*Eco*RV Restriction Endonuclease: Communication between Catalytic Metal Ions and DNA Recognition†

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ABSTRACT: In the absence of magnesium ions, the *Eco*RV restriction endonuclease binds all DNA sequences with equal affinity but cannot cleave DNA. In the presence of Mg^{2+}, the *Eco*RV endonuclease cleaves DNA at one particular sequence, GATATC, at least a million times more readily than any other sequence. To elucidate the role of the metal ion, the reactions of the *Eco*RV restriction enzyme were studied in the presence of $MnCl_2$ instead of $MgCl_2$. The reaction at the *Eco*RV recognition site was slower with Mn^{2+}. This was caused partly by reduced rates for phosphodiester hydrolysis but also by the translocation of the enzyme along the DNA after cleaving it in one strand. In contrast, alternative sites that differ from the recognition site by one base pair were cleaved faster in the presence of Mn^{2+} relative to Mg^{2+}. When located at an alternative site on the DNA, the *Eco*RV enzyme bound Mn^{2+} ions readily but had a very low affinity for Mg^{2+}. The *Eco*RV nuclease is thus restrained from cleaving DNA at alternate sites in the presence of Mg^{2+}, but the restraint fails to operate with Mn^{2+}. A discrimination factor, which measures the ratio of the activity of the *Eco*RV nuclease at its recognition site over that at an alternative site, had values of 3×10^5 in $MgCl_2$ and 6 in $MnCl_2$.

Type II restriction endonucleases recognize specific sequences on DNA, typically 4–8 bp[1] long, and cleave both strands of the DNA at their recognition sites (Modrich & Roberts, 1982; Bennett & Halford, 1989). The only cofactor that they need for DNA cleavage is Mg^{2+}. In general, these enzymes are extremely specific for their recognition sites. A change of just 1 bp in the recognition sequence can reduce k_{cat}/K_m for DNA cleavage by a factor of a million or more (Taylor & Halford, 1989; Lesser et al., 1990). Under standard conditions in vitro, it is difficult to detect the reaction of a restriction nuclease at a noncognate site, one that differs from the recognition site by 1 bp, unless high concentrations of the enzyme are used (Luke et al., 1987). Moreover, in vivo, a restriction enzyme is unlikely to ever cut both strands of DNA at a noncognate site, on account of the proofreading of restriction activity by DNA ligase (Taylor et al., 1990). However, in vitro, the activity of many restriction enzymes at noncognate sites can be enhanced by special reaction conditions: high pH and low ionic strength, or organic solvents such as glycerol of DMSO (Polisky et al., 1975; Tikchonenko et al., 1978; George & Chirikjian, 1982; Halford et al., 1986; Barany, 1988).

In the absence of divalent metal ions, restriction enzymes cannot cleave DNA but they can bind to it (Halford & Johnson, 1980; Terry et al., 1983; Taylor et al., 1991; Aiken et al., 1991). Hence, the metal ion is required for catalysis rather than DNA binding. For several nucleases that have either Ca^{2+} or Mg^{2+} cofactors (Saenger, 1991), the role of the metal ion(s) is now well established; for example, the 3'–5' exonuclease of DNA polymerase I (Derbyshire et al., 1991; Beese & Steitz, 1991). But equivalent information is not yet available for any restriction enzyme. The activities of many restriction enzymes have been measured with either Mn^{2+} or another ion in place of Mg^{2+} [reviewed by Modrich and Roberts (1982)]. Typically, Mn^{2+} causes about a 20-fold reduction in activity and Co^{2+} causes an even larger reduction, while Ca^{2+} fails to support any activity. But the alterations in reaction mechanism, which cause the reduced rates, have yet to be identified. Moreover, while Mn^{2+} reduces the activity of the *Eco*RI endonuclease at its recognition site, this ion increases activity at noncognate sites for *Eco*RI (Hsu & Berg, 1978; Tikchonenko et al., 1978): similar observations have been reported for several other restriction enzymes [reviewed by Bennett and Halford (1989)].

In binding to DNA in the absence of metal ions, different restriction enzymes show very different levels of specificity for their recognition sites. The *Eco*RI nuclease binds more tightly to its recognition sequence than to any other DNA sequence (Halford & Johnson, 1980; Terry et al., 1983). The difference in binding energies makes a major contribution to the specificity of DNA cleavage by *Eco*RI (Thielking et al., 1990; Lesser et al., 1990). In contrast, the *Eco*RV restriction enzyme seems to generate all of its specificity from the catalytic reaction (Newman et al., 1990). The *Eco*RV enzyme shows no preference for binding to a DNA with the *Eco*RV recognition site over one without the site: it has the same affinity for all DNA sequences and it translocates readily from one site to another along the DNA (Taylor et al., 1991). However, when the *Eco*RV enzyme is located at its recognition site, the protein has a high affinity for Mg^{2+} ions; when located at a noncognate site, it has a low affinity for Mg^{2+} (Taylor & Halford, 1989). At least part and maybe all of the specificity of the *Eco*RV nuclease stems from the way in which the affinity of the EcoRV–DNA complex for Mg^{2+} varies with the DNA sequence.

In this study, we report on the perturbation of this system by using Mn^{2+} ions with the *Eco*RV restriction enzyme, in order to find out whether the substitution of Mg^{2+} by another metal ion has any effect on the specificity of DNA cleavage. In the following study (Vermote et al., 1992), we describe the converse to this approach: the perturbation of DNA recognition functions in the *Eco*RV protein, by amino acid sub-

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[1] Abbreviations: bp, base pair(s); BME, β-mercaptoethanol; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; K_D, equilibrium dissociation constant; $t_{1/2}$, reaction half-time.

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stitutions, followed by an analysis of the interactions of the mutant proteins with metal ions. Both approaches show that the reason why improper DNA–protein interactions in the *Eco*RV system lead to reduced nuclease activity is that they alter the interaction between the protein and the catalytic metal ion. This applies to both the right protein on the wrong DNA sequence and vice versa.

Experimental Procedures

Concentrations of reactants against time were fitted to zero-order or first-order rate equations by nonlinear regression using ENZFITTER (Biosoft, Cambridge, U.K.). This was also used to fit variations of rates with reagent concentrations to rectangular hyperbolas. More complicated functions were fitted by FACSIMILE (Chance et al., 1977), version 101010, mounted on a MicroVax 3900. This program fits kinetic data (the concentrations of each species in the reaction against time) to any chosen scheme, by searching iteratively for the set of rate constants that gives the minimal sum of the squares of the deviations between the model curves and the complete set of experimental data.

