Biochemistry

 Volume 31, Number 15 April 21, 1992

Accelerated Publications

Mg^{2+} Confers DNA Binding Specificity to the *Eco*RV Restriction Endonuclease[†,‡]

Vera Thielking, Ursel Selent, Eleonore Köhler, Axel Landgraf,[§] Heiner Wolfes, Jürgen Alves, and Alfred Pingoud*
Zentrum Biochemie, Medizinische Hochschule Hannover, Konstanty-Gutschow-Strasse 8, D-3000 Hannover 61, Germany
Received December 23, 1991; Revised Manuscript Received February 5, 1992

ABSTRACT: The *Eco*RV mutant D90A which carries an amino acid substitution in its active center does not cleave DNA. Therefore, it is possible to perform DNA binding experiments with the *Eco*RV–D90A mutant both in the absence and in the presence of Mg^{2+}. Like wild-type *Eco*RV [Taylor et al. (1991) *Biochemistry 30*, 8743–8753], it does not show a pronounced specificity for binding to its recognition site in the absence of Mg^{2+} as judged by the appearance of multiple shifted bands in an electrophoretic mobility shift assay with a 377-bp DNA fragment carrying a single *Eco*RV recognition sequence. In the presence of Mg^{2+}, however, only one band corresponding to a 1:1 complex appears even with a high excess of protein over DNA. This complex most likely is the specific one, because its formation is suppressed much more effectively by a 13-bp oligodeoxynucleotide with an *Eco*RV site than by a corresponding oligodeoxynucleotide without an *Eco*RV site. The preferential interaction of the *Eco*RV–D90A mutant with specific DNA in the presence of Mg^{2+} was also demonstrated directly: a 20-bp oligodeoxynucleotide with an *Eco*RV site is bound with $K^{Ass} = 4 \times 10^8\ M^{-1}$, while a corresponding oligodeoxynucleotide without an *Eco*RV site is bound with $K^{Ass} \leq 1 \times 10^5\ M^{-1}$. From these data it appears that Mg^{2+} confers DNA binding specificity to this mutant by lowering the affinity to nonspecific sites and raising the affinity to specific sites as compared to binding in the absence of Mg^{2+}. It is concluded that this is also true for wild-type *Eco*RV.

Restriction endonucleases must efficiently discriminate between nonspecific and specific DNA sequences. The recognition process can be envisaged as a molecular trial and error approach which involves (I) many nonspecific binding events presumably dominated by electrostatic contacts and (II) facilitated diffusion along the DNA until the specific target sequence is reached, which is identified by forming additional contacts, in particular, hydrogen bonds to the heteroatoms of the nucleobases [for recent reviews on restriction endonucleases, see Bennett and Halford (1989), Rosenberg (1991), and Winkler (1992); for a recent overview on protein–nucleic acid interaction, see Steitz (1990)]. The specific interaction then activates the catalytic center by a mechanism which is not yet known but could involve a conformational transition that allows the binding of the essential cofactor Mg^{2+} (Pingoud et al., 1991; Taylor et al., 1991). In this scheme, specific binding differs from nonspecific binding in a qualitative and quantitative manner. This was experimentally confirmed for several restriction endonucleases, for example, *Eco*RI or its isoschizomer *Rsr*I, which bind by 4–5 orders of magnitude more firmly to specific than to nonspecific DNA (Clore et al., 1982; Terry et al., 1983; Thielking et al., 1990; Lesser et al., 1990; Aiken et al., 1991) provided the DNA is sufficiently long to occupy all of the protein's DNA binding site. For *Eco*RI it was demonstrated, in addition, that this enzyme makes use of different protein–DNA contacts to achieve stringent discrimination between specific and nonspecific sites (Lesser et al., 1990).

These binding studies were carried out in the absence of the essential cofactor Mg^{2+} to prevent cleavage of the DNA. Rapid kinetic experiments with *Eco*RI indicated that there is strong specific binding also in the presence of Mg^{2+} (Alves et al., 1989). In contrast to *Eco*RI, *Eco*RV, another restriction endonuclease which has also been studied extensively in mechanistic and structural terms, does not show preferential binding to specific sequences in the absence of Mg^{2+}. This is most easily seen in electrophoretic mobility shift assays, in

[†] This work has been supported by grants from the Deutsche Forschungsgemeinschaft (Pi 122/2-4, Pi 122/5-2, and Ma 465/11-6).
[‡] This paper is dedicated to Prof. Dr. Ernst Bayer, University of Tübingen, on the occasion of his 65th birthday.
* To whom correspondence should be addressed.
[§] Present address: Beckman Instruments, Ostracherstrasse 15, D-7000 Stuttgart 80, Germany.

