Site-Directed Mutagenesis of Putative Active Site Residues of *Mun*I Restriction Endonuclease: Replacement of Catalytically Essential Carboxylate Residues Triggers DNA Binding Specificity†

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ABSTRACT: Mapping of the conserved sequence regions in the restriction endonucleases *Mun*I (C/AATTG) and *Eco*RI (G/AATTC) to the known X-ray structure of *Eco*RI allowed us to identify the sequence motif 82PDX$_{14}$EXK as the putative catalytic/Mg^{2+} ion binding site of *Mun*I [Siksnys, V., Zareckaja, N., Vaisvila, R., Timinskas, A., Stakenas, P., Butkus, V., & Janulaitis, A. *Gene* (1994) 142, 1−8]. Site-directed mutagenesis was then used to test whether amino acids P82, D83, E98, and K100 were important for the catalytic activity of *Mun*I. Mutation P82A generated only a marginal effect on the cleavage properties of the enzyme. Investigation of the cleavage properties of the D83, E98, and K100 substitution mutants, however, *in vivo* and *in vitro*, revealed either an absence of catalytic activity or markedly reduced catalytic activity. Interestingly, the deleterious effect of the E98Q replacement *in vitro* was partially overcome by replacement of the metal cofactor used. Though the catalytic activity of the E98Q mutant was only 0.4% of WT under standard conditions (in the presence of Mg^{2+} ions), the mutant exhibited 40% of WT catalytic activity in buffer supplemented with Mn^{2+} ions. Further, the DNA binding properties of these substitution mutants were analyzed using the gel shift assay technique. In the absence of Mg^{2+} ions, WT *Mun*I bound both cognate DNA and noncognate sequences with similar low affinities. The D83A and E98A mutants, in contrast, in the absence of Mg^{2+} ions, exhibited significant specificity of binding to cognate DNA, suggesting that the substitutions made can simulate the effect of the Mg^{2+} ion in conferring specificity to the *Mun*I restriction enzyme.

Type II restriction endonucleases cleave phosphodiester bonds in DNA, in the presence of Mg^{2+} ions, with remarkable specificity. Almost 2800 Type II restriction enzymes, representing more than 200 specificities, are known to date (Roberts & Macelis, 1997). The existence of such a variety of restriction enzymes, with little primary sequence similarity, is suggestive of the possibility that these enzymes might employ diverse mechanisms in their interaction with DNA.

Comparison of the crystal structures of the restriction enzymes *Eco*RI and *Eco*RV reveals that despite variations in recognition sequence and cleavage position, a common sequence motif is observed, PDX$_n$(D/E)XK, which lies in close proximity to the scissile phosphodiester bond (Winkler et al, 1993). The acidic residues within this motive are thought to be components of the active site and are thought to chelate Mg^{2+} ions, a necessary cofactor in catalysis. The putative catalytic/Mg^{2+} binding site in *Bam*HI (Newman et al., 1994, 1995) and *Pvu*II (Athanasiadis et al., 1994; Cheng et al., 1995) seems to be similar to those documented in *Eco*RI and *Eco*RV, though some variation in the PDX$_n$(D/E)XK sequence motif is noted (Aggarwal, 1995). A second Mg^{2+} binding site has also been identified in *Eco*RV on the basis of crystallographic (Kostrewa & Winkler, 1995) and biochemical (Vipond & Halford, 1995) evidence. However, such a site has not yet been identified in *Eco*RI. Structural comparison of *Pvu*II (Athanasiadis et al., 1994; Cheng et al., 1995) and *Cfr*10I (Bozic et al., 1996) with *Eco*RV, however, suggests that a second metal binding site may well be manifest in both *Pvu*II and *Cfr*10I. Therefore, much is still to be clarified with respect to the features of conserved and nonconserved active site structures and the roles of particular residues in phosphodiester bond cleavage.

X-ray and sequence data currently available suggest that the sequence motif PDX$_n$(D/E)XK might represent a weak active site signature motif of restriction enzymes (Anderson, 1993; Siksnys et al., 1995). The recent mutational studies of *Fok*I (Waugh & Sauer, 1993) and *Nae*I (Holtz & Topal, 1995) would support such an idea. However, more mutational experiments backed up by X-ray analysis are required to confirm the significance of this motif in the restriction enzymes in which it is manifest.

We have focused our studies on the analysis of the mechanisms sequence discrimination and cleavage by *Mun*I restriction endonuclease. This enzyme recognizes the C/AATTG sequence and cleaves it as indicated (Siksnys et al., 1994). There is overlap in this sequence and the recognition sequence of the *Eco*RI enzyme (G/AATTC), which is one of the best-studied restriction enzymes to date. Alignment of the protein sequence of *Mun*I and that of *Eco*RI therefore, guided by the X-ray structure of *Eco*RI, allowed us previously to identify the sequence motif 82PDX$_{14}$EXK as the putative *Mun*I catalytic/Mg^{2+} ion binding site (Siksnys et al., 1994).

In this report we describe the catalytic and binding properties of putative active site mutants of *Mun*I. *In vivo*

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and *in vitro* studies indicated that the D83, E98, and K100 residues of *Mun*I were critical for DNA cleavage. In the absence of Mg^{2+}, gel shift experiments indicated little specific DNA binding by WT *MunI*. D83A and E98A replacements, however, conferred DNA binding specificity under the same conditions. In addition, the E98Q mutant manifest a change in sensitivity to the metal ion cofactor used, suggesting a specific role of this residue in metal ion coordination. The experimental evidence therefore suggests that metal ion chelation by the D83 and E98 residues may be responsible for the development of specificity in WT *Mun*I.

