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Site-Directed Mutagenesis Studies with Restriction Endonuclease *Eco*RV to Identify the Role of Ile91 in Recognition and Catalysis

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Abstract: Site-directed substitutions were made to change the Ile91 of restriction endonuclease *Eco*RV to either Val, Ala or Gly to identify the role of Ile91 in recognition and catalysis, since substitution of Ile91 with Leu afforded dramatic effects on the activity and properties of restriction endonuclease *Eco*RV. These changes alter the size of the hydrophobic side chain at position 91 and thus might have revealed the reason for the altered phenotype of Ile91Leu. However, the properties of Ile91Val and Ile91Ala mutants were much like wild type *Eco*RV, in both activity and metal ion preference. Ile91Gly had very little activity with either Mg^{2+} or Mn^{2+} as cofactors. To try to understand the unusual Mn^{2+} profile of the Ile91Leu mutant, two double mutants, Ile91Leu;Asp90Asn and Ile91Leu;Glu45Met were created. Both double mutants were seriously disabled by the second amino acid change. Ile91Leu;Glu45Met had some residual activity in the Mn^{2+} reaction buffer, whereas the Ile91Leu;Asp90Asn displayed no detectable activity.
Key words: restriction endonuclease *Eco*RV, site-directed mutagenesis.

*Eco*RV is a type II restriction endonuclease (Bennet and Halford, 1989). It recognizes and cleaves DNA at the GATATC sequence, leaving blunt-ended fragments (Kholmina *et al.*, 1980). *Eco*RV exists as a dimer of 2 identical subunits and it requires Mg^{2+} for cleavage. The structures of the free protein bound to both specific and non-specific DNA molecules have been solved by X-ray crystallography (Winker, 1992). The complexes were crystallized in the absence of Mg^{2+}, which is an essential cofactor for DNA cleavage conditions. From the crystal structures obtained, it was seen that the DNA in the specific complexes lies in the cleft between the 2 subuints of the dimer with its minor groove facing the bottom of the cleft and major groove facing the entrance. One peptide loop centered on Asn 70 approaches the minor groove, and all its interactions with the DNA are with the phosphates rather than the bases. Another loop centered on Asn185 penetrates deep into the major groove and interacts with the bases. The amino acids located in these two loop regions are responsible for DNA recognition (Theiking *et al.*, 1991). Crystallographic analysis has revealed the presence of a cluster of 3 acidic amino acids (Glu45, Asp 74 and Asp90) and 1 basic amino acid (Lys92) in close proximity to the scissile phosphodiester bond. When *Eco*RV binds to the specific DNA, the DNA distorts and the conformation of the protein changes (Winker, 1992). This brings the amino acids in the catalytic center close to the scissile phosphodiester bond. Asp74 and Asp90 are now oriented in a position favorable for binding Mg^{2+}. Mg^{2+} polarizes the scissile P-O bond, enhancing its susceptibility to nucleophilic attack. The hypothesis put forward by Pingoud and his coworkers (Jeltsch *et al.*, 1992) suggests that an acidic amino acid (either Asp74 or Asp90) activates a water molecule which then attacks the scissile bond. Mg^{2+} may be involved in binding the attacking water molecule. The extra negative charge of the pentacovalent phosphorus formed upon attack by the water molecule is compensated for by Lys92. Lys92 therefore serves to stabilize the transition state. The hydrolytic reaction proceeds with inversion of the configuration around phosphorous (Grasy and Connolly, 1992). The hypothesis accounts for why *Eco*RV binds all DNA sequences (both specific and non-specific) with equalaffinity in the absence of Mg^{2+}, but cleaves its recognition site faster than other DNA sequences when Mg^{2+} is present. There is coupling between recognition and cleavage via formation of a Mg^{2+} binding site.

Random mutagenesis studies (Vipond *et al.*, 1994) and site-directed mutagenesis studies (Moon *et al.*,

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1995) have identified that the substitution of Ile91 by Leu leads to enzyme activity being decreased a thousandfold compared to wild type. In addition to low activity, its metal ion dependency was also altered. Considering the fact that Ile91 does not take part directly in catalytic activity and a conservative substitution could not yield such drastic consequences on the activity and properties of the enzyme, we carried out a further investigation on *Eco*RV. Further site-directed substitutions were made to change the Ile to either Val, Ala or Gly. These changes alter the size of the hydrophobic side chain at position 91 and thus might have revealed the reason for the altered phenotype of Ile91Leu.