0006-2960/92/0431-3727$03.00/0

which *Eco*RV (Taylor et al., 1991) unlike *Eco*RI (Terry et al., 1985) produces multiple shifted bands with large DNA fragments containing one specific and many nonspecific sites. The apparently nonspecific interaction underlying the DNA binding process of *Eco*RV sharply contrasts with its high cleavage specificity toward the canonical recognition site. Landgraf (1987) and Taylor and Halford (1989) have shown that in pAT153 the canonical *Eco*RV site is cleaved by several orders of magnitude more rapidly than the next best site. We have shown more recently in experiments similar to those reported for *Eco*RI (Thielking et al., 1990) by employing oligodeoxynucleotides as substrates that *Eco*RV is at least as accurate as *Eco*RI: DNA sequences that differ in one base pair from the recognition sequence are cleaved by at least 5 orders of magnitude more slowly than DNA containing the canonical *Eco*RV site, depending on the sequence (Alves et al., manuscript in preparation). One must conclude from the two sets of data, binding data on one side and cleavage data on the other, that *Eco*RV requires Mg^{2+} for specific binding or that binding per se does not contribute to discrimination but rather processes that follow binding and lead to the transition state.

To distinguish between these possibilities, we have analyzed the DNA binding behavior of an *Eco*RV mutant defective in catalysis due to a single amino acid exchange in the active center. Our results demonstrate that this mutant, as wild-type *Eco*RV, shows hardly any specificity in binding to DNA in the absence of Mg^{2+} but in its presence binds to DNA containing the recognition site with strong preference and discriminates against nonspecific sequences. We suggest that wild-type *Eco*RV, like *Eco*RI and other restriction enzymes which have been studied in this respect, preferentially binds to its recognition site but, unlike *Eco*RI, requires the presence of the essential cofactor Mg^{2+} for specific binding. While our results suggest that DNA binding by *Eco*RV is specific, they do not exclude that processes following binding also contribute to the specificity of this enzyme.

Materials and Methods

Site-Directed Mutagenesis and Purification of the D90A Mutant. Site-directed mutagenesis of the *Eco*RV gene was performed according to the gapped-duplex protocol. For a description of the *Escherichia coli* strains and plasmid vectors used, as well as for details of the mutagenesis procedure, see Thielking et al. (1991).

The fermentation of wild-type *Eco*RV and the D90A mutant as well as the purification of the proteins was performed essentially as described earlier for other *Eco*RV mutants (Thielking et al., 1991). For details of the D90A preparation, cf. Selent et al. (1992).

Gel Electrophoretic Mobility Shift Experiments. The 2.5 nM 5′-[^{32}P]-labeled 377mer (the HPLC-purified *Eco*RI/*Bam*HI fragment of pAT153) or 25–2500 nM 5′-[^{32}P]-labeled 20mers, viz.

(I) CATTGTTAGATATCATACAC
TAACAATCTATAGTATGTGC

(II) CATTGTTAGAATTCATACAC
TAACAATCTTAAGTATGTGC

were incubated with wild-type *Eco*RV or the D90A mutant in binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM 2-mercaptoethanol, 2 mM spermine, 0.1 mg/mL bovine serum albumin) containing either 5 mM EDTA or $MgCl_2$ for at least 15 min at room temperature. To 20 μL of this binding mixture was added 5 μL of gel loading buffer [50% (v/v) glycerol, 0.25% (w/v) xylene cyanole, 0.15% (w/v) azorubin in binding buffer]. Electrophoresis was carried out on 20 × 20 cm 6% polyacrylamide gels at room temperature. Samples containing EDTA were run on "normal" TBE-buffered gels (50 mM Tris–borate, pH 8.0, 1.25 mM EDTA), whereas samples containing Mg^{2+} were analyzed on gels with Tris–borate and 5 mM $MgCl_2$ instead of EDTA. Some gel shift experiments were carried out in the presence of unlabeled competitor DNA, d(TATAGAATTCTAT) and d(TATAGATATCTAT), in varying concentrations (0, 0.06, 0.18, 0.54, 1.62, 4.86, and 14.58 μM double-stranded oligodeoxynucleotide, respectively).

The double-stranded 20mers used in the shift experiments were obtained by mixing equimolar amounts of the individual single strands. Due to an uncertainty of the calculated extinction coefficients and small pipetting errors, usually one single strand was in slight excess over the other.

Bands were visualized by autoradiography. For quantitative analysis, the radioactive bands were cut out, the gel slices were submerged in scintillation liquid, and the radioactivity was measured in a scintillation counter. Binding constants were determined using the PC program FTITMER (C. Urbanke, this department), which fits the binding curve to the experimental data using a nonlinear least-squares procedure. For the determination of binding parameters from gel shift experiments, it was assumed that saturation is achieved when all DNA in the assay mixture is shifted. This is a necessary assumption for the evaluation of binding experiments where 100% binding is not achieved.

