Restriction Endonuclease *Eco*RV Mutants that Switch Metal Ion Requirement

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Divalent metal ions, normally Mg^{2+}, are essential for both DNA cleavage by the *Eco*RV restriction endonuclease at its recognition sequence, GATATC[1], and also for the enzymes discrimination between this particular sequence and all other sequences.[2] In the absence of divalent metal ions, *Eco*RV demonstrates no catalytic activity, though it can still bind to DNA in a nonspecific manner with no preference in its recognition sequence.[3] The complex of *Eco*RV and its cognate DNA, however, has a high affinity for Mg^{2+} due to the distortion of the bound DNA, creating a metal-binding site between the protein and the DNA.[4] In contrast, *Eco*RV bound to nonspecific DNA has low affinity for Mg^{2+}. In this case, the lack of distortion leaves the DNA too far away from the active site to allow a metal ion to be liganded by both protein and DNA.[5] Thus, the metal ion effectively creates the specificity of *Eco*RV for its recognition site by locking the protein onto DNA at this sequence.[6] Other metal ions in place of Mg^{2+} perturb both the activity and the specificity of *Eco*RV.[7] In the presence of Mn^{2+}, *Eco*RV has a lower reaction rate (k_{cat}) at its recognition site but a higher rate at noncognate sites, with the result that the ratio of the DNA cleavage rate at cognate and noncognate sites alternates from a value of 1×10^6 with Mg^{2+} to 6 with Mn^{2+}.[8] The lack of discrimination with Mn^{2+} stems from both cognate and noncognate complexes having high affinities for this ion, but why the noncognate complex should have so much higher an affinity for Mn^{2+} than for Mg^{2+} is yet to be explained. The switch from Mg^{2+} to Mn^{2+} also perturbs both the mechanism and specificity of other restriction enzymes such as TaqI.[9] The *Eco*RV endonuclease is thought to require two metal ions per active site in order to catalyze phosphodiester hydrolysis. One Mg^{2+} (or Mn^{2+}) is located between Asp90 and Asp74 and the second ion between Asp74 and Glu45.[2] It has also been shown that an Ile91 to Leu mutation switches metal ion specificty from Mg^{2+} to Mn^{2+}.[12]

A mutant restriction endonuclease that recognizes a novel DNA sequence would be more useful than one recognizing a novel metal ion, but this is yet to be achieved.[10] The conversion of a restriction enzyme to a new sequence specificity *in vivo* demands the parallel conversion of the modification methyltransferase to the same sequence.[11] A mutant Ile91Leu *Eco*RV restriction endonuclease that switches the cofactor requirement from Mg^{2+} to Mn^{2+} suggests the circumvention of the need for a methyltransferase, inactivity in vivo coupled to high activity under non-physiological conditions *in vitro*.[12] In a search for enzymes that recognize a novel DNA sequence or a novel metal ion, we carried out site directed mutagenesis of *Eco*RV restriction endonuclease. We report here the mutant enzymes that showed novel metal ion specificity. Several mutations were created by changing amino acid residues in the vicinity of the scissile phosphodiester bond in the *Eco*RV-DNA complex by site-directed mutagenesis. Table 1 shows the results of cleavage of plasmid pAT153 by mutant enzymes in the presence of a variety of added metal ions. Wild type enzyme is most active with Mg^{2+} as the cofactor than with any other divalent ion including Mn^{2+}. Under standard conditions (10 mM $MgCl_2$, 100mM NaCl, pH 7.5), all the mutants were much less active than wild type. Two mutant enzymes, however, showed different metal ion specificity. Asp90Cys mutant in which aspartate at position 90 was replaced by cystein was synthesized. Initially, we attempted to have a mutant that has a high activity with Zn^{2+} since the thiol group is known as a very good ligand for Zn^{2+}. But the mutant Asp90Cys preferred Mn^{2+} over Zn^{2+} as the cofactor. The mutant enzyme showed 100 times less activity than wild type at standard reaction conditions. In the presence of Zn^{2+}, the mutant enzyme showed activity that was close to that of the wild type enzyme.