EXPERIMENTAL PROCEDURES

Strains and Plasmids. *Escherichia coli* ER2267 [*recA1 lacI*^{q} *lacZ*ΔM15 *zzf::*mini-Tn10(Kan^r)] strain was used for cloning and expression of WT and mutant genes. λ_{vir} was used to test restriction of infecting λ_{vir} bacteriophages by the cells harboring the WT *Mun*I and mutant proteins. The plasmid p*Mun*IR 4.8 (Ap^r)[1] (A. Lagunavicius, unpublished), a derivative of p*Mun*IR 6.0 (Ap^r) (Siksnys et al., 1994) obtained by deletion of 1.2 kb *Nco*I−*Ecl*136II fragment, was used as an expression vector of WT and mutant genes. The p*Mun*IR 4.8 contained the *mun*I-R gene under the control of the WT promoter. The compatible plasmid p*Mun*IM 6.2 (Tc^r, Cm^r) (Siksnys et al., 1994), containing the WT *mun*I-M gene under the control of the WT promoter, was used to transform the *E. coli* ER2267 strain in generating the host strain for the cloning of p*Mun*IR 4.8 (Ap^r) containing the WT and mutant *Mun*I-R genes.

Mutagenesis. Site-directed mutagenesis of the *mun*I-R gene was performed by the two-step "megaprimer'' method (Barik, 1993). A double-stranded plasmid p*Mun*IR 4.8 was used as a template for the polymerase chain reaction, using a primer containing the appropriate mispair in the codon designating the amino acid subjected to mutagenesis and a second primer upstream of the *Mun*I gene. The following mispaired primers were used:

5′-GCGAAATCTGCAGAAACACC
(P82A replacement)

5′-GCGAAAGCGGGAGAAACACC
(D83A replacement)

5′- CCATCTTGTCTTTTAATTTGGCCAAAAG
(E98Q replacement)

5′- CCATCTTGTCTTTTAATGGCGCCAAAAG
(E98A replacement)

5′- CCCATCCATCTTGTCTTTCAATTTCACT
(K100E replacement)

5′- CCCATCCATCTTGTCTCGCGATTTCACT
(K100A replacement)

The resulting DNA products were purified from agarose gels and used in a second round of mutagenesis with a primer downstream from the *munI* gene. The resulting product was cleaved by *Bgl*II and *Xba*I and ligated into the p*Mun*IR 4.8 (Ap^r) vector precleaved with *Bgl*II and *Xba*I.

Cell Transformation and Analysis of Transformants. Competent host cells were prepared and transformed with heterologous DNA using the $CaCl_2$ method. The plasmids were purified from different clones by the miniprep plasmid purification method (Sambrook et al., 1989) and analyzed by restriction analysis.

DNA Isolation, Purification, and Sequencing. The plasmid DNA was isolated by the alkaline lysis procedure (Sambrook et al., 1989) and purified using silica powder (MBI "Fermentas"). The purified DNA was then subjected to sequencing. The entire sequence of each mutant endonuclease gene was subsequently determined, using the Sanger dideoxy sequencing method, directly from DNA templates.

Analysis of Strain Phenotypes Containing WT and Mutant Enzymes. The phenotypes conferred by WT and mutant enzymes of the *Mun*I restriction endonuclease were assessed by transforming the *E. coli* strain ER2267 with plasmids containing or lacking *Mun*I methylase *in trans.* Growth of the cells was tested by streaking on LB agar plates supplied with Ap and Cm. The effect of mutant genes on cell growth was evaluated by comparing colony size.

Restriction of Infecting Phages by Wild Type and Mutant MunI Restriction Endonuclease. Restriction of infecting λ_{vir} bacteriophages was assessed by measuring the titer of λ_{vir} phage on the strain ER2267 and comparing it with the titer of phage on the strain ER2267 expressing the *Mun*I methylase and WT or mutant restriction endonuclease genes. The extent of phage restriction was determined quantitatively by spotting portions of serially diluted phage stock on a lawn of bacteria (Sambrook et al., 1988). The strains were grown on LB plates.

Purification of WT and Mutant MunI Proteins. Purification of WT enzyme and mutant proteins was performed according to the technique described by (Stakenas et al., 1993). The cells were grown into late logarithmic phase in LB medium containing 50 mg/L Ap and 30 mg/L Cm, with aeration, and were harvested by centrifugation. Crude cell extract was obtained by sonication; it was then applied to a heparin-Sepharose column and eluted using a NaCl gradient, with subsequent chromatography on blue-Sepharose and phosphocellulose. Polyclonal antibodies raised against *Mun*I were used to monitor purification of mutant proteins. The fractions containing WT or mutant enzymes were pooled and dialysed against the storage buffer (10 mM K_3PO_4, pH 7.4; 1 mM EDTA, 7 mM 2-mercaptoethanol, 50% glycerol) and stored at −20 °C. The enzyme and mutant proteins were >99% homogeneous by SDS gel analysis. Protein concentrations were determined spectrophotometrically at 280 nm using the monomer extinction coefficient of 45 720 M^{-1} cm^{-1}. The concentrations of *Mun*I are given in terms of dimeric protein.