Mg^{2+} Dependence of Gel Electrophoretic Mobility Shifts. In order to show that the number of retarded bands with a given 377mer and D90A concentration only depends on the amount of Mg^{2+} in binding mixture and gel, a 20 × 20 cm gel with a linear Mg^{2+} gradient for 0 to 5 mM perpendicular to the electric field was poured using a gradient mixer. The two solutions both contained 6% polyacrylamide, 50 mM Tris–borate, pH 8.0, and 0 or 5 mM $MgCl_2$, respectively. To the first solution was added 7.5% Ficoll 400 in order to increase its viscosity and thereby stabilize the gradient while the gel was poured. After polymerization, the gel was turned by 90°, fresh gel solution poured on top, and the comb inserted in such a way that it touched the gradient gel. All samples contained the same concentration of 377mer (2.5 nM) and D90A (20 nM) but varied in their Mg^{2+} concentration (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5 mM $MgCl_2$).

Mg^{2+} Dependence of the EcoRV-Catalyzed Cleavage of DNA. The 16.7 nM plasmid pATRV DNA (a derivative of pAT153 containing an additional *Eco*RV cleavage site replacing the *Ava*I site) was incubated with 0.5 nM *Eco*RV at 37 °C in a buffer containing 20 mM Tris-HCl, pH 7.2, and varying concentrations of $MgCl_2$ and NaCl. After short time intervals, aliquots were stopped by adding agarose gel loading buffer, and reaction products were separated by electrophoresis on 1.2% agarose gels. For quantification of the remaining amount of supercoiled DNA, the ethidium bromide stained gels were photographed and the negatives scanned with an LKB Ultroscan Laser densitometer. The scans were digitized and numerically integrated using the PC program INTEG (J. Greipel, this department). Initial rates of cleavage were calculated from these data.

Results

Binding of the EcoRV–D90A Mutant to a 377-bp Fragment in the Absence and Presence of Mg^{2+}. The 377-bp *Eco*RI–*Bam*HI fragment of pAT153 contains a single *Eco*RV site in a central position. It was used previously by Taylor et al.

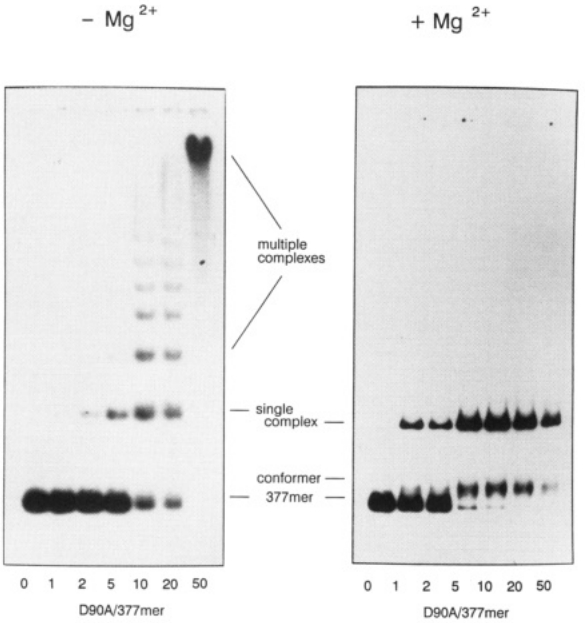

FIGURE 1: Gel electrophoretic mobility shift experiments with D90A and a 377-bp DNA fragment. The 2.5 nM [^{32}P]-labeled 377-bp fragment was incubated with a 0-, 1-, 5-, 10-, 20-, and 50-fold molar excess of D90A and separated in a 6% polyacrylamide gel. The experiment was carried out in the absence (left) and presence (right) of Mg^{2+}.

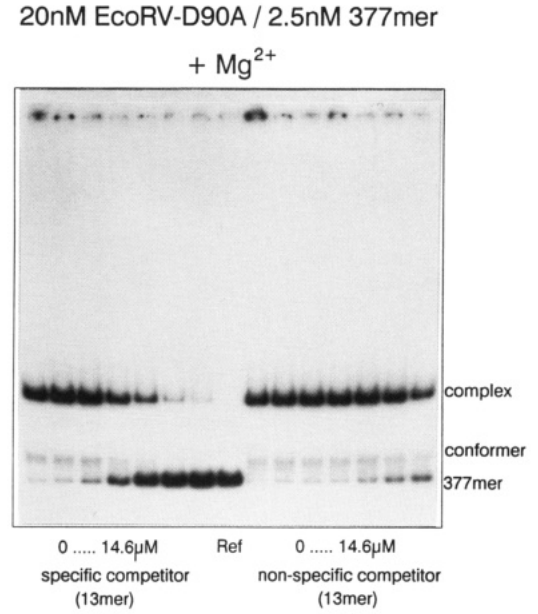

FIGURE 2: Gel electrophoretic mobility shift experiments with D90A and a 377-bp DNA fragment in the presence of competing oligodeoxynucleotides. The 2.5 nM [^{32}P]-labeled 377-bp fragment and an 8-fold molar excess of D90A were incubated with 0, 0.06, 0.18, 0.54, 1.62, 4.86, and 14.58 μM double-stranded specific (left half) or nonspecific (right half) 13mer. In the middle, a reference is shown representing the uncomplexed 377mer.