But it showed much lower activity with Zn^{2+} than with Mg^{2+} or Mn^{2+}. The Mn^{2+} activity profile of the mutant enzyme showed a decrease in activity with increasing concentration of Mn^{2+} (data not shown). The same Mn^{2+} activity profile has been shown with the reported Ile91Leu mutant.[12] Interestingly, the mutant enzyme showed higher sequence specificity than wild type enzyme. Plasmid pAT153 contains, in addition to one *Eco*RV recognition site, 12 noncognate sites that can be cleaved by *Eco*RV under star conditions with high concentration of the enzyme.[13] The wild type *Eco*RV cuts noncognate sites at high concentration of enzyme or under star conditions, but Asp90Cys mutant enzyme did not cut noncognate sites even at high concentra-

Table 1. Realtive DNA Cleavage Activities of Mutant *Eco*RV enzymes

	Relative Activity			
Enzyme	with Mg^{2+}	with Mn^{2+}	with Cu^{2+}	with Zn^{2+}
wild type	1	0.05	none detected	0.0001
Ile91Leu	0.001	1.9	none detected	none detected
Asp90Cys	0.01	0.4	none detected	0.0001
Asp90Cys+ Ile91Leu	0.01	0.1	1[a]	none detected

[a]The activity was measured by observing of the changes in the concentrations of the supercoiled and open-circle DNA.

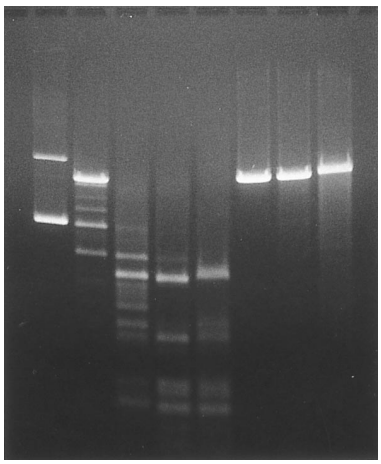

Figure 1. Reactions at noncognate sites on pAT153 by wild type *Eco*RV and Asp90Cys mutant. Reactions in buffer contained 10 nM DNA with Mn^{2+} instead of Mg^{2+}. The concentrations of *Eco*RV (lane 1-5) were 0, 1, 10, 100, 1000 nM and the concentration of the mutant (lane 6-8) were 10, 100, 1000 nM, respectively. The top bands (lane 2, 6,7,8) are initial full length linear form from super coiled DNA. Lanes 2, 3, 4, 5 show several small fragments produced from initial products.

tion of the enzyme or under star conditions (Figure 1). The mutant appeared very similar to Ile91Leu mutant in properties of Mn^{2+} dependency and sequence specificity under star conditions.[12]

Since substitution either at position Asp90 or Ile91 created the mutant that switches the cofactor requirement from magnesium to manganese (Table 1), two further simultaneous mutation at position Asp and Ile were created in order to examine whether simultaneous mutations at the two position has a synergetic effect or not. A double mutant Asp90Cys +Ile91Leu was synthesized. Under standard reaction condition, the double mutant showed about 100 times less activity than wild type enzyme. In the presense of Mn^{2+}, the double mutant had higher activity than the wild type enzyme, but the mutant showed much less activity than the single mutant, Ile91Leu or Asp90Cys. We did not observe a synergetic effect of double mutation at position Asp90 and Ile91. On the contrary, interestingly the double mutant showed very high activity with Cu^{2+}. In reference to the reported divalent metal ion effect on acivities of *Eco*RV,[7] wild type *Eco*RV does not possess any activity in the presence of Cu^{2+}. The double mutant showed another interesting property, with the double mutant enzyme cutting only one strand of double strand plasmid DNA. Figure 2 shows that conversion of supercoiled pAT (I) to nicked DNA (II) by the double mutant enzyme without formation of linear form DNA is apparent in the presence of Cu^{2+} (Figure 2). Some mutants that cut one strand of double strand DNA, generating open-circle DNA have been described before.[14] All previous mutants, however, generate open-circle DNA as an intermediate by cutting one strand of double strand DNA and then cutting the other strand by stepwise reactions to give a linear form DNA. This is the first enzyme to cut one strand of double strand DNA generating an open-circle DNA. It has been

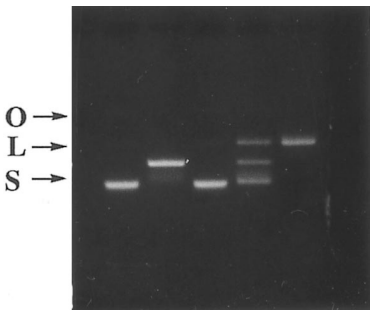

Figure 2. Cleavage of pAT by wild type *Eco*RV and Asp90Cys+ Ile91Leu mutant on pAT153 in the presence of Mg^{2+} and Cu^{2+}. Reactions in buffer contained 10 nM DNA with either Mg^{2+} or Cu^{2+}. Lane 1 is supercoiled DNA without enzyme. The DNA was digested by wild type *Eco*RV (lane 2) and Asp90Cys+Ile91Leu mutant (lane 4) in the presence of Mg^{2+} and by wild type *Eco*RV (lane 3) and Asp90Cys+Ile91Leu mutant (lane 5) in the presence of Cu^{2+}, respectively. The mobilities of the supercoiled, open-circle, and linear forms of the plasmids are marked on the left of the gel as S, O, L, respectively.

