Alves & A. Pingoud, personal communication; M. Szczelkun & B. Connolly, personal communication).

When the *Eco*RV endonuclease was mixed with the 381-mer in the presence of $CaCl_2$ and the DNA was then analyzed by electrophoresis through polyacrylamide, no cleavage of the DNA was detected, even though this fragment contains an *Eco*RV site. However, the electrophoretic mobility of the DNA was reduced (Figure 1b). In contrast to the multiple complexes seen with this DNA in the absence of Ca^{2+} (Figure 1a), a titration of the 381-mer with increasing concentrations of *Eco*RV in the presence of Ca^{2+} resulted in virtually all of the DNA being initially converted into just one DNA–protein complex (Figure 1b). As the concentration of the enzyme was raised further, the amount of the initial complex decreased progressively, but in this reaction at 5 mM $CaCl_2$, the decrease was not accompanied by the formation of a set of additional complexes that could be resolved from each other. Instead, a heterogeneous smear of DNA was observed on the gels between the initial complex and the loading wells (Figure 1b). At the highest concentrations of *Eco*RV tested, the majority of the DNA failed to enter the gel and remained in the well.

Specificity of Binding. The gel-shift technique was also used to monitor the binding of *Eco*RV to the nonspecific 389-mer in the presence of $CaCl_2$ (Figure 1c). This DNA fragment failed to give the initial complex that had been seen with the specific 381 bp DNA (Figure 1b). However, at high concentrations of the protein, the amount of the free form of the 389-mer decreased progressively with increasing concentrations of *Eco*RV and, simultaneously, a smear of DNA appeared on the gels, running between the free form and the top of the gel (Figure 1c). The concentration of *Eco*RV required to convert the free form of the 389-mer to a smear was the same as that needed to convert the initial complex on the 381-mer to a smear.

To test whether DNA methylation at the *Eco*RV site would perturb binding by *Eco*RV, the specific 381-mer was purified from a sample of pAT153 that had been co-transformed into *E. coli* with a plasmid that encodes the *Eco*RV modification methyltransferase, pMetB (Vermote et al., 1992). The latter plasmid expresses the methyltransferase from its native promoter: this results in the *Eco*RV modification enzyme methylating all of the *Eco*RV sites in the cellular DNA but giving no detectable methylation at other sites (Taylor et al.,

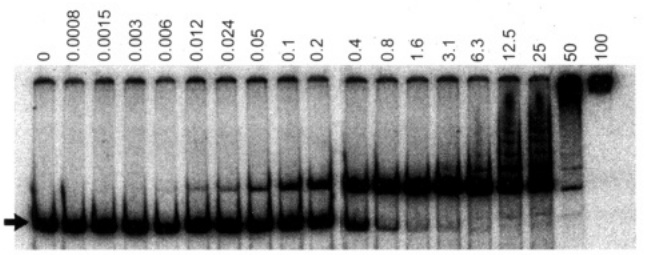

FIGURE 2: DNA binding by *Eco*RV in 1 mM $CaCl_2$. The binding reaction, in Ca^{2+}-binding buffer with 1 mM $CaCl_2$, contained the 381 bp DNA fragment at about 10 pM and *Eco*RV endonuclease at the concentrations (nM) noted above each lane of the gel (the lane marked 0 had no enzyme). After electrophoresis of the samples through polyacrylamide gels containing 1 mM $CaCl_2$, the gels were analyzed on a Phosphorimager. An arrow on the left side of the gel marks the position of free DNA.

1990). Methylation of alternative sites in vivo is seen only after overproduction of the enzyme (Nwosu et al., 1988; Taylor et al., 1990). Thus, the only difference between this form of the 381-mer and that used above will be methylation at the *Eco*RV site: no other site on the DNA is likely to have been methylated. In control experiments, the methylated form of pAT153 was resistant to cleavage by the *Eco*RV endonuclease, while the unmethylated form was readily cleaved (data not shown).