(1991) and Selent et al. (1992) in electrophoretic mobility shift assays with wild-type *Eco*RV and the enzymatically inactive *Eco*RV mutant D90A. It was demonstrated in these experiments that, in the absence of the essential cofactor Mg^{2+}, the wild-type enzyme as well as the D90A mutant binds to the 377mer in a mainly nonspecific manner, as indicated by several shifted bands at moderate excess of protein over DNA. We have now used the same DNA substrate to investigate the binding of the D90A mutant to DNA in the presence of Mg^{2+} in order to study whether Mg^{2+} confers DNA binding specificity to *Eco*RV. This experiment can only be carried out with a catalytically inactive mutant like D90A, not, however, with the wild-type enzyme which would rapidly cleave the DNA. Figure 1 shows the electrophoretic mobility shift assays with D90A in the absence and in the presence of Mg^{2+}. It can be seen that a band shift appears at a lower concentration of D90A in the presence of Mg^{2+} than in its absence. This implies that Mg^{2+} increases the affinity of the D90A mutant toward DNA. In addition, Mg^{2+} suppresses nonspecific binding of the D90A mutant to DNA, because with Mg^{2+} multiple shifted bands do not appear even at a 50-fold excess of protein over DNA.

In all electrophoretic mobility shift assays with the 377mer and the D90A mutant (see also Figure 2), we have noticed that in the presence of Mg^{2+}, but not, however, in its absence, the main shifted band is accompanied by a second shifted band of higher mobility. As the intensity of this band is always proportional to the intensity of the main shifted band, we believe that it is due to a conformational isomer of the protein–DNA complex (Fried, 1989). It must be emphasized that the "satellite" bands were observed in the same relative intensities with another preparation of the D90A mutant, which argues against a small proteolytic fragment of D90A possibly present as a minor contaminant in the protein preparation to be responsible for the satellite bands. Using a permutation assay, where the position of the *Eco*RV recognition sequence is changed within the length of the DNA fragment, it should be possible to decide whether the conformational isomer is due to bending of the DNA, because this would alter the mobility of this species dependent upon the position of the GATATC site within the fragment. This is currently being investigated for the D90A mutant and, for comparison, other inactive *Eco*RV mutants.

Binding of the EcoRV–D90A Mutant to a 377-bp Fragment in the Presence of Competing Specific and Nonspecific Oligodeoxynucleotides. To demonstrate that the D90A mutant binds to the specific site of the 377mer in the presence of Mg^{2+}, electrophoretic mobility shift assays were carried out with increasing concentrations of competing oligodeoxynucleotides. Figure 2 shows that, in the presence of Mg^{2+}, a 13mer containing an *Eco*RV site, d(TATAGATATCTAT), is much more effective in suppressing the gel electrophoretic mobility shift of the 377mer than another similar 13mer which does not contain an *Eco*RV site, viz., d(TATAGAATTCTAT). From the concentration dependence of the two sets of competition experiments, it can be estimated that the specific oligodeoxynucleotide is by at least a factor of 100 more strongly bound by the D90A mutant than the nonspecific one. An identical competition experiment was also carried out in the absence of Mg^{2+}. Under these conditions, the specific oligodeoxynucleotide does not compete much more effectively in suppressing the appearance of (in this case) multiple shifted bands than the nonspecific one (data not shown). These results indicate that D90A interacts with much greater preference with its recognition sequence in the presence than in the absence of Mg^{2+}.

Mg^{2+} Concentration Dependence of Specific vs Nonspecific Binding of the EcoRV–D90A Mutant to a 377-bp Fragment. The experiments described so far were carried out in the absence or presence of 5 mM $MgCl_2$. As the band shift pattern is different under these conditions, it can be used to titrate the binding of Mg^{2+} to the 377mer–D90A complex. Figure 3 shows a gel electrophoretic mobility shift assay with increasing concentrations of Mg^{2+} using a Mg^{2+} gradient gel. The disappearance of the multiple band pattern between 1.5 and 2.5 mM $MgCl_2$ suggests that Mg^{2+} binds with an equilibrium constant $K_{Diss} \approx 2$ mM to the D90A–DNA complex in 50 mM Tris-HCl, pH 7.5, and 100 mM NaCl. A similar experiment