To try to understand the unusual Mn^{2+} dependency of the Ile91Leu mutant (Moon *et al.*, 1995), two further mutations were created at position 45 and 90. Two double mutants, Ile91Leu;Asp90Asn and Ile91Leu;Glu 45Met, were created, the proteins purified and their activities measured *in vitro*.

Materials and Methods

DNA manipulations

General manipulations of DNA were carried out as in Sambrook *et al.* (1989) DNA sequencing was by the dideoxy method (Sanger *et al.*, 1977). Oligonucleotide was synthesized at the Department of Biochemistry, University of Bristol, UK. Restriction enzymes and other DNA modifying enzymes were purchased and used as advised by the supplier. *Eco*RV was purified here.

Mutagenesis

Site-directed mutagenesis was performed by the modification of the Amersham mutagenesis system (code-RPN1523), which is based on the phosphorothioate method of Fritz Eckstein and his co-workers (Taylor *et al.*, 1985). The single strand template pRV18 was used. pRV18 is a derivative of pBluescript (Stratagene) carrying the *Eco*RV gene with a 30 base pair stuffer fragment to inactivate the gene (Vermote *et al.*, 1992). Positive clones were screened by restriction enzyme analysis and verified by sequencing the entire gene.

Enzyme overproduction and purification

The derivative of the pRV18 coding for the Ile91Leu mutant of *Eco*RV was used to transform *E. coli* CSH50 carrying pMetB. The transformant was grown in L-broth at 55℃ to an OD_{600} of 0.4. An equal volume of L-broth at 55℃ was added and the growth continued for 4 h at 42℃. The cell was harvested by centrifugation and stored at −20℃. The cells were resuspended and disrupted by sonication, and the mutant *Eco*RV enzyme was purified by chromatography, first on phosphocellulose and then on Blue-Sepharose, as described previously with wt enzyme (Luke *et al.*, 1987). The purifications were monitored by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; the gels were developed with Coomasive Brilliant Blue-R-250. Protein concentrations were determined by the method of Bradford (1976) after calibration of the method by amino acid analysis. Concentrations of both wild type and mutant *Eco*RV enzymes are given in terms of protein dimers of M_r 57000.

DNA binding

The binding of the wild type and mutant *Eco*RV enzymes to DNA was measured by gel-shift assays, as described by Taylor *et al.* (1991). The binding buffer was 50 mM Tris-HCl, 100 mM NaCl, 10 mM β-mercaptoethanol, 100 μg/ml BSA, and 0.1 mM EDTA, pH 7.5.

DNA cleavage

The substrate was the monomeric forms of pAT153 (Twigg and Sherratt, 1980), purified from transformants of *E. coli* HB101 that had been grown in M9 minimal salts with 1 mCi/l[methyl-^{3}H]thymidine(Halford and Goodall, 1988). The reactions with enzyme were carried out at 20℃ in buffer containing 50 mM Tris-HCl, 100 mM NaCl, 10 mM β-mercaptoethanol, 100 μg/ml bovine serum albumin, pH 7.5. with either $MgCl_2$ or $MnCl_2$ at the concentrations specified. During each reaction, the changes in the concentrations of the supercoiled, open-circle, and linear forms of the DNA were measured as described previously (Halford and Goodall, 1988; Taylor and Halford, 1989).

Results and Discussion

Mutagenesis

The mutant oligonucleotide is designed such that the third base in the codon on the coding strand coding for Ile89 is changed, destroying the *Cla*I site (ATCGAT) but Ile89 is retained owing to the degenerate nature of the genetic code. This allowed us to screen for cells that have been transformed by mutant DNA through a simple restriction analysis with *Cla*I. Absence of this cleavable *Cla*I site would mean that the cells have been transformed with mutant DNA. Also the codon for Ile 91 has been altered such that all the 3 homologous mutants, Ile91Val, Ile91Ala, Ile91Gly, can be obtained by substituting the second base of the codon for residue 91 with C, G or T gave 3 different residues, Ala, Gly and Val, respectively. There are 2 *Cla*I sites in the *Eco*RV cassette used. During mutagenesis a *Cla*I site was mutated and the absence of this cleavable