All other materials and methods were as described previously (Halford & Goodall, 1988; Taylor & Halford, 1989). In previous studies, the reactions of the *Eco*RV restriction endonuclease were carried out in either buffer A or buffer C. Buffer A is 50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM BME, 100 μg/mL BSA, pH 7.5; this is the standard buffer used for reactions of *Eco*RV at its recognition site. We refer here to buffer AM, an identical solution except for the omission of $MgCl_2$; the concentration of $MgCl_2$ or $MnCl_2$ is then noted separately. Buffer C is 50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM BME, 100 μg/mL BSA, 10% (v/v) DMSO, pH 8.5; the *Eco*RV enzyme cleaves noncognate sites more readily in buffer C than in buffer A (Taylor & Halford, 1989).

Results

Reaction at the Recognition Site. The recognition sequence for the *Eco*RV restriction endonuclease is GATATC (Schildkraut et al., 1984; D'Arcy et al., 1985). This sequence occurs once on pAT153 (Twigg & Sherratt, 1980). The plasmid was isolated from a *recA* strain of *Escherichia coli* (HB101) in its monomeric form (3658 bp), as a covalently closed circle of supercoiled DNA, and this was used as a substrate for the *Eco*RV endonuclease in the presence of $MnCl_2$ (Figure 1). Given a covalently closed circle of DNA that has one copy of the recognition sequence, a restriction enzyme can cleave the supercoiled DNA to produce first the open circle form, by cutting one strand of the duplex, and then the linear form, by subsequently cutting the recognition site in the second strand (Bennett & Halford, 1989). Alternatively, the restriction enzyme could convert the supercoiled substrate directly to the linear form, without generating an open circle intermediate, by cutting the recognition site in both strands at the same time.

Figure 1 delineates the changes in the concentrations of all three forms of pAT153 during an *Eco*RV reaction in the presence of $MnCl_2$. The reaction shown had a DNA concentration (in terms of molecules of pAT153; i.e., *Eco*RV recognition sites) in excess of the enzyme concentration (in terms of molecules of the dimeric form of *Eco*RV; D'Arcy et al., 1985). Under these conditions, a relatively rapid burst phase was observed in the first 10 min of the reaction, during which the amount of supercoiled DNA declined in an exponential manner. This was followed by steady-state phase, where the decline in supercoiled DNA was linear with time

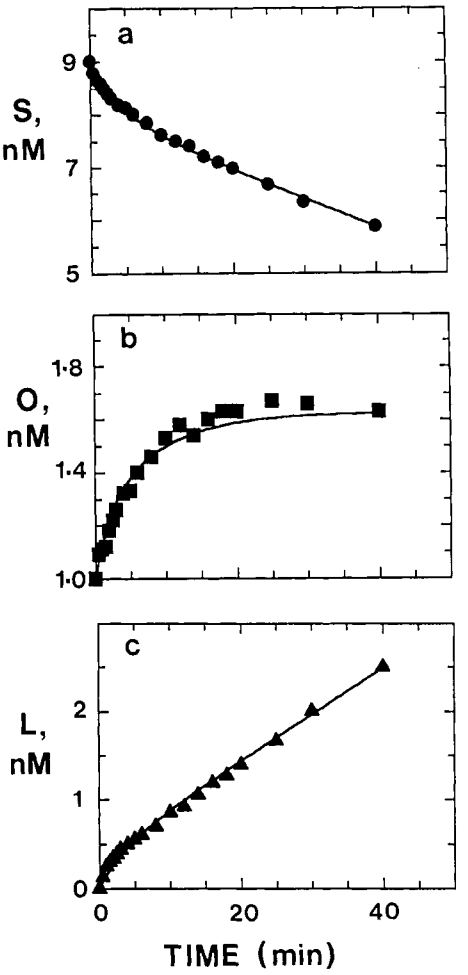

FIGURE 1: Cleavage of the recognition site on pAT153. The reaction at 20 °C, in buffer AM with 10 mM $MnCl_2$, contained 1.0 nM *Eco*RV restriction enzyme and 10 nM pAT153; the DNA had been labeled in vivo with [*methyl*-^{3}H]thymidine. Aliquots were withdrawn at timed intervals after the start of the reaction and mixed immediately with EDTA to stop DNA cleavage. The DNA in each sample was subsequently analyzed by electrophoresis through 1.2% (w/v) agarose to separate the supercoiled, open-circle, and linear forms of the DNA. The concentrations of the three forms were determined by scintillation counting on slices from the gel: panel a (●), supercoiled DNA (S); panel b (■), open-circle DNA (O); panel c (▲), linear DNA (L). All three panels are on the same time scale, but each has its own concentration scale. [At zero time, before the addition of enzyme, 90% of the DNA was supercoiled and 10% was open-circle.] The lines drawn in each panel are the theoretical lines from eq 3, with supercoiled DNA corresponding to [S] + [ES], open-circle to [EO] + [EO*], and linear to [EL] + [L], given the following rate constants: k_1 = 60 nM^{-1} min^{-1}; k_{-1} = 60 min^{-1}; k_2 = 0.36 min^{-1}; k_3 = 63 min^{-1}; k_4 = 0.25 min^{-1}; k_5 = 53 min^{-1}; k_{-5} = 0.075 min^{-1}. Values for k_1 and k_{-1} were assumed;[2] the other values are the optima found by simultaneously fitting the data in all three panels to eq 3.

(Figure 1a). The amount of open-circle DNA increased during the burst phase to a maximum level; this level was then maintained throughout the steady-state phase (Figure 1b). A small amount of linear DNA was also produced before the reaction reached the steady state (Figure 1c). The burst yielded more open-circle than linear DNA. In the steady-state phase, the increase in the amount of linear DNA matched the decrease in the amount of supercoiled DNA.