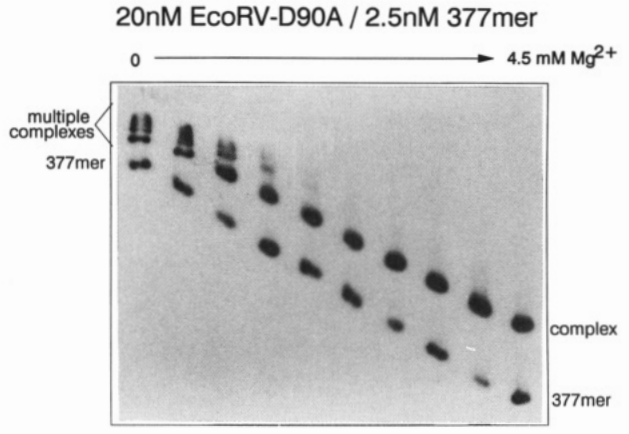

FIGURE 3: Mg^{2+} dependence of specific vs nonspecific binding of D90A to a 377-bp DNA fragment. The 2.5 nM [^{32}P]-labeled 377-bp fragment was incubated with an 8-fold molar excess of D90A in the presence of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5 mM Mg^{2+} and then separated on a 6% polyacrylamide gel with a linear Mg^{2+} gradient from 0 to 5 mM perpendicular to the electric field. The different mobilities of the DNA are not due to the different Mg^{2+} concentrations but mainly to the Ficoll gradient used to stabilize the Mg^{2+} gradient while the gel was poured.

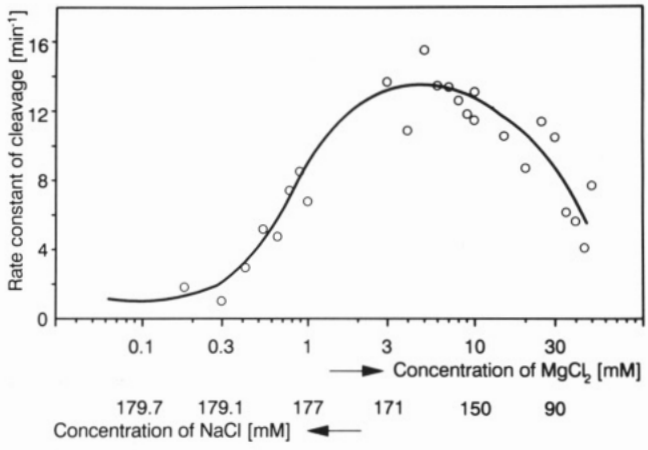

FIGURE 4: Mg^{2+} dependence of pATRV DNA cleavage by wild-type *Eco*RV. 16.7 nM pATRV and 0.5 nM *Eco*RV were incubated at 37 °C in 20 mM Tris-HCl, pH 7.5, and 0.1 up to 50 mM $MgCl_2$; the varying ionic strength due to the increasing amount of $MgCl_2$ was compensated by NaCl.

cannot be done with wild-type *Eco*RV; we know, however, the apparent K_M value for Mg^{2+}, which is ~2 mM in 20 mM Tris-HCl, pH 7.2, and 50 mM NaCl (data not shown) and ~0.7 mM in 20 mM Tris-HCl, pH 7.2, and 178 mM NaCl (Figure 4). Inasmuch as indirect binding experiments (Mg^{2+}-dependent DNA binding of D90A) and steady-state DNA cleavage experiments (Mg^{2+}-dependent DNA cleavage by wild-type *Eco*RV) can be compared, these results suggest that Mg^{2+} binds similarly or slightly more weakly to the D90A–DNA complex than to the wild-type *Eco*RV–DNA complex. It must be emphasized that we have not demonstrated by these experiments binding of Mg^{2+} to wild-type *Eco*RV or to the D90A mutant but only to the respective complexes with DNA. It can be expected that the free proteins bind Mg^{2+} considerably less firmly, as the phosphate oxygen of the substrate is needed as a ligand for strong binding, similarly as described for the exonuclease domain of the Klenow polymerase (Freemont et al., 1988; Mullen et al., 1990; Beese & Steitz, 1991; Han et al., 1991).

Gel Electrophoretic Mobility Shift Assays with D90A and Synthetic 20mers in the Absence and Presence of Mg^{2+}. For direct determination of the affinity of the D90A mutant to