When the binding of *Eco*RV to the methylated 381-mer was measured by gel shift in the absence of divalent metal ions, the results (not shown) were identical to those with the unmethylated form of this DNA; again, a series of retarded complexes was observed due to the binding of 1, 2, 3, ..., *n* molecules of protein per molecule of DNA (i.e., as in Figure 1a). However, the methylated and unmethylated forms of the 381-mer behaved differently from each other in the presence of $CaCl_2$: the methylated DNA (Figure 1d) failed to give the single complex with *Eco*RV that had been found with the unmethylated DNA (Figure 1b). The 381 bp DNA methylated at the *Eco*RV site (Figure 1d) bound *Eco*RV in the presence of Ca^{2+} in the same way as the 389 bp DNA lacking an *Eco*RV site (Figure 1c).

Quantitative Analysis. The binding of *Eco*RV to the 381-mer was analyzed further by varying the concentration of $CaCl_2$ in the reactions from 1 mM (Figure 2) to 10 mM (results as in Figure 1b; data not shown). At each concentration of $CaCl_2$, the binding was examined across a wide range of concentrations of *Eco*RV endonuclease (Figure 2). Both these data and those from parallel gel-shift experiments on the binding of *Eco*RV to the 381-mer in the absence of Ca^{2+} (as in Figure 1a) were analyzed quantitatively by using the Phosphorimager to measure the radioactivity from the ^{32}P-labeled DNA; the amounts of the free DNA remaining at each concentration of *Eco*RV were determined (Figure 3a), as were the amounts of the first retarded complex (Figure 3b). The quantitative analysis revealed that the binding of *Eco*RV to DNA in the presence of Ca^{2+} differed from that in the absence of Ca^{2+} in three major respects, but only one of these three depended on the concentration of Ca^{2+} ions.

The first difference was that the concentration of *Eco*RV enzyme required to convert the free DNA to complex(es) was lower in the presence of Ca^{2+} than in its absence: this was observed at all concentrations of $CaCl_2$ tested (Figure 3a). At either 1, 5, or 10 mM $CaCl_2$, about half of the free DNA had been converted to the initial complex at 0.2 nM *Eco*RV. If the initial complex is due to the binding of

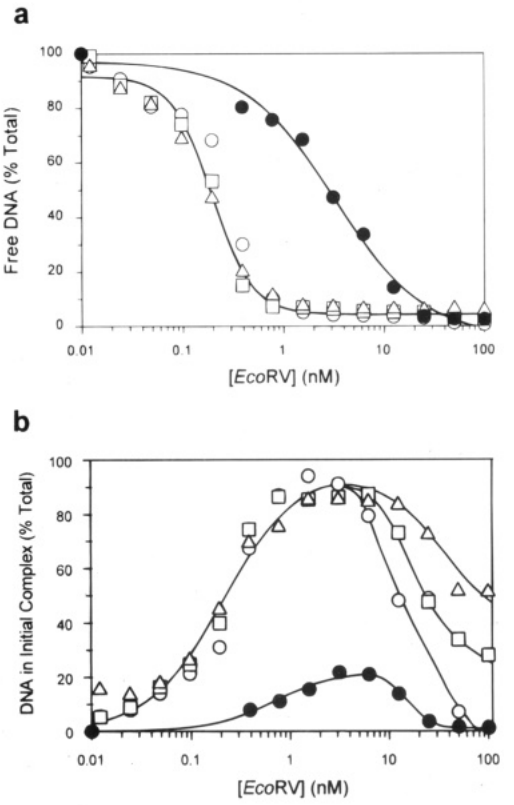

FIGURE 3: Quantitative analysis. The binding of varied concentrations of the *Eco*RV restriction endonuclease to the 381 bp DNA fragment (^{32}P-labeled) was analyzed by gel shift, as shown in Figure 2, in either EDTA-binding buffer or Ca^{2+}-binding buffer containing varied concentrations of $CaCl_2$. Electrophoresis for each set of samples was carried out with the same concentration of $CaCl_2$ as in the binding reaction. For each lane on the gel, the amounts of the free DNA and the DNA in the first retarded complex were determined as a percent of the total amount of DNA in that lane, by measuring with the Phosphorimager the radioactivity from the appropriate areas of the gels. The amount (% of total) of the free DNA at each concentration of *Eco*RV is shown in panel a; the amount (% of total) of the first retarded complex is in panel b. In both panels, the concentration of *Eco*RV (nM) is given on a logarithmic scale; binding reactions in EDTA (●) and 1 (○), 5 (□), and 10 mM $CaCl_2$ (△).