DNA Cleavage Assay. Activities of the *Mun*I endonuclease and each of its mutants in crude cell lysates were assayed by incubating aliquots of cell lysates in 50 μL of standard reaction buffer [containing 10 mM Tris-HCl (pH 7.4 at 37 °C), 10 mM $MgCl_2$ (or $MnCl_2$), 50 mM NaCl, and 1 μg of λ DNA] for 0.5 h at 37 °C. The reaction was terminated by addition of a stop solution (30% glycerol, 0.25% bromphenol blue, 50 mM EDTA), and DNA was analyzed by electrophoresis in agarose gels.

Preparation of DNA Fragments for Gel Shift Assay. A ^{33}P-labeled DNA fragment was obtained by PCR, using 2

[1] Abbreviations: Ap and Ap^r, ampicillin and ampicillin resistance; bp, base pair(s); Cm, chloramphenicol; and Cm^r, chloramphenicol resistance; EDTA, ethylenediaminetetraacetic acid; Kan, kanamycin; and Kan^r, kanamycin resistance; PAAG, polyacrylamide gel; SDS, sodium dodecyl sulfate; LB, Luria broth; TAE, Tris-acetate buffer; Tc and Tc^r, tetracyclin and tetracyclin resistance; WT, wild-type endonuclease.

units of *Taq* polymerase, primers (0.5 μM final concentration), the DNA template (0.2 ng/μL final concentration), [α^{33}P]ATP (1.5×10^{17}Bq/mol), and three other unlabeled dNTPs (1 μM final concentration of every labeled or unlabeled deoxynucleotide during first 15 PCR cycles) in 50 μL of reaction buffer [67 mM Tris-HCl, pH 8.3 at 25 °C, 10 mM 2-mercaptoethanol, 16.7 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$]. After 15 cycles the concentration of dNTP was increased to 20 μM using unlabeled dNTPs, and an additional 15 PCR cycles were run.

A specific 166-mer fragment (specific DNA) was obtained as described above, using plasmid p*Mun*IR as the template DNA and primers 5′-GTAAGGATCCTAGCGCTGGCAGGGG and 5′-CTCTTTTGGGATCTCGAG. The fragment contained a single *Mun*I site in the middle of the sequence.

A nonspecific 174-mer fragment was obtained, using the p*Mun*IR plasmid as template DNA, which was precleaved through the unique *Mun*I site, blunted using DNA polymerase, and religated to yield a plasmid lacking the *Mun*I recognition site. The same primers (see above) were used during amplification of the nonspecific DNA fragment. After PCR unincorporated label and cold dNTPs were removed, using a microcentrifuge filter (Sigma, M-0411). The retained DNA was washed twice with MilliQ water. Concentrations of the labeled DNA fragments were determined spectrophotometrically. The presence of the recognition sequence in the specific fragment was confirmed by *Mun*I cleavage. The noncognate DNA fragment was not cleaved under similar conditions.

Gel Shift Assay. The labeled DNA fragments (in a final concentration of 0.5 nM) were incubated with different amounts of the WT or mutant enzymes in 20 μL of binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10% glycerol) at room temperature for 15 min. The stock solutions of the WT or mutant enzymes were diluted to the appropriate concentrations directly before use in the same binding buffers. The samples were loaded directly onto an 8% acrylamide gel (29:1 acrylamide/bisacrylamide), and run in 1× TAE (40 mM Tris-acetate, pH 8.3, 1 mM EDTA) for 2 h at 100 V (10 V/cm). The gels were prerun for 1 h at 100 V. After electrophoresis, the gels were fixed in 1:9 (v/v) acetic acid/water, washed in water and ethanol, dried, and subjected to autoradiography.

RESULTS

Alignment of the protein sequences of *Mun*I and *Eco*RI restriction endonucleases, guided by the X-ray structure of the *Eco*RI restriction enzyme, allowed us to identify the sequence motif $82PDX_{14}EXK$ as a putative catalytic/Mg^{2+} ion binding site of *Mun*I (Siksnys et al., 1994). In order to test whether amino acids P82, D83, E98, and K100 were important for the catalytic activity of *Mun*I, replacement mutants (P82A, D83A, E98Q, E98A, K100E, K100A) were obtained by site-directed mutagenesis (Barik, 1993). The DNA cleavage properties of these mutants were then assessed both *in vivo* and *in vitro*. First, intracellular activities of the mutants were assayed by plating phenotype and on the basis of restriction of infecting phages. Second, *in vitro* cleavage properties were tested in crude cell lysates.

Assay of Endonuclease Activity of Wild Type and Mutant MunI: Plating Phenotype. Immunological quantitation of *Mun*I mutant proteins showed that all proteins were present at a level equal to that of WT (M. Leckiene, A. Lagunavicius, A., unpublished). Plasmids carrying either WT or mutant *Mun*I genes were used to transform *E. coli* ER2267 strains with or without the *Mun*I methylase gene (*in trans*). The effect of mutant genes on cell growth was then evaluated by comparing colony size as described under Experimental Procedures (above). Only two (K100E and K100A) of the seven mutant strains survived in the absence of methylase (Table 1). Mutant strains (P82A, E98Q, and E98A) failed to grow under these conditions but survived in the presence of the *Mun*I methylase, yielding colonies of larger size than the strain expressing the WT enzyme. Substitution mutants of the D83 residue however, led to lethality (D83N) or decreased growth (D83A) of the host strain, even in the presence of the *Mun*I methylase.