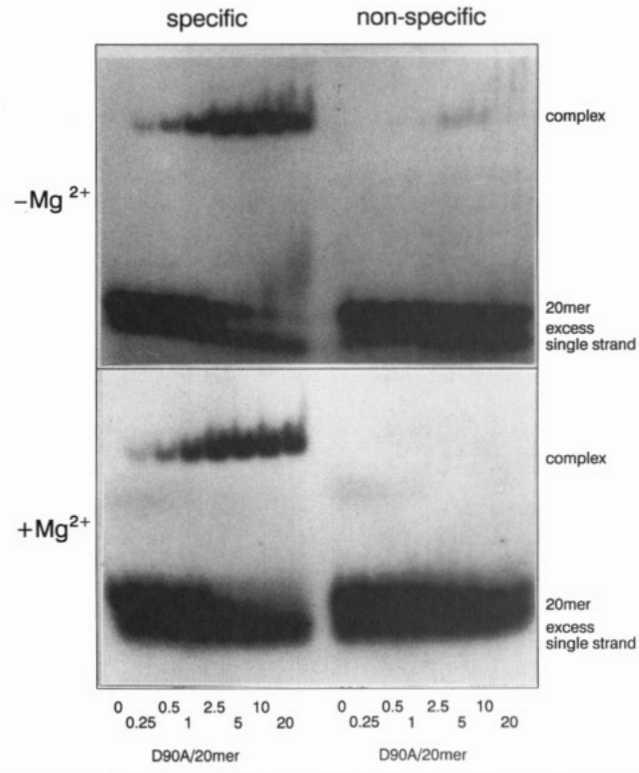

FIGURE 5: Gel electrophoretic mobility shift experiments with D90A and 20mer oligodeoxynucleotides with and without an *Eco*RV recognition site. The 25 nM [^{32}P]-labeled 20mers were incubated in the absence (top) or presence (bottom) of Mg^{2+} with a 0-, 0.25-, 0.5-, 1-, 2.5-, 5-, 10-, and 20-fold molar excess of D90A and separated in a 6% polyacrylamide gel (left, specific 20mer; right, nonspecific 20mer).

Table I: Equilibrium Constants K^{Ass} (M^{-1}) for the Binding of Wild-Type *Eco*RV and the D90A Mutant to 20mer Oligodeoxynucleotides with and without an *Eco*RV Recognition Sequence

	wt *Eco*RV		D90A	
	specific	nonspecific	specific	nonspecific
EDTA	4×10^4	5×10^4	1×10^8	$\sim 1 \times 10^6$
Mg^{2+}			4×10^8	$<10^5$

specific and nonspecific DNA, we have carried out gel electrophoretic mobility shift assays with a synthetic oligodeoxynucleotide that contains an *Eco*RV recognition site (I, specific; *Eco*RV site underlined) or a similar oligodeoxynucleotide with two base-pair exchanges (II, nonspecific), viz.

(I) CATTGTTAGATATCATACAC
TAACAATCTATAGTATGTGC

(II) CATTGTTAGAATTCATACAC
TAACAATCTTAAGTATGTGC

Figure 5 shows gel electrophoretic mobility shift assays with the D90A mutant and these two oligodeoxynucleotides in the absence and presence of Mg^{2+}, respectively. It is obvious that the specific oligodeoxynucleotide is bound much more strongly than the nonspecific one, both in the absence and in the presence of Mg^{2+}. The quantitative analysis shows that the specific oligodeoxynucleotide is bound more efficiently in the presence than in the absence of Mg^{2+}. The nonspecific oligodeoxynucleotide is hardly bound at all in the presence of Mg^{2+}, while it shows some binding in the absence of Mg^{2+} which cannot be quantitated with precision, as at high enzyme concentrations the band shifts become blurred and 100% binding is not achieved. A summary of the quantitative analysis of these data is given in Table I.

Gel Electrophoretic Mobility Shift Assays with Wild-Type EcoRV and Synthetic 20mers in the Absence of Mg^{2+}. Under the same conditions as employed for D90A, wild-type *Eco*RV does not show detectable binding to either the specific or nonspecific oligodeoxynucleotide. With a 100-fold more concentrated solution of the oligodeoxynucleotide, a band shift was observed which allowed an estimate to be given for the equilibrium constant for the binding of wild-type *Eco*RV to specific and nonspecific DNA in the absence of Mg^{2+} (Table I). In agreement with Taylor et al. (1991), wild-type *Eco*RV seems to bind specific and nonspecific DNA with similar affinity. However, with the 20mers used here the affinity determined is by a factor of 28 lower than that observed by Taylor et al. (1991) for a 55mer.

Discussion

Restriction enzymes unlike DNA *binding* proteins (e.g., repressors) can discriminate specific and nonspecific sequences not only by binding but also during the catalytic process. While this is plausible, it is hard to be demonstrated experimentally, because binding and catalysis are connected processes. One way of characterizing the binding process itself is to prevent catalysis to take place, for example, by not supplying the essential cofactor Mg^{2+}. This means, of course, that the effect of Mg^{2+} on the binding process is neglected. With *Eco*RI, the absence of Mg^{2+} does not prevent highly preferential binding to its cleavage site, which allowed identification of potential contacts between the enzyme and its recognition sequence by footprinting and cross-linking techniques (Lu et al., 1981; Wolfes et al., 1986; Kuwabara et al., 1986; Fox, 1988; Becker et al., 1988) as well as determination of protein-induced distortions of the DNA by the specific interaction (Kim et al., 1984; Douc-Rasy et al., 1989; Thompson & Landy, 1989) and study of specific complex formation by spectroscopic (Goppelt et al., 1980; Jhon et al., 1988), electron microscopic (Johannsen et al., 1984), and crystallographic techniques (Kim et al., 1990). Nevertheless, it is likely that Mg^{2+} has an influence on specific complex formation, presumably by allowing additional contacts to be formed via Mg^{2+} which in the *Eco*RI–product complex is sandwiched between Glu111 and the postreactive phosphate (Rosenberg, 1991). The Mg^{2+}-dependent contacts seem to increase the strength of interaction. This is concluded from the finding that *Eco*RI mutants in which Glu111 and Asp91 are replaced by neutral amino acids bind DNA more strongly in the absence of Mg^{2+} than wild-type *Eco*RI (King et al., 1989; Wright et al., 1989; Selent et al., 1992), presumably because Glu111 and Asp91 repel the DNA in the absence of the bridging Mg^{2+} ion.