Further reactions of the *Eco*RV restriction enzyme on pAT153 were carried out as in Figure 1, except that the enzyme concentration was varied from 0.5 nM to 2 nM. For these experiments, the concentration of the plasmid was held at 10 nM and the $MnCl_2$ concentration was held at 10 mM. The amplitudes of the burst phase, measured from either the

Table I: *Eco*RV Kinetics with $MnCl_2$ or $MgCl_2$

substrate[a]	kinetic parameter	metal ion[b] Mn^{2+}	metal ion[b] Mg^{2+}
GATATC	k_{cat} (min^{-1})	0.04 (∓0.003)[c]	0.9 (∓0.05)
GATATC	K_m (nM)	1.7 (∓0.2)	0.5 (∓0.1)
GATATC	k_{cat}/K_m (M^{-1} s^{-1})	4×10^5	3×10^7
GTTATC	$k_a/[E_0]$ (M^{-1} s^{-1})	$7 (\mp 2) \times 10^4$	$1 (\mp 0.3) \times 10^2$

[a] The substrates are either the *Eco*RV recognition site on pAT153 or the preferred noncognate site on pAT153b. [b] Reactions were at 20 °C in buffer AM with either 10 mM $MnCl_2$ or 10 mM $MgCl_2$. [c] The ∓ values in parentheses are the standard errors for primary determinations.

decline in supercoiled DNA or the increments in open-circle and linear DNA, increased linearly with increasing enzyme concentrations: likewise, so did the steady-state reaction velocities (data not shown).

The linear DNA from the Mn^{2+}-dependent reactions was analysed by restriction digests, to determine where the cleavage of pAT153 had occurred. With 1 nM *Eco*RV enzyme, all of the DNA appeared to have been cut solely at the *Eco*RV recognition site (data not shown).

Steady-State Kinetics. Steady-state velocities were measured from reactions of the *Eco*RV restriction enzyme, with pAT153 as the substrate and $MnCl_2$ as the cofactor. The velocities were determined from the steady-state phase of the reaction, after the completion of the burst phase (Figure 1). In this phase, the zero-order rate for the utilization of supercoiled DNA was equal to that for the production of linear DNA. A range of substrate concentrations were used, from 0.5 to 20 nM; steady-state conditions were maintained by adjusting the enzyme concentration so that it was always at least 5 times lower than that of the substrate. The reaction velocities, once normalized to a fixed enzyme concentration, followed a conventional Michaelis–Menten curve against the concentration of pAT153 (data not shown). The hyperbolic curve yielded values for both k_{cat} and K_m and hence the ratio k_{cat}/K_m (Table I).

Parallel experiments were also carried out with the normal cofactor, $MgCl_2$, in place of $MnCl_2$ (Table I). They gave the same values for both k_{cat} and K_m as in previous studies (Halford & Goodall, 1988). The replacement of Mg^{2+} by Mn^{2+} increased the K_m for the recognition site 3-fold, but it reduced k_{cat} by more than 20-fold. The concentration of $MgCl_2$ used here (10 mM) is in the range where the velocity of the *Eco*RV reaction is at its maximum; the Mg^{2+}-dependent rate cannot be increased by raising the level of $MgCl_2$ (Halford & Goodall, 1988). The same applies to the Mn^{2+} reaction (see below, Figure 4).

Pre-Steady-State Kinetics. The reaction of the *Eco*RV restriction enzyme at its recognition site on pAT153 involves a burst phase of substrate utilization/product formation, both with Mn^{2+} as the cofactor (Figure 1) and with Mg^{2+} (Halford & Goodall, 1988). However, the burst phase with Mg^{2+} differs in two respects from that with Mn^{2+}. First, the burst phase was completed in <1 min with Mg^{2+} [Figure 4 in Halford and Goodall (1988)] while it took >10 min with Mn^{2+}. Second, the reaction with 10 mM $MgCl_2$ produced none of the open-circle form of the DNA, either before or during the steady state; in both phases, supercoiled DNA was converted directly to the linear form (Halford & Goodall, 1988). The Mg^{2+}-dependent reaction is consistent with the mechanism

$$E + S \rightleftharpoons ES \rightarrow EO \rightarrow EL \rightarrow E + L \qquad (1)$$

where S, O, and L represent the supercoiled, open-circle, and linear forms of the DNA, though the concentration of each form that is determined experimentally (as in Figure 1) is the sum of both free and enzyme bound DNA.[2] This scheme can accommodate the kinetics with Mg^{2+}, provided that the rate constant for ES → EO is smaller than that for EO → EL but larger than that for EL → E + L; the latter is the rate-limiting step for the complete reaction (Halford & Goodall, 1988). The concentration of EO will then be extremely low throughout the reaction, and the burst phase reflects the accumulation of EL, at a rate limited by ES → EO, prior to the steady-state dissociation of EL → E + L.

Attempts were made to fit the data for the Mn^{2+}-dependent reaction to the scheme in eq 1. However, FACSIMILE was unable to find any combination of rate constants for eq 1 that was consistent with the experimental data. If any step preceding the formation of EO is rate-limiting, the model predicts that there should be no burst phase for either open-circle or linear DNA. If EO → EL is rate-limiting, eq 1 predicts the burst of EO but linear DNA should then appear only after an initial lag phase. If EL → E + L is rate-limiting, the scheme predicts that there will be a burst in linear DNA but that the steady-state level of open-circle DNA will be close to zero. The experimental data in Figure 1 contradict all three possibilities.

Another mechanism that has been used previously to account for the kinetics of restriction enzymes is shown in by

$$\begin{array}{ccccccccc} E + S & \rightleftharpoons & ES & \rightarrow & EO & \rightarrow & EL & \rightarrow & E + L \\ & & & & \updownarrow & & & & \\ & & & & E + O & & & & \end{array} \qquad (2)$$

This differs from that in eq 1 by allowing the enzyme to dissociate reversibly from the DNA after it cuts one strand (Terry et al., 1987; Hemsley et al., 1990). The mechanism in eq 2 was tested by using FACSIMILE to find the set of rate constants that produced the best fit to the experimental data in Figure 1 (and also similar experiments at different enzyme concentrations). The observed changes in the concentrations of either supercoiled or linear DNA could be matched fairly closely to the model. However, the set of rate constants that gave the optimal fit between model and data predicted that the amount of open-circle DNA (i.e., [EO] + [O]) should increase linearly with time over the initial portion of the steady-state phase: i.e., from 10 to 40 min for the reaction in Figure 1b. The complete data set could not be reconciled to eq 2 with a single set of rate constants.