Table 1: Plating Phenotypes of Strains Harboring WT and Mutant Genes of *Mun*I Restriction Endonuclease[a]

strain	with *Mun*I methylase	without *Mun*I methylase
WT	++	−
P82A	+++	−
D83A[b]	+	−
E98Q	+++	−
E98A	+++	−
K100A	+++	+
K100E	+++	+++

[a] The phenotypes conferred by WT and mutant enzymes of the *Mun*I restriction endonuclease were assessed by transforming strain ER2267 with plasmids containing or lacking *Mun*I methylase *in trans.* Growth of the cells was tested by streaking on LB agar plates supplied with Ap and Cm. Effect of mutant genes was evaluated by comparing colony size. The number of plus signs refers to colony size: +++, normal colony size; ++, slightly reduced colony size; +, greatly reduced colony size; −, no colonies. [b] The cells containing this mutant grew better at 30 °C than at 37 °C.

Table 2: Phage Restriction in Cells Harboring WT and Mutants of *Mun*I Restriction Endonuclease[a]

strain	restriction (folds)
ER2267	1
ER2267(*Mun*I-M)	1.2
ER2267 (*Mun*I-RM; WT)	1.1×10^4
ER2267 (*Mun*I-RM; P82A)	7.1×10^2
ER2267 (*Mun*I-RM; D83A)	not determined[b]
ER2267 (*Mun*I-RM; E98Q)	6.2×10^1
ER2267 (*Mun*I-RM; E98A)	1.2
ER2267 (*Mun*I-RM; K100A)	8.5
ER2267 (*Mun*I-RM; K100E)	1.2

[a] Restriction of infecting λ_{vir} bacteriophages was assessed by measuring the titer of λ_{vir} phage on the strain ER2267 and comparing it with the titer of phage on the strain ER2267 expressing the *Mun*I methylase and WT or mutant restriction endonuclease genes. The extent of phage restriction was determined quantitatively by spotting portions of serially diluted phages stock on a lawn of bacteria. The strains were grown on LB plates. [b] Restriction was not evaluated since the strain ER2267 containing the D83A mutant gene was unable to form an entire lawn on an LB plate at 37 °C.

Assay of Endonuclease Activity of WT and Mutant MunI: λ_{vir} Restriction. Ability to restrict λ_{vir} was assessed by measuring the titer (evaluated from three independent measurements) of λ_{vir} phage on the strain ER2267 expressing the WT and mutant *Mun*I genes in the presence of the *Mun*I methylase and comparing it with a titer of phage on the strain expressing only the methylase gene (Table 2). WT *Mun*I restricted the phage growth by 4 orders of magnitude. *Mun*I mutants differed in their ability to restrict phage (Table 2). Two *Mun*I mutants (E98? and K100E) failed to restrict phage, while three other mutants (P82A, E98Q, and K100A)

Table 3: Catalytic Activities of the WT and Mutant *Mun*I Endonucleases in Crude Cell Lysates[a]

enzyme	Mg^{2+}	Mn^{2+}
wild type	+++	+
P82A	++	+
D83A	−	−
E98Q	+	++
E98A	−	+
K100A	−	−
K100E	−	−

[a] Activities of the *Mun*I endonuclease and each of its mutants in crude cell lysates were assayed incubating aliquots of cell lysates for 1 h in 50 μL of standard reaction buffer containing 10 mM Tris-HCl (pH 7.4 at 37 °C), 10 mM $MgCl_2$ (or $MnCl_2$), 50 mM NaCl, 1 μg of λ DNA. Number of plus signs indicates relative levels of activity: +++, WT activity; ++, reduced activity; +, highly reduced activity; −, activity was not detectable under conditions of this assay.

Table 4: Specific Activities of *Mun*I Mutants[a]

*Mun*I protein	Mg^{2+}	Mn^{2+}
WT	660 000	66 000
E98Q	2 700	27 000
E98A	<300[b]	2 400
D83A	−	−

[a] Specific activities of the *Mun*I endonuclease and mutants were assayed incubating varying amounts of purified proteins in 50 μL of standard reaction buffer containing 10 mM Tris-HCl (pH 7.4 at 37 °C), 10 mM $MgCl_2$ (or $MnCl_2$), 50 mM NaCl, 1 μg of λ DNA for 1 h. [b] Complete cleavage was not obtained.

showed diminished but still significant activity, 15-, 180-, and 1300-fold less, respectively, than the WT enzyme.

Characterization of the DNA Cleavage Activity in WT and the Mutant MunI in Vitro. The cell lysates obtained from the strains expressing *Mun*I mutant proteins were tested for λ DNA cleavage activity in the presence of Mg^{2+} and Mn^{2+} ions (Table 3). The catalytic activities of mutant proteins *in vitro* in the presence of Mg^{2+} correlated with effects *in vivo*. Strains harboring genes of the most active mutants *in vivo*, P82A and E98Q, also manifest the highest activities *in vitro*. Analysis of the effects of Mg^{2+} and Mn^{2+} on the DNA cleavage properties of the WT *Mun*I indicated that Mg^{2+} promotes WT enzyme activity. In contrast, the cleavage activity of the E98Q mutant was promoted in the presence of Mn^{2+}. Under these conditions DNA cleavage properties of the E98Q mutant were comparable to those of the WT enzyme. Similarly, the E98A mutant cleavage activity was promoted in a buffer containing Mn^{2+}. The catalytic activity of the three remaining mutants (D83A, K100A, and K100E) was not detectable in crude lysates using buffer containing either Mg^{2+} or Mn^{2+}.