*Eco*RV to a single site on the DNA, this corresponds to a K_D value of about 0.2 nM for that particular site; this value is independent of Ca^{2+} concentrations ≥ 1 nM. In the absence of Ca^{2+}, the concentration of *Eco*RV required to convert one-half of the free DNA to complexes was about 2.5 nM (Figure 3a). But in the absence of divalent metal ions, *Eco*RV appears to be able to bind equally well to any of the 367 potential sites on the 381 bp DNA fragment (Taylor et al., 1991). The amount of *Eco*RV needed for binding to half of the DNA molecules thus translates to a K_D value of 0.9 μM for the binding of *Eco*RV to any one site on the DNA. This value agrees closely with that from previous determinations under the same reaction conditions, 0.8 μM (Taylor et al., 1991).

Secondly, the amount of the first DNA−protein complex produced by titrating the DNA with *Eco*RV in the presence of Ca^{2+} reached a maximal level of about 90% of the input DNA: the same level was observed, within experimental error, at each concentration of $CaCl_2$ tested (Figure 3b). However, among the multiple complexes formed from the DNA in the absence of Ca^{2+} (Figure 1a), no more than 20% of the DNA was ever converted to the first retarded complex,

the one with one molecule of protein bound to the DNA (Figure 3b).

Thirdly, at all three concentrations of $CaCl_2$ tested and in the absence of $CaCl_2$, the amount of the initial complex decreased progressively from its maximum value as the concentration of *Eco*RV endonuclease was raised to high levels, but the steepness of this decline depended on the concentration of $CaCl_2$: the steepest decline was with 1 mM $CaCl_2$, and it became progressively more shallow with increasing concentrations of $CaCl_2$ (Figure 3b). Moreover, while the decrease in the amount of the initial complex in binding reactions at 5 (or 10) mM $CaCl_2$ was accompanied by the appearance of a smear of DNA on the gels running above the first complex (Figure 1b), the decrease at either 0 or 1 mM $CaCl_2$ was accompanied by the appearance of a large number of discrete bands on the gel with incrementally reduced mobilities (Figures 1a and 2).[3] High concentrations of Ca^{2+} ions thus seem to weaken nonspecific binding, as judged by the conversion of the initial complex to the subsequent additional complexes at high protein concentrations and by the stability of the additional complexes.

DNA Bending. The strategy of Wu and Crothers (1984) was employed to determine whether the initial binding of *Eco*RV endonuclease to a DNA with an *Eco*RV site induces a bend in the DNA. A permuted series of DNA fragments, each of 141 bp, was prepared by digesting a derivative of pBend3 (Zwieb & Adhya, 1994) with different restriction enzymes (see Experimental Procedures). Each fragment contained an *Eco*RV site, but this was located at different positions along the DNA: the distance from the center of the *Eco*RV site to the left-hand end of the DNA varied from 6 to 129 bp. All of the fragments in the set had the same electrophoretic mobilities in the absence of *Eco*RV (data not shown). If an intrinsic bend had been located at one point in the 141 bp sequence, each fragment in the set would have had a different mobility: a fragment with a bend at the center should be less mobile than one with a bend close to the end of the DNA (Hagerman, 1990). Hence, it is unlikely that this sequence possesses any static curvature in its helical axis.