A modification to the mechanism in eq 2, showin in eq 3, is that the EO intermediate, instead of dissociating to E + O, isomerizes to a state (EO*) that can no longer cleave the second strand of the DNA.

$$\begin{array}{ccccccccccc} E + S & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & ES & \xrightarrow{k_2} & EO & \xrightarrow{k_3} & EL & \xrightarrow{k_4} & E + L \\ & & & & \underset{k_{-5}}{\overset{k_5}{\updownarrow}} & & & & \\ & & & & EO^* & & & & \end{array} \qquad (3)$$

When this scheme was tested with FACSIMILE, a set of rate constants were found that fitted the experimental data for all three forms of the DNA, not only for the data shown in Figure

[2] The assay used here makes no distinction between enzyme bound and free DNA. Consequently, it provides no information about the initial association of the enzyme with the supercoiled substrate. For all of the data-fitting described here, it was assumed that the rate constant for E + S → ES was 1×10^9 M^{-1} s^{-1}, a typical value for DNA–protein associations (von Hippel & Berg, 1989), and that for ES → E + S was 1 s^{-1}. However, the fitting was insensitive to the values chosen for these two rate constants, provided that they were large enough to ensure that the equilibration between E + S and ES was not rate-determining. The data were inconsistent with all models tested where the equilibration was slow.

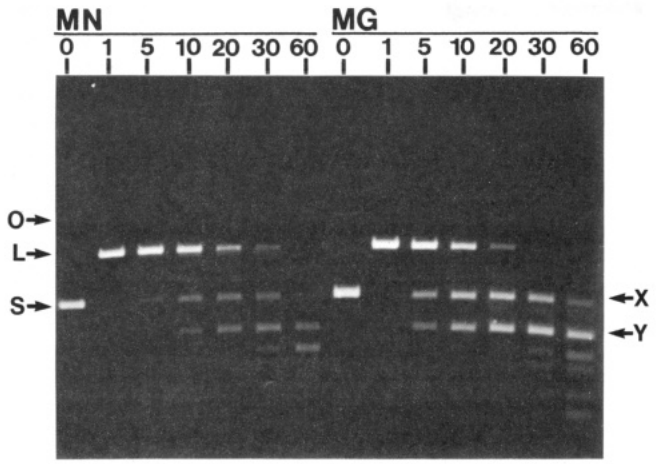

FIGURE 2: Cleavage of noncognate sites on pAT153. The reactions at 20 °C contained 10 nM pAT153 in either buffer AM with 10 mM $MnCl_2$ and 100 nM *Eco*RV nuclease or buffer C and 1 μM *Eco*RV nuclease. (Buffer C contains 10 mM $MgCl_2$.) Samples were withdrawn from the reactions at timed intervals after the addition of the enzyme, the reaction in each sample was stopped immediately, and the samples were then analyzed by electrophoresis through 1.2% agarose. Samples from the $MnCl_2$ and the $MgCl_2$ reactions are marked above the gel as MN and MG, respectively, and the times of withdrawal of the samples (in minutes) are noted above each lane: lane 0 is before the addition of enzyme. The supercoiled, open-circle, and linear forms of pAT153 are marked as S, O, and L on the left of the gel, and the two products, X and Y, are identified on the right of the gel.

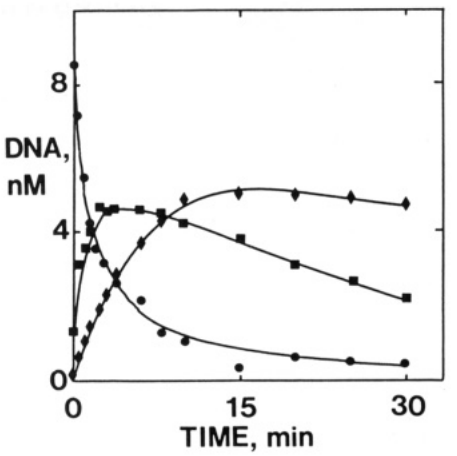

FIGURE 3: Cleavage of the preferred noncognate site on pAT153b. The reaction contained 100 nM *Eco*RV restriction enzyme and 10 nM pAT153b (^{3}H-labeled) in buffer AM with 10 mM $MnCl_2$ at 20 °C. The concentrations of the supercoiled (●), open-circle (■), and linear (♦) forms of the DNA were measured at timed intervals during the reaction as in Figure 1.

1 (where the lines drawn are the theoretical lines for eq 3 with the optimized set of rate constants)[2] but also for similar experiments at different *Eco*RV concentrations (data not shown). The optimal set of rate constants contain unique values for the three smallest rate constants, k_2, k_4, and k_{-5}. For each of these constants, the boundary values for 5–95% confidence limits were within 15% of the values given in Figure 1. The curve-fitting demanded that k_3 and k_5 were large compared to k_2, k_4, and k_{-5}, but these values were poorly defined by the fitting procedure; the data were equally consistent with a range of values for k_3 provided that the value of k_5 was altered in parallel so that the ratio k_3/k_5 stayed between 1.0 and 1.2.

Reactions at Noncognate Sites. In addition to one *Eco*RV recognition site, pAT153 contains another 12 sites that can be cut by the *Eco*RV restriction enzyme. All of the additional sites are at sequences that differ from the canonical site for *Eco*RV by 1 bp (Halford et al., 1986). To date, DNA cleavage by *Eco*RV at noncognate sites has been observed only in reactions containing DMSO or in standard reactions with enzyme concentrations > 1 μM (Halford et al., 1986; Taylor & Halford, 1989). Under these conditions, one of the noncognate sites on pAT153, GTTATC at position 1734, is cleaved more rapidly than any other (Taylor & Halford, 1989, 1992). The Mn^{2+}-dependent reactions of *Eco*RV at its noncognate sites were studied with either pAT153 or pAT153b as the substrates; the latter differs from pAT153 only by the disruption of the *Eco*RV site with an 8-bp *Bgl*II linker (Taylor & Halford, 1989).

With pAT153 as the substrate, in the presence of either $MgCl_2$ or $MnCl_2$ as the cofactor, and with comparatively high concentrations of *Eco*RV enzyme (Figure 2), the supercoiled DNA was cleaved first at the *Eco*RV recognition site to produce the linear form of pAT153 (as in Figure 1). But the full-length linear form was then cut again, first to two products (marked X and Y in Figure 2) and then to a series of smaller DNA fragments. However, with Mn^{2+}, less of the nuclease was needed to make the additional cuts on the *Eco*RV-linearized pAT153. The two reactions shown in Figure 2 are on the same time scale but that with Mg^{2+} was in a buffer containing DMSO, which enhances *Eco*RV activity at noncognate sites (Halford et al., 1986), yet it still needed 10 times more enzyme.