The ability of Mn^{2+} ions to partly suppress the cleavage defect observed in the E98Q and E98A mutants, in the presence of Mg^{2+}, encouraged us to study the catalytic and binding properties of the mutant proteins more thoroughly, using purified preparations of proteins. WT *Mun*I and the D83A, E98A, and E98Q mutants were purified to apparent homogeneity, as described in the Experimental Procedures (above). The specific activities of the purified WT and mutant proteins were assessed in the presence of Mg^{2+} and Mn^{2+} ions using λ DNA as the substrate (Table 4). The specific activity of the WT *Mun*I in the presence of Mg^{2+} was 10 times higher than its specific activity in the presence of Mn^{2+} ions. E98Q replacement reduced this specific activity approximately 250 times in the presence of Mg^{2+},

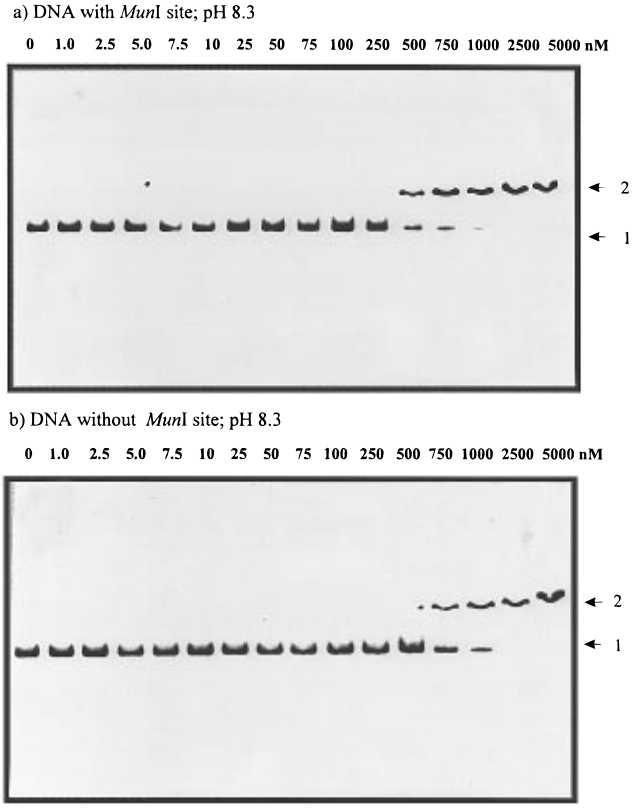

FIGURE 1: Binding of *Mun*I to specific and nonspecific DNA. The 166 bp fragment containing the recognition sequence of *Mun*I (panel a) or the 174 bp fragment lacking the recognition sequence of *Mun*I (panel b) was used in binding experiments as specific and nonspecific DNA, respectively. The binding mixture contained 0.5 nM of ^{33}P-labeled DNA and *Mun*I at concentrations indicated above each lane of the gel. After PAAG electrophoresis in TAE buffer (pH 8.3), gels were dried and subjected to autoradiography. Arrows on the right side of the gel indicate positions of DNA forms with different mobility.

however when Mn^{2+} ions were added to the buffer instead of Mg^{2+} ions, the E98Q mutant exhibited approximately 40% of the WT enzyme activity. The E98A mutant failed to cleave λ DNA to completion in the presence of Mg^{2+} ions, indicating that its specific activity was less than 300 units/mg. In the presence of Mn^{2+}, however, complete cleavage of λ DNA was observed with a specific activity of 2400 units/mg.

Effect of Mutations on the DNA Binding Properties of MunI and Mutant Proteins. To determine whether the mutations used impaired the implicated residues' ability to bind substrate, the DNA binding of WT and mutant proteins was analysed using the gel mobility shift assay. Two DNA fragments obtained by PCR were used in binding experiments: the first, a 166 bp DNA fragment containing a single *Mun*I recognition sequence embedded almost in the middle of the fragment (specific fragment) and the second, a 174 bp fragment lacking the *Mun*I recognition sequence (nonspecific fragment). Increasing concentrations of WT *Mun*I or mutant proteins were preincubated with 0.5 nM DNA in the absence of Mg^{2+} and loaded on a PAAG, and electrophoresis was run as described under Experimental Procedures (above). The gel shift assay of WT *Mun*I binding with the specific fragment (Figure 1a) revealed shifted DNA bands (band 2) only at high concentrations ($\geq$500 nM) of protein. A qualitatively similar binding pattern was observed when the noncognate DNA was used in the binding experiment (Figure 1b), suggesting that the shifted band in both cases