reported that metal activated nonenzymatic cleavage of DNA gives conversion of supercoiled plasmid DNA to nicked DNA.[15] In contrast to nonspecific cleavage of DNA by metal ion,[15] the cleavage of DNA by the double mutant is specific. To test specificity of the mutant Asp90Cys+ Ile91Leu, plasmid pAG DNA, which does not have *Eco*RV recognition site (GATATC), but does have *Eco*RI recognition site (GAATTC), was treated with the mutant in the presence of Cu^{2+}. As shown in Figure 3, the plasmid supercoiled DNA was cleaved to linear form DNA by *Eco*RI restriction endonuclease, but the supercoiled DNA was not digested by either *Eco*RV or the Asp90Cys+Ile91Leu mutant. These results indicate that the mutant enzyme recognizes and cuts the *Eco*RV recognition site of DNA. Considering the mode of DNA cleavage by *Eco*RV, which is a concerted reaction on both strands of the DNA at recognition sequence, leading directly to a double strand break, the mutant Asp90Cys+ Ile91Leu may have a different mode of DNA cleavage from

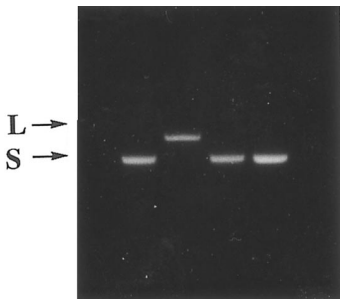

Figure 3. Reactions on pAG by wild type *Eco*RV and Asp90Cys+ Ile91Leu mutant in the presence of Cu^{2+}. Reactions in buffer contained 10 nM DNA with either Mg^{2+} or Cu^{2+}. Lane 1 is supercoiled DNA without enzyme. The DNA was digested by *Eco*RI (lane 2) and *Eco*RV (lane 3) in the presence of Mg^{2+} and by Asp90Cys+Ile91Leu mutant (lane 4) in the presence of Cu^{2+}, respectively. The mobilities of the supercoiled, and linear forms of the plasmids are marked on the left of the gel as S and L, respectively.

wild type enzyme.

In conclusion, mutants that recognize other metal ions have been made by a site directed mutagenesis. Mutant Asp90Cys switches the cofactor requirement from Mg^{2+} to Mn^{2+} and double mutant Asp90Cys+Ile91Leu requires Cu^{2+} as the cofactor. Double mutant Asp90Cys+Ile91Leu cuts site-specifically only one strand of double strand DNA. Yet we do not know the mechanism of DNA cleavage by the double mutant and switches in cofactor requirement of the mutants. They may be usefully applied to elucidate the mechanism of natural endonucleases and in the development of artificial restriction enzyme. An attempt to apply Asp90Cys+Ile91Leu mutant to generate single strand plasmid DNA chemically is in progress.

Experimental Section

Mutagenesis. Site-directed mutagenesis was performed by the modification of the Amersham mutagenesis system (codeRPN1523), which is based on the phosphorothioate method of Eckstein and his co-workers.[16] 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.[14] Positive clones were screened by restriction enzyme analysis and verified by sequencing the entire gene.

Enzyme Overproduction and Purification. The derivative of 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 °C to an OD_{600} of 0.4. An equal volume of L-broth at 55 °C was added and the growth continued for 4 h at 42 °C. The cell was harvested by centrifugation and stored at -20 °C. 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 wild type enzyme.[17] 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. Bradford method was used to determine protein concentrations after the method calibrated 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 Cleavage. The substrate was the monomeric forms of pAT153,[18] purified from transformants of *E. coli* HB101 which were grown in M9 minimal salts with 1mCi/l[methyl-^{3}H]thymidine.[19] The reactions with enzyme were carried out at 25 °C in buffer A(standard reaction condition) or in buffer B, with either $MgCl_2$ or $MnCl_2$ at the concentrations specified. Buffer A is 50 mM Tris-HCl, 100 mM NaCl, 10 mM β-mercaptoethanol, 100 μg/mL bovine serum albumin, pH 7.5. Buffer B is 50 mM Tris-HCl, 100 mM NaCl, 10 mM β-mercaptoethanol, 100 μg/mL bovine serum albumin, 10%(v/v) DMSO, pH 8.5. During each reaction, the changes in the concentrations of the supercoiled, open-circle, and linear forms of the DNA were measured as described previously.[19,20]

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