In the presence of Mg^{2+}, it had been shown previously that the two products, X and Y, stem from the cleavage of pAT153 both at the *Eco*RV recognition site and at the noncognate site at position 1734 (Taylor & Halford, 1989). To identify the noncognate site that was cut preferentially in the presence of Mn^{2+}, the initial products from the Mn^{2+}-dependent reaction were characterized by restriction analysis using *Nsp*HI, which cleaves pAT153 at position 1769. The first noncognate site to be cleaved was again GTTATC at position 1734 (data not shown). However, the Mn^{2+} reaction generated less of X and Y than the Mg^{2+} reaction (Figure 2). The degree of preference for this noncognate site is lower in Mn^{2+} than in Mg^{2+}.

With pAT153 as the substrate, the products from reactions at noncognate sites were detected only after both strands of the *Eco*RV-linearized DNA had been cleaved at the same site (Figure 2). In contrast, pAT153b lacks an *Eco*RV recognition site so the first reaction of the nuclease is at the preferred noncognate site. Hence, it was possible to monitor the cleavage of each strand of the DNA at this noncognate site, by observing the conversion of the supercoiled form of pAT153b to open-circle and linear forms (Figure 3). With $MnCl_2$ as the cofactor, the reaction of the *Eco*RV nuclease (with the enzyme in excess of the DNA) resulted in an exponential decrease in the amount of supercoiled DNA, a transient formation of open-circle DNA and, subsequently, the production of linear DNA (Figure 3). Later, the full-length linear DNA was cut at other noncognate sites to produce the smaller fragments.

Reactions of the type in Figure 3 were analyzed by evaluating the first-order rate constant for the conversion of supercoiled to open-circle DNA (k_a). [In previous studies with Mg^{2+} (Taylor & Halford, 1989), a rate constant had also been determined for the conversion of open-circle to linear DNA (k_b). But k_b was ill-defined in the Mn^{2+} reactions: the difference between the noncognate site at position 1734 and the others on the DNA was not as marked as in Mg^{2+}, so the full-length linear form of pAT153b was soon cleaved to smaller fragments.] The dependence of k_a on the concentration of the *Eco*RV enzyme was determined in two series of reactions on pAT153b (10 nM) in buffer AM: in one series, $MnCl_2$ (10

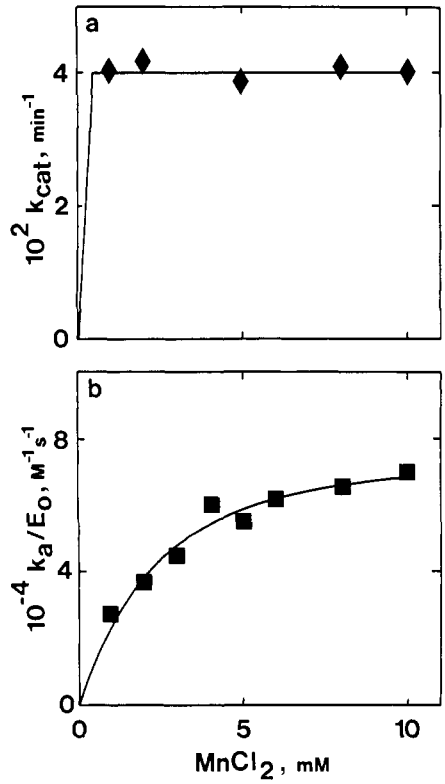

FIGURE 4: Manganese concentration dependencies at the recognition site (a) and at the noncognate site (b). Panel a: values for k_{cat} were measured from steady-state velocities as in Figure 1; the reactions contained the *Eco*RV restriction enzyme (0.5 nM) and pAT153 (10 nM) in buffer AM with varied concentrations of $MnCl_2$. Panel b: values for $k_a/[E_0]$ were determined from the exponential decline in supercoiled DNA as in Figure 3; the reactions contained the *Eco*RV restriction enzyme (50 nM) and pAT153b (10 nM) in buffer AM with varied concentrations of $MnCl_2$. The line drawn in (b) in the theoretical line (a rectangular hyperbola) for an apparent K_D of 2.3 mM.

mM) was used as the cofactor and the concentration of the *Eco*RV nuclease was varied from 20 to 100 nM; in the second series, with $MgCl_2$ (10 mM), the concentration of the nuclease was varied from 1 to 3 μM. In both series, the values for k_a increased linearly with increasing levels of enzyme (data not shown). The rate constants could thus be normalized against the enzyme concentration to yield a parameter, $k_a/[E_0]$, that is related to k_{cat}/K_m for the reaction of *Eco*RV at a noncognate DNA sequence.[3]

In buffer AM, with Mn^{2+} as the cofactor, $k_a/[E_0]$ was found to be 7×10^4 M^{-1} s^{-1} while that with Mg^{2+} was 1×10^2 M^{-1} s^{-1} (Table I). The replacement of Mg^{2+} with Mn^{2+} thus increases the activity of the *Eco*RV restriction endonuclease at the noncognate site by a factor of 700. Previously, the activity of the *Eco*RV nuclease at noncognate sites had been enhanced by adding DMSO to the Mg^{2+} buffer and by raising

[3] If a reaction of an enzyme that obeys Michaelis–Menten kinetics is carried out under the conditions, [S] < [E] < K_m, the utilization of substrate follows an exponential progress curve: the first-order rate constant from the exponential divided by $[E_0]$ is equal to k_{cat}/K_m. The reactions of the *Eco*RV enzyme at its noncognate sites on pAT153b meet these conditions, but the rates of these reactions are inhibited by the remainder of the DNA molecule (Taylor et al., 1991), so $k_a/[E_0]$ is related to but not equal to k_{cat}/K_m. In previous studies (Taylor & Halford, 1989), *Eco*RV activity at noncognate sites had been measured from the rate of double-strand breaks (i.e., $k_b/[E_0]$) while the values given here are from the first strand to be cut (i.e., $k_a/[E_0]$). Reactions at the *Eco*RV recognition site on pAT153 are not detectably inhibited by the remainder of the DNA molecule (Taylor et al., 1991), so these yield values for k_{cat}/K_m; the value is for double-strand breaks.