*Cla*I site meant that the cell had been successfully transformed with mutated DNA. Positive clones were selected by *Cla*I restriction analysis. For the double mutant Ile91Leu;Asp90Asn, the mutant oligonucleotide is designed such that the first base, G in the codon for Asp 90 is substituted with A, changing the codon for Asp to Asn and destroying the *Cla*I site. The codon for Ile91 is substituted has been altered such that the first base of the codon for residue 91 with C for the Leu residue. Another double mutant Ile91Leu;Glu45Met was obtained by substituting the first twobases (GA) of the codon for residue 45 with AT in the codon on the coding strand coding for Ile91Leu. The codon for Ser 48 has been altered such that the third base in the codon was changed to generate the *Xho*I site for restriction analysis. Within the *Eco*RV cassette we used there is a stuffer fragment (Vermote *et al.*, 1992) containing 6 stop codons to prevent overexpression of the protein which would otherwise kill the cells. It was removed using *Pst*I, and the fragments ligated back. There is an *Eco*RV site present in the stuffer fragment and *Eco*RV was added then to select for molecules that have religated back without the stuffer fragment. The colonies from activated clones were grown overnight and the cultures were then inoculated into fresh medium, grown until turbid and later induced to overexpress *Eco*RV mutants.

Purification of *Eco*RV mutants

The purification of mutant proteins from the cell lysates was carried out essentially as described previously with wild type enzyme (Luke *et al.*, 1987). The purifications of the proteins by chromatography on phosphocellulose gave reasonably pure proteins by polyacrylamide gel electrophoresis.

Futrher purification by affinity chromatography on Blue-Sepharose afforded homogeneous proteins. However, the protein Gly at position 91 was unstable and could only be purified in low yield. SDS-Polyacrylamide gel electrophoresis shows smaller fragment bands below the main band.

DNA cleavage experiments with the mutants

The DNA cleavage activities of *Eco*RV and *Eco*RV mutants were determined with pAT153 as substrate, in the presence of Mg^{2+} or Mn^{2+}, respectively. Relative DNA cleavage activity of *Eco*RV mutants are shown in Table 1. Under standard reaction condition, the Ile 91Leu mutant shows over 1000-fold less activity than wild type *Eco*RV. But surprisingly, Ile91Val and Ile91 Ala mutants showed relatively strong cleavage activity. They showed only 3-fold less activity than wild type *Eco*RV. When we consider hydrophobic side chains of Leu, Val, and Ala, it is difficult to explain the above results on the basis of size of the side chain alone. The Mg^{2+} dependency of the reaction was also examined. As reported before (Moon *et al.*, 1995), the Mg^{2+} dependency of the Ile91Leu mutant was different from that of the wild type *Eco*RV where the reaction rates were invariant with increasing concentration of $MgCl_2$ (Halford and Goodall, 1988). The activity of the Ile91 Leu mutant enzyme increased with the magnesium ion concentration, indicating that the mutant was not fully saturated with the metal ion over the range studied (Vermote and Halford, 1992). But the Mg^{2+} dependency Ile91Val and Ile91Ala mutants were much like wild type *Eco*RV (Fig. 1). Maximal activity was obtained at the lowest concentration of $MgCl_2$ tested (1 mM). It is due to high affinity of the mutant-DNA complex for Mg^{2+} ions. These results indicate that the Ile to Val or Ala substitution has somehow less perturbed the metal binding site of the enzyme than to Leu. In contrast to wild type enzyme, the Ile91Leu mutant prefers Mn^{2+} to Mg^{2+} as the cofactor. In the Mn^{2+} buffer, the mutant is as active as the wild type enzyme in Mg^{2+} buffers. However, the rates of DNA cleavage by Ile91 Val or Ile91Ala mutant were slower in $MnCl_2$ than in $MgCl_2$ (Table 1). The Mn^{2+} activity profiles of Ile91Val and Ile91Ala were very similar to wild type enzyme. In contrast to Ile91Leu, of which the activity was decreased with increasing concentration of Mn^{2+}, the maximal activity of Ile91Val or Ile91Ala was obtained at a low concentration of $MnCl_2$ (1 mM) and was not decreased with increasing concentration of Mn^{2+} tested (10 mM). Ile91Gly mutant had very little activity with either Mg^{2+} or Mn^{2+} as cofactors. Therefore, the effect of Ile91Leu substitution is difficult to reconcile on the basis of the size of the side chain alone.