The series of fragments was used for gel-shift experiments on the binding of *Eco*RV in the presence of Ca^{2+} ions. The concentration of *Eco*RV endonuclease was adjusted so that a fraction of the DNA remained unbound while another fraction was converted to the first retarded complex with one molecule of *Eco*RV per molecule of DNA (data not shown). With each fragment, the distances travelled through the gel by the first retarded complex and by the free DNA were both measured, and the relative electrophoretic mobility of the initial complex was calculated from the ratio of the two distances. The relative electrophoretic mobility of the initial complex varied from fragment to fragment: the fragments carrying the *Eco*RV site close to the center had the least mobility, and the fragments with the *Eco*RV site at either end had the greatest mobility (Figure 4).

[3] The majority of the experiments described here employed the same concentration of $CaCl_2$ in the binding reaction, in the polyacrylamide gel, and in the electrophoresis buffer. But in some instances, binding reactions containing 5 mM $CaCl_2$ were loaded onto polyacrylamide gels lacking $CaCl_2$. These experiments yielded results similar to those in Figure 2 as opposed to those in Figure 1b, i.e., one initial complex formed at low concentrations of *Eco*RV followed by additional complexes at high protein concentrations that ran on the gel as a series of discrete bands rather than as a smear.

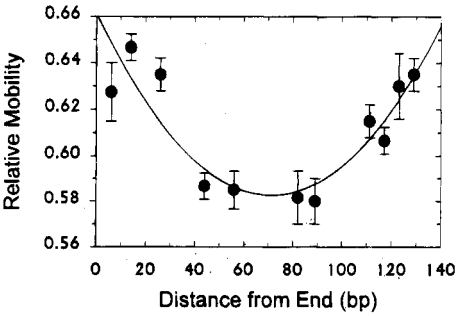

FIGURE 4: DNA bending. The binding of *Eco*RV restriction endonuclease to 141 bp DNA fragments was monitored by gel shifts in 5 mM $CaCl_2$. All of the fragments contained an *Eco*RV site; the number of base pairs between the center of the *Eco*RV site and the left-hand end of the fragment is shown on the *x*-axis. For each fragment, the distances that the free DNA and the first retarded complex migrated through the gel were measured. The relative electrophoretic mobilities that are given on the *y*-axis denote the ratio of the distance travelled by the first retarded complex over that for the free DNA. The values for relative electrophoretic mobility are averages from ≥3 separate experiments, and the error bars mark the standard deviations from the repeat experiments.

In the initial complex formed with a DNA containing an *Eco*RV site in the presence of Ca^{2+}, the DNA thus appears to be bent at (or close to) the *Eco*RV recognition site. The degree of bending (α) was estimated from the data in Figure 4 by using the empirical relationship of Thompson and Landy (1988),

$$\mu_M/\mu_E = \cos(\alpha/2)$$

where μ_M and μ_E are the mobilities for the DNA−protein complexes with the protein located at either the middle or the end of the DNA, respectively. This equation gave a value of 53° ± 4° for the bend angle, α. The margin of error in this estimate was calculated by averaging the standard deviations for the relative electrophoretic mobilities with each fragment and then applying this average to both μ_M and μ_E.

DISCUSSION

We have observed that the addition of *Eco*RV endonuclease and Ca^{2+} to a DNA molecule with an *Eco*RV recognition site results in the initial formation of a single DNA−protein complex (Figures 1b and 2), in contrast to the multiple complexes detected in reactions lacking divalent metal ions (Figure 1a). In the latter case, the distinct complexes are due to the binding of 1, 2, 3, ..., *n* molecules of the restriction enzyme to each molecule of DNA, with the same equilibrium constant for each association (Taylor et al., 1991). Consequently, the first molecule of *Eco*RV to bind to the DNA in the absence of divalent metal ions will not necessarily be located at the *Eco*RV recognition site. Instead, it could be located at any one of the large number of alternative (nonspecific) sites on the DNA. Indeed, the protein will probably be in a rapid dynamic equilibrium between the alternative sites. However, three lines of evidence indicate that the initial complex generated with Ca^{2+} is a specific complex with *Eco*RV bound at its recognition site on the DNA. Firstly, an equivalent complex was not formed with an isogenic DNA molecule, in which the only difference from the substrate DNA was an 8 bp insertion at the *Eco*RV recognition site (Figure 1c). Secondly, the formation of the initial complex on the DNA with an *Eco*RV

site was blocked by prior exposure of the DNA to the *Eco*RV modification methyltransferase in vivo (Figure 1d), under conditions where the only alteration to the DNA will be adenine methylation at the *Eco*RV site (Taylor et al., 1990). Thirdly, the bend generated by the binding of *Eco*RV with Ca^{2+} to DNA containing an *Eco*RV sites appears to be located at the *Eco*RV site (Figure 4).