Table II: *Eco*RV Reactions with both $MnCl_2$ and $MgCl_2$

metal ion[a]		substrate[b]	
$MnCl_2$ (mM)	$MgCl_2$ (mM)	GATATC k_{cat} (min^{-1})	GTTATC $k_a/[E_0] \times 10^{-4}$ (M^{-1} s^{-1})
0	10	0.90	0.01
1	0	0.040	2.8
1	9	0.036	2.6
2	0	0.042	3.8
2	8	0.039	3.0
5	0	0.038	5.6
5	5	0.044	5.3
8	0	0.041	6.5
8	2	0.037	6.3
10	0	0.040	7.0

[a] Reactions were at 20 °C with 10 nM DNA in buffer AM with either $MnCl_2$ alone, at the concentrations indicated, or $MnCl_2$ and $MgCl_2$ together at a combined concentration of 10 mM. [b] Substrates were either the *Eco*RV recognition site on pAT153 (steady-state reaction velocities were measured with 0.5 nM enzyme) or the preferred noncognate site on pAT153b (exponential progress curves were measured with 50 nM enzyme).

the pH, to give buffer C (Taylor & Halford, 1989). But the value for $k_a/[E_0]$ in buffer C, 7×10^3 M^{-1} s^{-1}, is 10 times lower than that in the standard buffer with Mn^{2+} in place of Mg^{2+}.

Manganese Concentration Dependencies. The rates of DNA cleavage by *Eco*RV, at its recognition site on pAT153 and at its preferred noncognate site on pAT153b, were measured across a range of $MnCl_2$ concentrations (Figure 4). The reactions at the recognition site were carried out as in Figure 1, with a DNA concentration that was higher than both the enzyme concentration and the K_m for the recognition site. The rates (k_{cat} values) were determined from the zero-order steady-state phase of the reactions, after the completion of the burst phase. The reactions at the noncognate site were carried out as in Figure 3, with the enzyme in excess of the DNA, and the kinetic parameter determined at each $MnCl_2$ concentration was $k_a/[E_0]$. In both types of reactions, the relevant parameter, either k_{cat} or $k_a/[E_0]$, should increase with increasing levels of Mn^{2+} in a hyperbolic fashion; an apparent K_D for the binding of the metal ion to the enzyme–DNA complex can then be calculated from the ion concentration that gives half the maximal rate (Halford & Goodall, 1988; Taylor & Halford, 1989).

In the reaction at the *Eco*RV recognition site, no variation in k_{cat} was observed over the range of $MnCl_2$ concentrations tested (Figure 4a). This shows that, when the *Eco*RV restriction endonuclease is located at its recognition site on the DNA, it has an apparent K_D for Mn^{2+} that is $\ll$ 1 mM. This is similar to previous experiments with Mg^{2+} as the cofactor, at pH 7.5, no variation in k_{cat} was observed as the concentration of $MgCl_2$ was varied from 1 to 10 mM (Halford & Goodall, 1988). At the recognition site, the apparent K_D for Mg^{2+} is also $\ll$ 1 mM.

The rate at which the *Eco*RV enzyme cleaved the noncognate site on pAT153b showed a hyperbolic dependence on the concentration on $MnCl_2$ (Figure 4b). This result differs markedly from equivalent experiments with Mg^{2+}, in which the rate for cutting the noncognate site increased *linearly* as the concentration of $MgCl_2$ was increased from 1 to 10 mM (Taylor & Halford, 1989). Hence, when located at the noncognate site, the *Eco*RV enzyme has apparent K_D values of 2.3 mM for Mn^{2+} (Figure 4b) and $\gg$ 10 mM for Mg^{2+}.

To test whether the *Eco*RV enzyme really had a higher affinity for Mn^{2+} over Mg^{2+}, further experiments were carried out with either $MnCl_2$ alone or with mixtures of $MnCl_2$ and

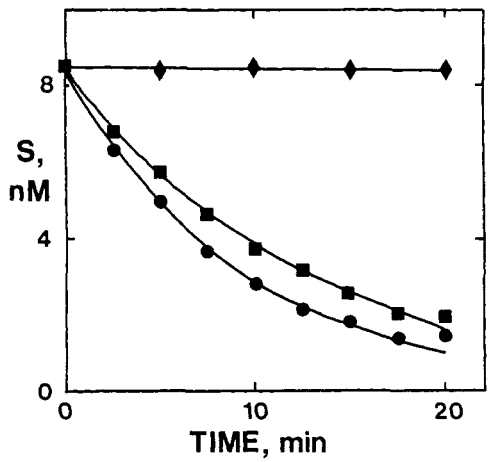

FIGURE 5: Mn^{2+}/Mg^{2+} competition. The reactions at 20 °C contained the *Eco*RV restriction enzyme (50 nM) and pAT153b (10 nM) in buffer AM with either 8 mM $MgCl_2$ (♦) or 2 mM $MnCl_2$ (●) or both 8 mM $MgCl_2$ and 2 mM $MnCl_2$ (■). Initially, 85% of the pAT153b was supercoiled and the decline in the concentration of the supercoiled form (S) was measured at timed intervals during the reactions, as in Figure 3.

$MgCl_2$ (Table II). In each mixture, the amount of $MgCl_2$ brought the total concentration of divalent metal ions to 10 mM. At each concentration of $MnCl_2$ tested, the addition of $MgCl_2$ made no difference to the reaction rate observed in the parallel reaction containing only $MnCl_2$ (Table II). The rates for cleaving either the *Eco*RV recognition site on pAT153 or the noncognate site on pAT153b are therefore determined solely by the concentration of $MnCl_2$. For example, the *Eco*RV reaction on pAT153b with 2 mM $MnCl_2$ and 8 mM $MgCl_2$ proceeded at a rate similar to that with 2 mM $MnCl_2$ alone, and this rate was much faster than that with 8 mM $MgCl_2$ alone (Figure 5). Since the reaction at the noncognate site on pAT153b is 700 times slower with Mg^{2+} compared to Mn^{2+} (see above), any binding of Mg^{2+} to the enzyme–DNA complex should have resulted in a marked reduction in the rate of DNA cleavage. Conversely, the reaction at the *Eco*RV recognition site is faster with Mg^{2+} than with Mn^{2+} (Table I). In this case, it might have been expected that the addition of $MgCl_2$ would have accelerated DNA cleavage, but this was not observed (Table II).