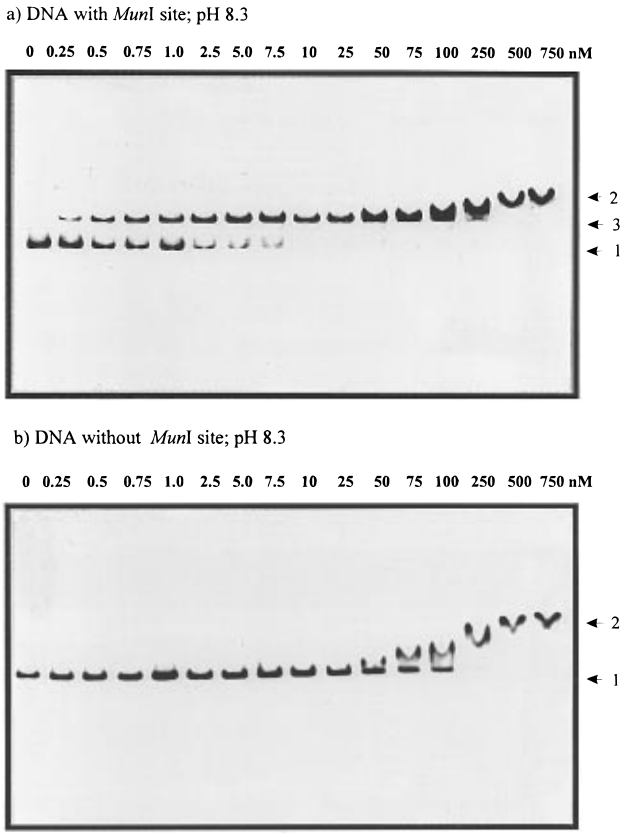

FIGURE 2: Binding of E98A *Mun*I mutant to specific and nonspecific DNA. 166 bp fragment containing the recognition sequence of *Mun*I (panel a) or 174 bp fragment lacking the recognition sequence of *Mun*I (panel b) were used in binding experiments as specific and nonspecific DNA, respectively. The binding mixture contained 0.5 nM ^{33}P-labeled DNA and *Mun*I at concentrations indicated above each lane of the gel. After PAAG electrophoresis in TAE buffer (pH 8.3), gels were dried and subjected to autoradiography. Arrows on the right side of the gel indicate positions of DNA forms with different mobility.

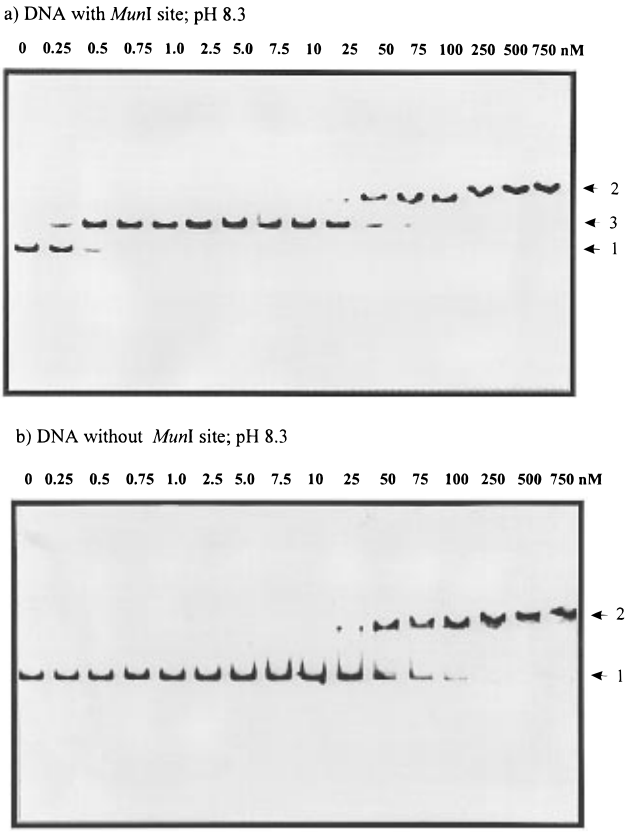

FIGURE 3: Binding of D83A *Mun*I mutant to specific and nonspecific DNA. The 166 bp fragment containing the recognition sequence of *Mun*I (panel a) or the 174 bp fragment lacking the recognition sequence of *Mun*I (panel b) was used in binding experiments as specific and nonspecific DNA, respectively. The binding mixture contained 0.5 nM of ^{33}P-labeled DNA and *Mun*I at concentrations indicated above each lane of the gel. After PAAG electrophoresis in TAE buffer (pH 8.3), gels were dried and subjected to autoradiography. Arrows on the right side of the gel indicate positions of DNA forms with different mobility.

represented nonspecific complexes of *Mun*I and DNA. The slight differences in *Mun*I concentration required to obtain shifted bands using the nonspecific fragment may reflect the differences in sequence context of the cognate and noncognate DNA fragments used. Gel shift analysis of the E98Q mutant revealed that the binding properties of the mutant were similar to those of the WT enzyme both when specific and nonspecific DNA fragments were used (data not shown). Under similar binding conditions, however, the D83A and E98A mutants exhibited different binding patterns. In contrast to the WT enzyme, experiments designed to evaluate the binding of the D83A and E98A mutants to the cognate DNA revealed a shifted band (band 3, Figures 2a and 3a) even at low protein concentrations (0.25 nM). The amount of the initial complex (band 3) decreased progressively as protein concentration increased. This decrease was accompanied by the appearance of a slowly migrating DNA species (band 2, Figures 2a and 3a) which was characteristic of the binding of the WT *Mun*I (Figure 1a,b). Similar binding studies of the D83A and E98A mutants with the noncognate DNA revealed only a slowly migrating complex (band 2, Figures 2b and 3b) at the top of the gel which was characteristic of WT enzyme binding at high protein concentrations. The concentration of the D83A and E98A mutants required to generate this complex, however, was approximately 10 times lower than the concentration of WT enzyme required.