To try to understand the unusual Mn^{2+} profile of Ile91Leu, two double mutants, Ile91Leu;Asp90Asn and Ile91Leu;Glu45Met were created. Two proteins were purified and their activities were measured. If the Ile91

Table 1. Relative DNA cleavage activity of *Eco*RV mutants

Enzymes	Relative activity	
	with Mg^{2+}	with Mn^{2+}
wild type	100	5
Ile91Leu	0.1	190
Ile91Val	35	2
Ile91Ala	30	1
Ile91Gly	0.05	0.05
Ile91Leu;Glu45Met	no detectable	0.03
Ile91Leu;Asp90Asn	no detectable	no detectable

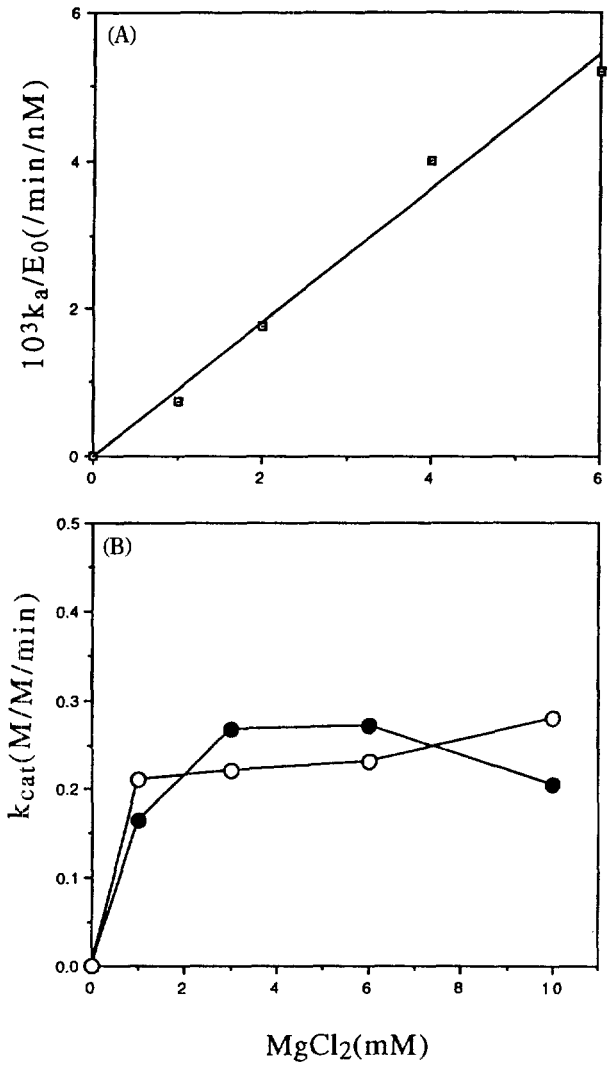

Fig. 1. Magnesium concentration dependencies. (A) Ile91Leu *Eco*RV mutant. For each reaction, a first-order rate constant (ka) was evaluated from the decline in the amount of supercoiled DNA. The rate constants were normalized against the concentration of the enzyme, to produce values for k_a/[Eo]. (B) Ile91Val and Ile91Ala *Eco*RV mutants. Values for kcat were determined from the steady-state reaction velocities. The open circles (○) represent the magnesium dependecy curve for Ile91Val and the closed circles (●) represent the magnesium dependecy curve for Ile91Ala.