Discussion

The principal observations reported here are that the substitution of Mg^{2+} by Mn^{2+} reduces not only the activity of the *Eco*RV restriction enzyme at its recognition sequence, in accord with other studies (Luke et al., 1987; Selent et al., 1992), but also its specificity (i.e., its ability to discriminate against other DNA sequences). For the *Eco*RV nuclease, a discrimination factor can be derived by comparing the value of k_{cat}/K_m from reactions at the recognition site with the value of $k_a/[E_0]$ from reactions at noncognate sites. Though the discrimination factor provides an indication of the relative activities at cognate and noncognate sites, the former has been measured in this study from the rate of double-strand breaks and the latter has been measured from single-strand breaks.[3] In addition, the precise numbers for the discrimination factors vary with the metal ion concentration: a change in the concentration of the metal ion alters *Eco*RV activity at the noncognate site without altering activity at the cognate site (Figure 4).

In the presence of 10 mM $MgCl_2$, k_{cat}/K_m and $k_a/[E_0]$ have values of 3×10^7 M^{-1} s^{-1} and 1×10^2 M^{-1} s^{-1}, respectively (Table I), so the discrimination factor comes to 300 000. With 10 mM $MnCl_2$, the equivalent parameters are 4×10^5 M^{-1} s^{-1} and 7×10^4 M^{-1} s^{-1}, which yields a discrimination factor of 6. The switch from Mg^{2+} to Mn^{2+} thus produces a 50 000-fold change in the discrimination factor for *Eco*RV. By itself, this shows that the metal ion cofactor plays a key role in determining the specificity of DNA cleavage by this restriction enzyme.

Manganese Ions at the EcoRV Recognition Site. If an enzyme has a k_{cat} of around 100 s^{-1}, as is the case for very many enzymes (Fersht, 1985), the $t_{1/2}$ for a single turnover will be about 7 ms. In this situation, the pre-steady-state kinetics can be measured only by stopped-flow or other rapid reaction methods (Gutfreund, 1972). However, restriction endonucleases generally cleave their recognition sites with much lower turnover numbers: the value of 0.9 min^{-1} for *Eco*RV in the presence of Mg^{2+} is similar to that for most other restriction enzymes (Bennett & Halford, 1989). In the presence of Mn^{2+} ions, k_{cat} for the reaction of *Eco*RV at its recognition site is reduced to 0.04 min^{-1} (Table I), which corresponds to a $t_{1/2}$ of over 15 min for a single turnover. The time base for the Mn^{2+}-dependent reaction of *Eco*RV shown in Figure 1 is 40 min, yet the data are exactly analogous to stopped-flow data and can be analyzed by the standard methods for the treatment of pre-steady-state kinetics (Gutfreund, 1972; Fersht, 1985).

The pre-steady-state kinetics from the Mn^{2+}-dependent reaction of the *Eco*RV nuclease at its recognition site were tested against three mechanisms (eqs 1–3). But only the mechanism in eq 3 could fully accommodate the experimental data. In the presence of Mg^{2+}, the *Eco*RV enzyme cleaves the two strands of the DNA in a concerted fashion (Halford & Goodall, 1988), and the same applies in the presence of Mn^{2+}. In both cases, the rate constant for cutting the first strand (k_2) is smaller than that for the second strand (k_3) though both values are much lower with Mn^{2+} than Mg^{2+}. The concerted mechanism demands that the specific DNA–protein complex is fully loaded with the metal ion. Otherwise, if only one subunit of the dimeric protein had bound the metal, only one strand would be cleaved (Luke et al., 1987; Halford & Goodall,1988). This requirement is met. The *Eco*RV enzyme, when located at its recognition sequence on the DNA, has a high affinity for Mn^{2+} (apparent $K_D \ll 1$ mM; Figure 4a), even higher than that for Mg^{2+} (Table II).

The reduction in the rate of the hydrolytic reactions is not, however, the reason why the change from Mg^{2+} to Mn^{2+} causes such a large reduction in k_{cat}. To accommodate the pre-steady-state kinetics from the reaction with Mn^{2+}, it was necessary to propose that, after the enzyme had cut one strand of the DNA, the EO intermediate could isomerize to a state (EO*) that was unable to cleave the second strand (eq 3). The slowest step in the overall reaction with Mn^{2+} is the recovery from EO* to EO (k_{-5} in eq 3), while that with Mg^{2+} had been the final dissociation of the DNA cut in both strands (k_4; Halford & Goodall, 1988).

What is the nature of EO*? When bound to DNA in the absence of divalent metal ions, the *Eco*RV restriction enzyme translocates along the DNA and has no preference for one site over another: its occupancy of any given site can only be given as a time average (Taylor et al., 1991). Hence, a plausible model for the EO → EO* isomerization is that it reflects the translocation of the enzyme from the recognition site to nonspecific DNA in the time interval between cutting the first and second strands of the DNA. If so, a similar translocation might have been expected during the Mg^{2+}-dependent reaction. Indeed, the rate constant for the EO → EO* isomerization (k_5) could have the same value with either Mn^{2+} or Mg^{2+}.

However, the rate constant for cutting the second strand of the recognition site (k_3 in eq 3) is large with Mg^{2+} than with Mn^{2+}. If k_3 is much larger than k_5, the isomerization never occurs; this seems to be the case in Mg^{2+} but not in Mn^{2+}.

An alternative model is that the isomerization from EO → EO* is a conformation change in the DNA–protein complex. Three crystal structures have been solved for the *Eco*RV restriction endonuclease: the free protein, the bound to its recognition sequence, and the protein bound to nonspecific DNA (Winkler et al., 1991; Winkler, 1992; F. K. Winkler, personal communication). The differences between these structures show that both binding to DNA and specific DNA recognition by this restriction enzyme involve major changes in both protein and DNA conformations. But it remains to be determined whether the observed conformation changes limit the kinetics of the reaction.

Manganese Ions at Noncognate Sites for EcoRV. In contrast to the reduced activity at the recognition site, the replacement of Mg^{2+} by Mn^{2+} increased the activity of the *Eco*RV endonuclease at DNA sequences that differ from its recognition sequence by 1 bp. The increase could be quantified because one out of the 12 noncognate sites on pAT153, GTTATC at position 1734, was cleaved more readily than the others with either Mg^{2+} or Mn^{2+} as the cofactors (Figure 2). The susceptibility of the site at 1743 is due to the DNA sequences that flank the site: these are sequences that confer flexibility to DNA conformation (Taylor & Halford, 1992).