DISCUSSION

X-ray studies of *Eco*RI, *Eco*RV, *Pvu*II, and *Bam*HI restriction enzymes (Aggarwal, 1995) have previously revealed that acidic residues were located near the scissile phosphate. Such residues could well contribute to the chelation of the Mg^{2+} ion, which is essential for cleavage. A conserved lysine residue has also been identified near the cleaved phosphate, though its role in catalysis is not yet clear. Indeed the sequence motif PDX$_n$(D/E)XK corresponding to the structurally conserved active sites of *Eco*RI and *Eco*RV has been suggested (Anderson, 1993) as a putative restriction enzyme active site signature motif. Accordingly, alignment of the protein sequences of *Mun*I and *Eco*RI indicate that the sequence motif 82PDX14EXK might be involved in metal ion coordination/catalysis in the *Mun*I restriction enzyme (Siksnys et al., 1994). Site-directed mutagenesis was therefore used to test whether the amino acids P82, D83, E98, and K100 were essential for the catalytic activity of *Mun*I.

The P82A mutant manifest reduced but still significant activity *in vivo* (Tables 1 and 2) and exhibited a moderate level of DNA cleavage activity in crude cell extracts (Table 3). Hence, the P82 residue seems to be important but not vital for the cleavage activity of *Mun*I. Replacement of the acidic D83 residue, however, had a much more pronounced effect on the ability of *Mun*I to cleave DNA, both *in vivo* and *in vitro* (Tables 1−3). Cleavage activity was lost *in*

vitro (Table 3), and in addition cell growth and viability were affected. The D83A mutant strain at 37 °C exhibited reduced colony size but grew more reliably at 30 °C, indicating an inhibitory effect of this mutant on the growth of the cell even in the presence of methylase (Table 1). This effect seemed to be enhanced in the case of the D83N substitution. Indeed, we were unable to obtain a D83N mutant of *Mun*I using the routine procedure. A number of attempts to transform competent cells with the plasmid containing the putative D83N mutant yielded no or only very few transformants. Sequencing of the DNA obtained from such transformants indicated that the desired D83N mutation was generally accompanied by additional mutations (e.g., W13amber, K47E). If this mutant retains the ability to cleave DNA, the lack of protection by the methylase appears to allow cleavage at sequences other than the canonical site. Alternatively, if this mutant lacks catalytic activity, it may display enhanced DNA affinity and affect cell growth as was previously shown for an active site mutant of *Hha*I methylase (Mi & Roberts, 1995).

Analysis of *Mun*I mutants carrying E98A and E98Q mutations also revealed the critical role of the E98 residue in catalysis by *Mun*I. E98A replacement abolished phage restriction ability (Table 3), and only trace activity was detectable in the *in vitro* assay (Tables 3 and 4). Conservative substitution of E98 with glutamine had a less pronounced effect on cleavage activity. The E98Q mutant still exhibited activity in the phage restriction assay but retained only 0.4% of the cleavage activity of the WT *Mun*I in the presence of Mg^{2+}. Mutational analysis of acidic residues within the putative *Mun*I catalytic site therefore revealed that mutations of the E98 residue had a less pronounced effect on the cleavage activity of *Mun*I than analogous mutations of the D83 residue.

K100A and K100E mutations within the putative *Mun*I catalytic site had the most dramatic effect on cleavage activity. The *Mun*I replacement mutant K100A exhibited slow growth in the absence of methylase activity and only weakly restricted incoming phage. The K100E mutant though, was completely inactive according to all activity tests employed: the strains harboring the gene of the K100E mutant grown in the absence of methylase were unable to restrict phage and had essentially no activity *in vitro*. Thus *in vivo* studies, supported by *in vitro* cleavage assays, allowed us to conclude that the D83, E98, and K100 residues, but not the P82 residue, are essential for catalytic activity of the *Mun*I restriction enzyme.

Interestingly, site-directed mutagenesis of equivalent active site residues from the $90PDX_{19}EXK$ motif of *Eco*RI established phenotypes similar to those described above for *Mun*I (Grabowski et al., 1995). Indeed, P90A replacement in *Eco*RI yielded a mutant that was 25 times less active than the WT enzyme, and the alanine and asparagine replacements at the D91 position of *Eco*RI were inactive. The E111Q mutant of *Eco*RI showed a highly reduced cleavage rate, though reports of residual catalytic activity vary (Wolfes et al., 1986; Wright et al., 1989). In addition, a number of K113 mutations in *Eco*RI, have been described which, except for the K113R mutant, yielded null phenotypes in *in vitro* cleavage assays (Grabowski et al., 1995). Thus similarity in impact of mutations in *Mun*I and *Eco*RI restriction enzymes is indicative of a similarity in catalytic/metal binding centers.