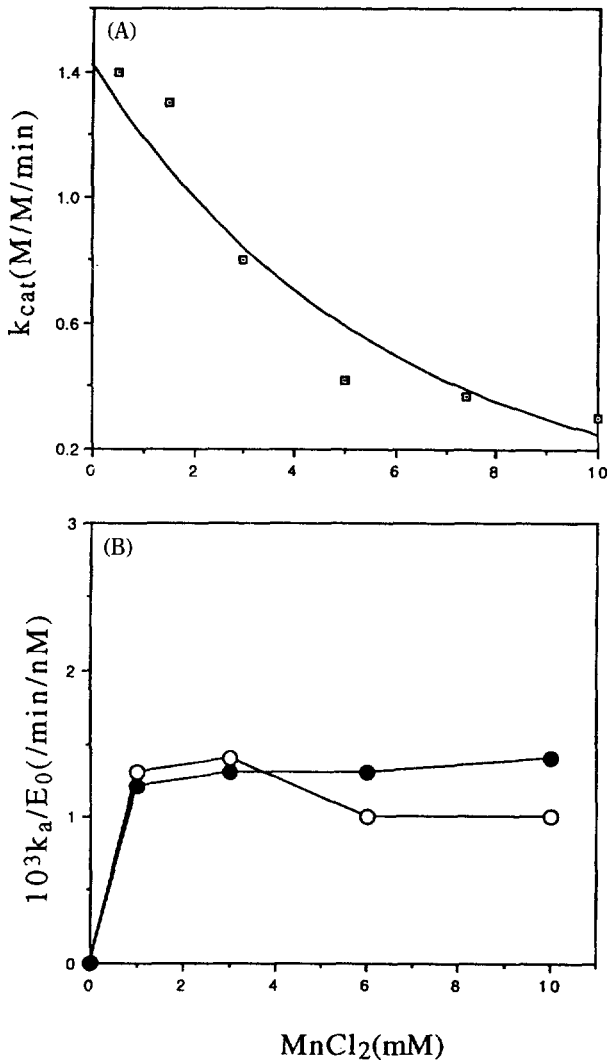

Fig. 2. Manganese concentration dependencies (A) Ile91Leu *Eco*RV mutant. Values for kcat were determined from the steady-state reaction velocities. (B) Ile91Val and Ile91Ala *Eco*RV mutants. For each reaction, a first-order rate constant (k_a) was evaluated from the decline in the amount of supercoiled DNA. The rate constants were normalized against the concentration of the enzyme, to produce values for k_a/[Eo]. The open circles (○) represent the manganese dependecy curve for Ile91Val and the closed circles (●) represent the manganese dependecy curve for Ile91Ala.

Leu mutant prefers a single metal ion in the active site, then the conversion of one of these residues to an amino acid incompatible with metal binding should not affect the activity of the enzyme. On the contrary, if there are two metal binding sites in the enzyme's active site and the Ile91Leu mutant has altered the geometry of the enzyme in a way that causes binding of the second metal ion to disrupt the active conformation, then the conversion of one of these residues to an amino acid incompatible with metal binding should affect the activity of the enzyme. The active site of *Eco*RV contains two potential sites for binding divalent metals: one between Asp90 and Asp74 and another between Asp74 and Glu45. We were especially interested in these mutants, since the mutants might give some kind of information on metal binding sites at the active site of wild type *Eco*RV. Two double mutants were seriously disabled by the second amino acid change. Ile91Leu;Glu45Met had some residual activity, being about 100 fold less active than Ile91Leu in Mn^{2+} reaction buffers, whereas the Ile91Leu;Asp90Asn double mutant displayed no detectable activity under all conditions tested. The Ile91Leu mutant thus needs the right side chains at positions 45 and 90 as much