Previously, qualitative experiments with *Eco*RI and other restriction enzymes had shown that their activities at noncognate sites were also enhanced by Mn^{2+} (Hsu & Berg, 1978). But restriction nucleases are certainly not the only enzymes involved in DNA metabolism which lose their ability to discriminate against incorrect substrates with Mn^{2+} in place of Mg^{2+}. For example, Mn^{2+} enhances the rate at which DNA polymerases utilize either noncomplementary nucleotides or nucleotide analogues (Kunkel & Loeb, 1979; Taylor & Richardson, 1989). In general, reactions on DNA that are suppressed in the presence of Mg^{2+} ions seem to occur much more readily in the presence of Mn^{2+} ions. The data on the *Eco*RV restriction enzyme reported here provide a simple kinetic mechanism to account for why DNA cleavage at noncognate sites occurs more readily with Mn^{2+} as the cofactor than with Mg^{2+}.

When bound at a noncognate site, the *Eco*RV enzyme has an appreciable affinity for Mn^{2+} (Figure 4b). At the noncognate site, it has an apparent K_D of 2.3 mM for Mn^{2+} so, at 10 mM $MnCl_2$, over 80% of the protein will have bound Mn^{2+} ions. In the absence of divalent metal ions, the *Eco*RV endonuclease binds equally well to specific and nonspecific sites (Taylor et al., 1991), but the rate of DNA cleavage at each type of site will be a function of the fractional saturation of the system by the metal ion. In the presence of 10 mM $MnCl_2$, the enzyme is close to saturation with Mn^{2+} at both cognate and noncognate sites, and the two sorts of sites are indeed cleaved at similar rates (Table I). In contrast, the *Eco*RV enzyme at a noncognate site has a vanishingly low affinity for Mg^{2+} ions (Taylor & Halford, 1989). The affinity of the noncognate complex for Mg^{2+} is much lower than that for Mn^{2+} (Table II and Figure 5) and, at 10 mM $MgCl_2$, virtually none of the protein would have bound Mg^{2+} ions. Consequently, Mn^{2+} supports DNA cleavage at noncognate sites better than Mg^{2+}, despite the hydrolytic reactions being slower with Mn^{2+}.

When a DNA substrate containing an *Eco*RV site is mutiply bound by the *Eco*RV restriction enzyme and that complex is then challenged with a mixture of $MgCl_2$ and competitor DNA, the substrate is cleaved exclusively at the recognition site; the enzyme molecules at nonspecific sites either translocate to the recognition site or dissociate from the substrate without cleaving it (Taylor et al., 1991). The selection of the site for DNA cleavage is due to the binding of Mg^{2+} to the *Eco*RV enzyme having a negative ΔG when the enzyme is at the recognition site and a positive ΔG at all other sites. In contrast, the binding of Mn^{2+} has a negative ΔG at both cognate and noncognate sites, provided that the concentration of $MnCl_2$ is > 2.3 mM (Figure 4b). Mn^{2+} can thus trap the enzyme at either cognate or noncognate sites while Mg^{2+} locks the enzyme on to DNA only at the cognate site. This role for the metal ion is supported by the DNA binding properties of a mutant of *Eco*RV that had no catalytic activity: the protein bound DNA nonspecifically in the absence of Mg^{2+} but specifically in the presence of Mg^{2+} (Thielking et al., 1992).

The precise structural basis for the difference between Mg^{2+} and Mn^{2+} in the *Eco*RV reaction remains to be determined. The crystals of the EcoRV–DNA complexes that yielded the structures for both the specific complex at the recognition sequence and the nonspecific complex were grown in the absence of divalent cations (Winkler et al., 1991). Nevertheless, the specific and nonspecific complexes differ significantly in both DNA and protein conformations (Winkler, 1992). The catalytic center in the specific complex seems to be organized around the scissile bond in the DNA so that it is ready to bind Mg^{2+} and to carry out the catalytic reaction. The specific complex can also bind Mn^{2+}, with an even higher affinity than that for Mg^{2+}. In contrast, the nonspecific complex appears to lack the organization required for binding Mg^{2+}, though it can still accept Mn^{2+} ions. To create a binding site for Mg^{2+} between the protein and the DNA, it may be essential to distort the DNA in the way seen in the specific complex of *Eco*RV bound to its recognition sequence (Winkler, 1992). The lack of distortion of nonspecific DNA bound to EcoRV would leave the phosphate at the scissile bond too far away from the catalytic groups to permit the Mg^{2+} ion to be coordinated by both protein and DNA.

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*Eco*RV Restriction Endonuclease: Communication between DNA Recognition and Catalysis†

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ABSTRACT: A genetic system was constructed for the mutagenesis of the *Eco*RV restriction endonuclease and for the overproduction of mutant proteins. The system was used to make two mutants of *Eco*RV, with Ala in place of either Asn185 or Asn188. In the crystal structure of the *Eco*RV–DNA complex, both Asn185 and Asn188 contact the DNA within the *Eco*RV recognition sequence. But neither mutation affected the ability of the protein to bind to DNA. In the absence of metal ion cofactors, the mutants bound DNA with almost the same affinity as that of the wild-type enzyme. In the presence of Mg^{2+}, both mutants retained the ability to cleave DNA specifically at the *Eco*RV recognition sequence, but their activities were severely depressed relative to that of the wild-type. In contrast, with Mn^{2+} as the cofactor, the mutant enzymes cleaved the *Eco*RV recognition site with activities that were close to that of the wild-type. When bound to DNA at the *Eco*RV recognition site, the mutant proteins bound Mn^{2+} ions readily, but they had much lower affinities for Mg^{2+} ions than the wild-type enzyme. This was the reason for their low activities with Mg^{2+} as the cofactor. The arrangement of the DNA recognition functions, at one location in the *Eco*RV restriction enzyme, are therefore responsible for organizing the catalytic functions at a separate location in the protein.

The *Eco*RV restriction endonuclease cleaves DNA at its recognition sequence, GATATC (Schildkraut et al., 1984; D'Arcy et al., 1985). The only cofactor it needs for DNA cleavage is a divalent metal ion, either Mg^{2+}, Mn^{2+}, or Co^{2+} [reviewed by Luke et al. (1987)]. Yet, in binding to DNA in the absence of metal ion cofactors, the *Eco*RV restriction enzyme shows no preference for its recognition site over other sites: all DNA sequences yield the same equilibrium constant (Taylor et al., 1991). With Mn^{2+} as the cofactor for DNA

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