With *Eco*RV, there is no specific binding in the absence of Mg^{2+} (Taylor et al., 1991), although in the presence of Mg^{2+} this enzyme recognizes its target sequence as accurately as *Eco*RI (Halford et al., 1986; Landgraf, 1987; Taylor & Halford, 1989; Alves et al., manuscript in preparation). This implies that *Eco*RV needs Mg^{2+} ions for specific binding. To investigate this problem, an *Eco*RV mutant was produced which carries an amino acid exchange in the presumptive catalytic center of *Eco*RV (Thielking et al., 1991; Selent et al., 1992; Winkler, 1991). Since this mutant does not cleave DNA, its interaction with DNA can be studied not only in the absence but also in the presence of Mg^{2+}. Quantitative analysis of gel electrophoretic mobility shift assays demonstrates that, in the absence of Mg^{2+}, the D90A mutant binds to DNA in general more strongly than wild-type *Eco*RV, presumably because the replacement of Asp90 by Ala removes a repulsive interaction between the enzyme and the phosphodiester backbone. In contrast to wild-type *Eco*RV, the D90A mutant shows an approximately 100-fold preference for its specific site in the absence of Mg^{2+} (Table I). Presumably, the removal of the repellent group in the catalytic center allows the mutant enzyme to approach the DNA more closely and to form additional, discriminatory contacts. In the presence of Mg^{2+}, which is bound only slightly less firmly by the D90A mutant than by wild-type *Eco*RV, this effect is even more pronounced: the specific interaction of the D90A mutant becomes stronger, while the nonspecific interaction becomes weaker (Table I). We believe that this interaction resembles the interaction of wild-type *Eco*RV with DNA under conditions of catalytic turnover: *Eco*RV binds to DNA, and Mg^{2+} enters the complex by forming contacts both to the protein and to the DNA. This bridging interaction allows *Eco*RV to make additional interactions which are of a repulsive nature when a nonspecific sequence occupies the DNA binding site of *Eco*RV and are attractive ones when a specific sequence is there. These additional interactions are followed by slight readjustments of the protein–DNA interface and put the catalytically relevant amino acid residues in an optimal position with respect to the scissile phosphodiester bond. Mg^{2+}, therefore, confers DNA binding specificity to *Eco*RV by increasing the number of contacts and the binding energy, part of which may be used to distort the DNA, a prerequisite for cleavage to occur, and to activate the catalytic center. This sequence of events, which might occur in a concerted manner, differs in many respects from *Eco*RI, although *Eco*RI has a very similar catalytic center (Thielking et al., 1991; Selent et al., 1992; Winkler, 1992). The differences are highlighted by the fact that *Eco*RI binds specifically to its recognition sequence in the presence as well as in the absence of Mg^{2+}. One possibility is that these differences in binding behavior are related to the fact that *Eco*RV is a blunt-end cutter while *Eco*RI produces sticky ends. The implication of this distinction is that in *Eco*RV recognition occurs in the center of the catalytic site, while it occurs at the edges of the catalytic site in *Eco*RI. One could expect that blunt-end cutters in general are more likely to bind specific and nonspecific DNA sequences with equal affinity in the absence of Mg^{2+} than sticky-end cutters. Available information supports this notion, as for other sticky-end cutters, like *Bam*HI (Xu & Schildkraut, 1991) and *Rsr*I (Aiken et al., 1991), a behavior similar to *Eco*RI was reported, while for a blunt-end cutter, *Taq*I, a behavior similar to *Eco*RV was demonstrated (F. Barany, personal communication). We are currently testing this hypothesis with a large set of different restriction enzymes.

Acknowledgments

We are indebted to Dr. F. Winkler for supplying detailed information on the structure of the specific *Eco*RV–DNA complex. We thank Dr. G. Maass for stimulating discussions, Dr. B. Connolly and Dr. S. Halford for communicating information prior to publication, and Dr. J. Greipel, Dr. F. Peters, and Dr. C. Urbanke for providing computer programs. The technical assistance of Mrs. R. Kaysser, Mr. R. Mull-Grotefend, Ms. K. Wordelmann, and Mrs. K. Zander is gratefully acknowledged. Thanks are due to Mrs. E. Schuchardt for typing the manuscript.