The data in Figure 3 show that, in the presence of Ca^{2+}, the *Eco*RV enzyme binds its recognition site on a 381 bp DNA molecule with a K_D of about 0.2 nM. This value is similar to the K_m of 0.5 nM for the cleavage of plasmid substrates with Mg^{2+} as the cofactor (Taylor & Halford, 1989). In contrast, without Ca^{2+} in the binding reactions, the K_D value for any individual site on the 381-mer, including the recognition site, was estimated from the data in Figure 3 to be about 0.9 μM [see also Taylor et al. (1991)]. This 4500-fold increase in affinity for the recognition site was achieved by 1 mM $CaCl_2$; further increases in the concentration of $CaCl_2$ caused no further increase in affinity. The complex between the endonuclease and its recognition site therefore must have a high affinity for Ca^{2+} ions, so that 1 mM $CaCl_2$ is sufficient for saturation. Similarly, the maximal activity of *Eco*RV in cleaving plasmid substrates is achieved with 1 mM $MgCl_2$; further increases in the concentration of $MgCl_2$ cause no further increase in reaction rate (Halford & Goodall, 1988). These observations thus support the mechanism previously proposed for DNA recognition by *Eco*RV (Halford et al., 1993; Vipond & Halford, 1993). When the enzyme is located at its recognition site on the DNA, it has a high affinity for divalent metal ions, but when located at any other site, it has a very low affinity for metal ions. Consequently, the binding of metal ions to the *Eco*RV−DNA complex channels the protein to the recognition site. However, the metal ion in question can be either Mg^{2+}, in which case the enzyme−DNA complex proceeds to cleave the DNA, or Ca^{2+}, which leaves it as a stable ternary complex.

Prior to this study, specific binding of the *Eco*RV endonuclease to its recognition site on DNA had only been observed with a catalytically inactive mutant, D90A, in the presence of Mg^{2+} (Thielking et al., 1992). An alternative strategy with Mg^{2+} might appear to be an uncleavable DNA analog, with alterations to either the bases (Waters & Connolly, 1994), the phosphates (Grasby & Connolly, 1992) or the sugars (Hancox et al., 1993). But to date, no DNA analog has been found that binds to *Eco*RV better than nonspecific sequences (M. Szczelkun & B. Connolly, personal communication). Thus, the only current routes to a stable and specific ternary complex for *Eco*RV require either the protein or the metal ion to be altered to prevent catalysis. Moreover, DNA bending angles were determined for D90A with Mg^{2+} (Stöver et al., 1993) and for wild-type *Eco*RV with Ca^{2+} (Figure 4). They both yielded angles that were close to the value of 55° seen in the crystal structure (Winkler et al., 1993; Kostrewa & Winkler, 1995).

The role of Mg^{2+} in DNA binding by the D90A mutant remains to be determined since the mutant is unlikely to bind Mg^{2+} in the same manner as the wild-type protein. When crystals of the *Eco*RV endonuclease bound to its specific sequence were soaked with $MgCl_2$, the Mg^{2+} was located in the enzyme−DNA complex in between the carboxyl groups of Asp74 and Asp90 and one of the phosphoryl oxygens at the scissile bond in the DNA (Kostrewa & Winkler, 1995). A mutation at Asp90 thus is likely to perturb the binding of Mg^{2+} to the enzyme−DNA complex. However, soaking the crystals of the wild-type protein with $CaCl_2$ resulted in Ca^{2+} occupying the same site as Mg^{2+} in almost the same coordination geometry (Kostrewa & Winkler, 1995). The principal difference between the structures of the ternary complexes with either Mg^{2+} or Ca^{2+} is that a 0.8 Å shift in the DNA backbone caused by the binding of Mg^{2+} to the binary complex was not duplicated with Ca^{2+} (Kostrewa & Winkler, 1994). This may be part of the reason why Ca^{2+} fails to support DNA cleavage by *Eco*RV, but even with one Mg^{2+} ion at the active site, no DNA cleavage occurred within the crystals. Phosphodiester hydrolysis by *Eco*RV may require the binding of a second metal ion between Asp74 and Glu45 (Kostrewa & Winkler, 1995; Vipond et al., 1995; Baldwin et al., 1995).