DNA cleavage experiments revealed that a deleterious effect of E98Q and even E98A mutations on the catalytic activity of *Mun*I, in part, can be rescued by using Mn^{2+} ions rather than Mg^{2+} ions as a cofactor. In the presence of Mg^{2+} ions the E98Q mutant exhibited 0.4% of WT activity; however, its specific activity was 40% of the WT *Mun*I in the presence of Mn^{2+} ions (Table 4). This suggests that the E98Q replacement affects the binding of Mn^{2+} ions to a lesser extent than the binding of Mg^{2+} ions. Presumably, the glutamine residue at position 98 is still able to act as a ligand for Mn^{2+} at the active site of *Mun*I. EPR studies of Mn^{2+} binding at the active site of T7 RNA polymerase have previously demonstrated that the single replacement of an acidic metal ion chelating residue with asparagine reduced Mn^{2+} binding affinity two to five times (Woody et al., 1996). Since coordination complexes of Mn^{2+} are usually more stable, the E98Q mutant may still be able to bind Mn^{2+} ions under conditions when Mg^{2+} binding affinity is decreased. In the case of E98A replacement trace activities were observed with Mn^{2+} substitution suggesting that Mn^{2+} ions were still able to bind at the active site. The deleterious effect of the D83A mutation on the cleavage activity of *Mun*I, however, was not rescued by Mn^{2+} substitution. Nevertheless, it would seem clear that differences in binding affinity and the complexation mode in the presence of Mg^{2+} and Mn^{2+} ions might provide a basis for the variation in catalytic activities of WT *Mun*I observed in the presence of the two ions. Similar effects of metal ion replacement on *Eco*RV and *Bam*HI catalytic activities have previously been reported (Selent et al., 1992; Xu & Schildkraut, 1991). The results of mutations of putative catalytic site residues thought to be involved in the metal ion binding of the $3'-5'$ exonuclease domain of ϕ29 DNA polymerase (Esteban et al., 1994), RuvC resolvase (Saito et al., 1995) and transposase (Baker & Luo, 1994) have also been reported. In each of these the presence of Mg^{2+} and Mn^{2+} ions differentially affected cleavage rates. Finally, Vipond and Halford (1996) reported that single I91L replacement in *Eco*RV completely switched its preference from Mg^{2+} to Mn^{2+}.

In principle, therefore, the observed cleavage deficiency of the *Mun*I mutants described might result either from distortion of the metal binding site or loss of DNA binding ability. In order to discriminate between these two possibilities, the DNA binding properties of the WT *Mun*I and the mutant variants were studied using the gel shift procedure. Analysis of the binding properties of the WT *Mun*I indicated that the shifted bands appeared both when specific and nonspecific DNA fragments were used, only under conditions when protein was in significant excess (Figure 1a,b). We believe that the shifted bands (band 2, Figure 1a,b) represent nonspecific complexes of DNA and *Mun*I. Such nonspecific complexes were also observed at high protein concentrations in studies on the *Eco*RV (Taylor et al., 1989), *Taq*I (Zebala & Barany, 1992), and *Cfr*9I (Siksnys & Pleckaityte, 1993) restriction enzymes. The same binding pattern was observed for the E98Q mutant (data not shown). Interestingly, the analogous E111Q replacement in *Eco*RI yielded a cleavage deficient mutant that exhibited enhanced DNA binding properties (Wright et al., 1989).

In contrast to the WT enzyme, the D83A and E98A mutants of *Mun*I, in the absence of Mg^{2+}, bound DNA containing the recognition sequence with high affinity (Figures 2a and 3a). With noncognate DNA (Figures 2b and 3b) these mutants exhibited only weak binding, similar

to that of the WT *Mun*I. Since shifted bands at low protein concentration are observed only when the specific DNA fragment is used (band 3, Figures 2a and 3a), we believe that these bands represent specific complexes where E98A and D83A mutant proteins are bound primarily to recognition sites.

Why are such complexes not observed when WT *Mun*I is used? The experimental evidence presented above suggests that the D83 and E98 residues act as metal ion chelating residues in *Mun*I. Presumably, these residues are located at the surface of the protein close to the DNA binding site. In the absence of metal ion repulsive constraints between these residues and negatively charged phoshate oxygens may well interfere with the formation of tight complexes between DNA and protein. Replacement of the E98 or D83 residues with alanine then diminishes electrostatic repulsion and promotes formation of a tight site-specific complex. However, since the metal binding site is distorted by mutagenesis, no cleavage of DNA is observed. The increase of binding affinity with nonspecific DNA observed with the E98A and D83A mutants (see Figures 2b and 3b) might also be related to such suppressed repulsive constraints. Homologous mutations in *Eco*RI (Wright et al., 1989) and *Eco*RV (Kohler et al., 1993) also lead to an increased binding affinity with nonspecific DNA. The characteristics of the E98Q mutant however, do not fit directly into such a simplified model since no specific DNA binding is observed, despite the loss of the negative charge on the carboxylate residue. This mutation though, does not distort the metal binding site completely since the E98Q mutant maintained a residual catalytic activity (see discussion above). In this case partial negative charges present on the nitrogen amide group and the oxygen atoms of the amide moiety (Momany et al., 1975) may still interfere with tight DNA binding in the absence of metal. It seems reasonable, therefore, that Mg^{2+} ion binding might confer the specificity of the E98Q and WT *Mun*I enzymes during their transition states, as was previously suggested for *Eco*RV (Thielking et al., 1992). In contrast, gel shift experiments using the D83A mutant, in the presence of Mg^{2+} ions, indicated that Mg^{2+} ions had only a marginal effect on the binding affinity to specific DNA (data not shown), though Mg^{2+} ions were necessary to obtain specific binding by the D90A mutant of *Eco*RV (Selent et al., 1992). It seems therefore, that despite structural similarity active sites of restriction enzymes probably exhibit minor differences, that impact on their mechanisms of interaction with DNA.

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