Registry No. *Eco*RV, 83589-02-0; Mg^{2+}, 7439-95-4.

References

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

Alves, J., Urbanke, C., Fliess, A., Maass, G., & Pingoud, A. (1989) *Biochemistry 28*, 7879–7888.

Becker, M. M., Lesser, D., Kurpiewski, M., Baranger, A., & Jen-Jacobson, L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6247–6251.

Beese, L. S., & Steitz, T. A. (1991) *EMBO J. 10*, 25–33.

Bennet, S. P., & Halford, S. E. (1989) *Curr. Top. Cell. Regul. 30*, 57–104.

Clore, G. M., Gronenborn, A. M., & Davies, R. W. (1982) *J. Mol. Biol. 155*, 447–466.

Douc-Rasy, S., Kolb, A., & Prunell, A. (1989) *Nucleic Acids Res. 17*, 5173–5189.

Fox, K. R. (1988) *Biochem. Biophys. Res. Commun. 155*, 779–785.

Freemont, P. S., Friedman, J. M., Beese, L. S., Sanderson, M. R., & Steitz, T. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8924–8928.

Fried, M. G. (1989) *Electrophoresis 10*, 366–376.

Goppelt, M., Pingoud, A., Maass, G., Mayer, H., Köster, H., & Frank, R. (1980) *Eur. J. Biochem. 104*, 101–107.

Halford, S. E., Lovelady, B. M., & McCallum, S. A. (1986) *Gene 41*, 173–181.

Han, H., Rifkind, J. M., & Mildvan, A. S. (1991) *Biochemistry 30*, 11104–11108.

Jhon, N.-I., Casas-Finet, J. R., Maki, A. H., & Modrich, P. (1988) *Biochim. Biophys. Acta 949*, 189–194.

Johannsen, W., Schütte, H., Mayer, H., & Meyer, F. (1984) *Arch. Microbiol. 140*, 265–270.

Kim, R., Modrich, P., & Kim, S.-H. (1984) *Nucleic Acids Res. 12*, 7285-7292.

Kim, Y., Grable, J. C., Love, R., Greene, P. J., & Rosenberg, J. M. (1990) *Science 249*, 1307–1309.

King, K., Benkovic, S. J., & Modrich, P. (1989) *J. Biol. Chem. 264*, 11807–11815.

Kuwabara, M., Yoon, C., Gayne, T., Thederahn, T., & Sigman, D. S. (1986) *Biochemistry 25*, 7401–7408.

Landgraf, A. (1987) Diploma Thesis, Universität Hannover, Hannover, Germany.

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

Lu, A.-L., Jack, W. E., & Modrich, P. (1981) *J. Biol. Chem. 256*, 13200–13206.

Mullen, G. P., Serpersu, E. H., Ferrin, L. J., Loeb, L. A., & Mildvan, A. S. (1990) *J. Biol. Chem. 265*, 14327–14334.

Pingoud, A., Alves, J., Fritz, A., Köhler, E., Liedtke, M., Oelgeschläger, T., Rüter, T., Selent, U., & Thielking, V. (1991) *Biol. Chem. Hoppe-Seyler 372*, 731.

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

Selent, U., Rüter, T., Köhler, E., Liedtke, M., Thielking, V., Alves, J., Oelgeschläger, T., Wolfes, H., Peters, F., & Pingoud A. (1992) *Biochemistry* (submitted for publication).

Steitz, T. A. (1990) *Q. Rev. Biophys. 23*, 205–280.

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

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

Terry, B. J., Jack, W. E., Rubin, R. A., & Modrich, P. (1983) *J. Biol. Chem. 258*, 9820–9825.

Terry, B. J., Jack, W. E., & Modrich, P. (1985) *J. Biol. Chem. 260*, 13130–13137.

Thielking, V., Alves, J., Fliess, A., Maass, G., & Pingoud, A. (1990) *Biochemistry 29*, 4682–4691.

Thielking. V., Selent, U., Köhler, E., Wolfes, H., Pieper, U., Geiger, R., Urbanke, C., Winkler, F. K., & Pingoud, A. (1991) *Biochemistry 30*, 6416–6422.

Thompson, J., & Landy, A. (1989) *Nucleic Acids Res. 16*, 9687–9705.

Uchida, K., Pyle, A. M., Morii, T., & Barton, J. K. (1989) *Nucleic Acids Res. 17*, 10259–10279.

Winkler, F. (1992) *Curr. Opin. Struct. Biol. 2*, 93–99.

Wolfes, H., Fliess, A., Winkler, F., & Pingoud, A. (1986) *Eur. J. Biochem. 159*, 267–273.

Wright, D. J., King, K., & Modrich, P. (1989) *J. Biol. Chem. 264*, 11816–11821.

Xu, S.-Y., & Schildkraut, I. (1991) *J. Biol. Chem. 266*, 4425–4429.