At 1 mM $CaCl_2$ (Figure 2), high concentrations of *Eco*RV generated a series of additional DNA−protein complexes that could be resolved from each other by electrophoresis through polyacrylamide. These additional complexes are likely to be the DNA with one molecule of *Eco*RV at the recognition site and one, two, three, or more molecules of *Eco*RV bound elsewhere. However, at higher concentrations of $CaCl_2$ (Figure 1b), the additional complexes from the binding of *Eco*RV to nonspecific sites elsewhere on the DNA generally failed to run through the gel as discrete bands of DNA. Instead, they produced a heterogeneous smear of DNA. Gel-shift experiments with many DNA-binding proteins have revealed a ladder of discrete complexes from the nonspecific binding of one, two, three, or more molecules of protein per molecule of DNA: examples include, in addition to *Eco*RV under certain conditions (Figures 1a and 2), the *lac* repressor (Fried & Crothers, 1981) and CAP (Hudson et al., 1990). But gel-shift experiments on nonspecific DNA binding by many other proteins have yielded only smears of DNA: examples of the latter include, in addition to *Eco*RV under different conditions (Figure 1b−d), the *Taq*I endonuclease (Zebala et al., 1992) and the histone-like protein H-NS (Owen-Hughes et al., 1992).

The difference between these two types of behavior may be due to differences in the kinetics of association and dissociation of the protein from the DNA. If a DNA molecule carrying i molecules of protein is to remain as a single species throughout the electrophoresis, then any dissociation to give a DNA with $i - 1$ proteins must be followed by the immediate reassociation of the protein with the DNA before the two are physically separated from each other in the gel matrix. High concentrations of Ca^{2+} may inhibit the reassociation of *Eco*RV with DNA, so that each complex of the DNA with a given number of proteins at the start of the run gives rise to a series of complexes with fewer proteins during the run. Moreover, high concentrations of $CaCl_2$ weaken the nonspecific binding of *Eco*RV to DNA since the conversion of the single specific complex to additional complexes required progressively more *Eco*RV as the concentration of $CaCl_2$ was raised (Figure 3b). The effects of Ca^{2+} on both the kinetics and thermodynamics of nonspecific DNA binding by *Eco*RV may be simply a consequence of the neutralization of the negative charge on the DNA (Hagerman, 1990).

Given the impossibility of forming a ternary complex for *Eco*RV that is both kinetically competent and thermodynamically stable, the complex observed here between the endonuclease, its cognate DNA sequence, and Ca^{2+} ions is

perhaps the closest analog currently available. Moreover, the use of Ca^{2+} to mimic Mg^{2+} may be applicable to other enzymes involves in DNA metabolism. For example, the addition of Mg^{2+} to cocrystals of the *Eco*RI endonuclease with DNA resulted in the cleavage of the DNA: this permitted the determination of the structure of the enzyme−product−metal complex (Rosenberg, 1991). However, Ca^{2+} inhibits DNA cleavage by *Eco*RI (Vipond et al., 1994), so that the addition of Ca^{2+} instead of Mg^{2+} might reveal the structure of an enzyme−substrate−metal complex.

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REFERENCES

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

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

Fried, M., & Crothers, D. M. (1981) *Nucleic Acids Res. 9*, 6505−6525.

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

Hagerman, P. J. (1990) *Annu Rev. Biochem. 59*, 755−781.