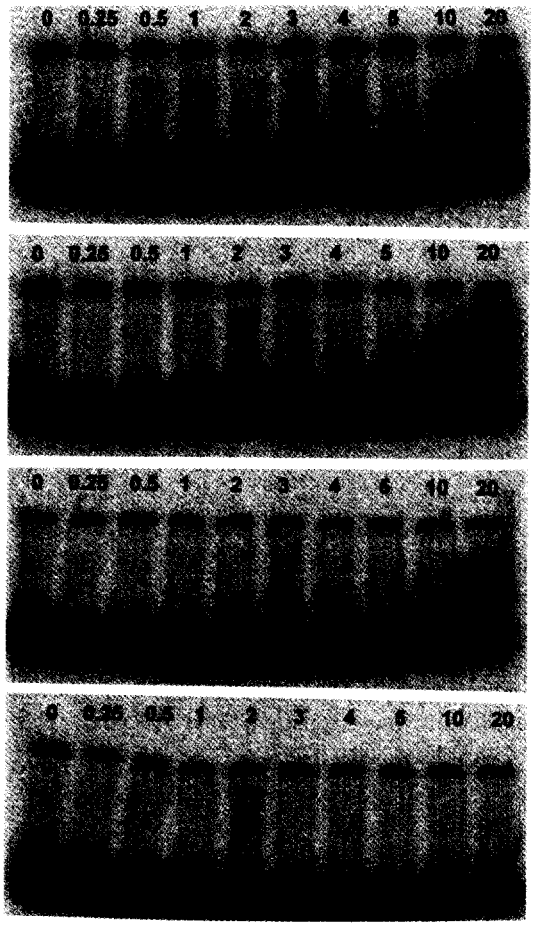

Fig. 3. Gel shift experiments with *Eco*RV-DNA complexes (A), Ile91Leu mutant-DNA complexes (B), Ile91Val mutant-DNA complexes (C), and Ile91Gly mutant-DNA complexes (D). The reactions contained the restriction endonucleases at concentrations (nM) indicated above each lane and approximately 0.1 nM ^{32}P-labeled DNA in binding buffer. Lane 0 is in the absence of the enzyme.

as the wild type enyzme, presumably because it also needs to bind metal ions at both the 90/74 and the 74/45 sites for catalytic activity. Recently, a mechanism for phosphodiester hydrolysis by *Eco*RV that invoves two metal ions was proposed (Viopnd *et al.*, 1995). The Ile91Leu mutant and wild type *Eco*RV seem to employ the same chemical mechanism for phosphodiester hydrolysis.

DNA binding by the *Eco*RV mutants

The binding properties of the *Eco*RV mutants to DNA were compared with those of the wild type *Eco*RV (Fig. 3). The binding studies were carried out by the gel shift method (Garner and Revzin, 1981; Taylor *et al.*, 1991), under conditions where the DNA cannot be cleaved due to the absence of divalent ions. The gel shift method is based on DNA-protein complexes having different electrophoretic mobilities from the free DNA. It can be used to separate the free DNA from DNA-protein complexes, but complexes with different protein stoichiometries on the same DNA can also be separated from each other (Fried, 1989). Consequently, if the low activity of the mutant is caused by low affinity of the enzyme to DNA, gel shift analysis would show a different pattern compared to that of wild type enzyme. The DNA used in the binding studies was the same 381 bp *Eco*RI-*Hin*dIII fragment from pAT153, ^{32}P-labeled at its 5′-ends, that had been used before for binding studies on wild type *Eco*RV (Taylor *et al.*, 1991; Thielking *et al.*, 1992). This fragment contains the *Eco*RV site from pAT153 and an additional 366 nonspecific site for a protein like *Eco*RV that covers about 15 bp, though only 25 or so of these sites can be occupied on each DNA molecule at any given time (Taylor *et al.*, 1991). Fig. 3 shows that both mutants Ile91Leu and Ile91Val yielded the same array of DNA-protein complexes as the wild type restriction enzyme. Like wild type *Eco*RV, both mutants gave the same pattern for non-specific binding as that shown for Ile91Leu. But Ile91Gly showed quite a different pattern of DNA binding. Gel-shifts with Ile91Gly failed to reveal any discrete DNA-protein complexes. We attribute the impaired DNA binding of the Ile91Gly mutant to be at least in part due to an altered protein structure. This result might explain the fact that the Ile91Gly mutant was too unstable to purify and had little activity and a different metal ion dependency.

Despite the availability of extensive X-ray crystallography data on *Eco*RV, both with and without specific and non-specific DNA (Winker, 1992), the precise role of Ile91 can only be guessed at. In crystal structures, the side chain of Ile91 points towards Glu45, so some subtle conformational packing at this metal binding site may be responsible for the characteristics of the mutated enzyme. The instability of Ile91Gly reveals that the bulk of the side-chain at position 91 helps maintain the correct overall structure of the enzyme. With Leu at position 91, the bulk of the side chain moved away from the peptidyl main chain of the protein, whereas Ala and Val retained space filling groups at the base of the side chain adjacent to the C-alpha position. However, the conformation of the *Eco*RV protein is highly flexible (Winkler *et al.*, 1993). These results suggest that minor alterations of the protein, at residues that have no direct role in either DNA recognition or catalysis, can thus have dramatic consequences on the activity and properties of the enzyme by perturbing the alignment of the functional groups that are essential for either DNA recognition or catalysis. The inactivity of the Asp90Asn;Ile91Leu variant confirms an essential role for Asp90 in the catalytic process. The Glu45Met; Ile91Leu mutant retains some trace activity, showing

that Glu45 also has a critical role in maintaining the activity of the Ile91Leu protein.

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