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

Halford, S. E., & Goodall, A. J. (1988) *Biochemistry 27*, 1771−1777.

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

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

Hudson, J. M., Crowe, L. G., & Fried, M. G. (1990) *J. Biol. Chem. 265*, 3219−3225.

Kong, H., Roemer, S. E., Waite-Rees, P. A., Benner, J. S., Wilson, G. G., & Nwankwo, D. (1994) *J. Biol. Chem. 269*, 683−689.

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

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

Luke, P. A., McCallum, S. A., & Halford, S. E. (1987) *Gene Amplif. Anal. 5*, 183−205.

McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469−489.

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

Owen-Hughes, T., Pavitt, G., Sanyos, D., Sidebotham, J., Hulton, C., Hinton, J., & Higgins, C. (1992) *Cell 71*, 255−265.

Roberts, R. J., & Halford, S. E. (1993) in *Nucleases*, 2nd ed. (Linn, S. M., Lloyd, R. S., & Roberts, R. J., Eds.) pp 35−88, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Rosenberg, J. M. (1991) *Curr. Opin. Struct. Biol. 1*, 104−113.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schildkraut, I., Banner, C. D., Rhodes, C. S., & Parekh, S. (1984) *Gene 27*, 327−329.

Selent, U., Rüter, T., Köhler, E., Liedtke, M., Thielking, V., Alves, J., Oelgeschläger, T., Wolfes, H., Peters, F., & Pingoud, A. (1992) *Biochemistry 31*, 4808−4815.

Shi, Y., Seto, E., Chang, L.-S., & Shenk, T. A. (1991) *Cell 67*, 377−388.

Siksnys, V., & Pleckaityte, M. (1993) *Eur. J. Biochem. 217*, 411−419.

Stöver, T., Köhler, E., Fagin, U., Wende, W., Wolfes, H., & Pingoud, A. (1993) *J. Biol. Chem. 268*, 8645−8650.

Taylor, J. D., & Halford, S. E. (1989) *Biochemistry 28*, 6198−6207.

Taylor, J. D., Goodall, A. J., Vermote, C. L. M., & Halford, S. E. (1990) *Biochemistry 29*, 10727−10733.

Taylor, J. D., Badcoe, I. G., Clarke, A. R., & Halford, S. E. (1991) *Biochemistry 30*, 8743−8753.

Taylor, J. D., Ackroyd, A. J., & Halford, S. E. (1994) in *Methods in Molecular Biology, Vol. 30: DNA-Protein Interactions; Principles and Protocols,* (Kneale, G. G., Ed.) pp 263−279, Humana Press, Totowa, NJ.

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.

Thielking, V., Selent, U., Köhler, E., Landgraf, A., Wolfes, H., Alves, J., & Pingoud, A. (1992) *Biochemistry 31*, 3727−3732.

Thompson, J. F., & Landy, A. (1988) *Nucleic Acids Res. 16*, 9687−9705.

Twigg, A. J., & Sherratt, D. J. (1980) *Nature (London) 283*, 216−218.

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., Baldwin, G. S., & Halford, S. E. (1995) *Biochemistry 34*, 697−704.

Waters, T. R., & Connolly, B. A. (1994) *Biochemistry 33*, 1812−1819.

Waugh, D. S., & Sauer, R. T. (1993) *Proc. Natl. Acad. U.S.A. 90*, 9596−9600.

Winkler, F. K., Banner, D. W., Oefner, C., Tsernoglou, D., Brown, R. S., Heathman, S. P., Bryan, R. K., Martin, P. D., Petratos, K., & Wilson, K. S. (1993) *EMBO J. 12*, 1781−1795.

Wu, H.-M., & Crothers, D. M. (1984) *Nature (London) 308*, 509−513.

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.

Zwieb, C., & Adhya, S. (1994) in *Methods in Molecular Biology, Vol. 30: DNA-Protein Interactions; Principles and Protocols* (Kneale, G. G., Ed.) pp 281−294, Humana Press, Totowa, NJ.

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