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The RecA Intein of *Mycobacterium tuberculosis* Promotes Cleavage of Ectopic DNA Sites

IMPLICATIONS FOR THE DISPERSAL OF INTEINS IN NATURAL POPULATIONS*

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The RecA intein of *Mycobacterium tuberculosis*, a novel double-stranded DNA endonuclease, requires both Mn^{2+} and ATP for efficient cleavage of the inteinless recA allele. In this study, we show that Mg^{2+} alone was sufficient to stimulate PI-*Mtu*I to cleave double-stranded DNA at ectopic sites. In the absence of Mg^{2+}, PI-*Mtu*I formed complexes with topologically different forms of DNA containing ectopic recognition sequences with equal affinity but failed to cleave DNA. We observed that PI-*Mtu*I was able to inflict double-strand breaks robustly within the ectopic recognition sequence to generate either a blunt end or 1–2-nucleotide 3′-hydroxyl overhangs. Mutational analyses of the presumptive metal ion-binding ligands (Asp^{122}, Asp^{222}, and Glu^{220}) together with immunoprecipitation assays provided compelling evidence to link both the Mg^{2+}- and Mn^{2+} and ATP-dependent endonuclease activities to PI-*Mtu*I. The kinetic mechanism of PI-*Mtu*I promoted cleavage of ectopic DNA sites proceeded through a sequential mechanism with transient accumulation of nicked circular duplex DNA as an intermediate. Together, these data suggest that PI-*Mtu*I, like group II introns, might mediate ectopic DNA transposition and hence its lateral transfer in natural populations.

Mobile inteins and introns are genetic elements capable of self-propagation by "homing" into host genes in a wide variety of organisms: eubacteria, eukarya, archaea, and viruses (reviewed in Ref. 1). The process is promoted by a homing endonuclease, which is encoded by an open reading frame embedded within the genetic element. The genes for homing endonucleases are found among group I and group II introns, archaeal introns, intein-coding sequences, and free standing open reading frames (reviewed in Refs. 1–6). Inteins are genetic elements present within protein-coding sequences with dual function: protein-splicing and homing endonuclease activities. They are believed to play a central role in rearrangement of organelle as well as nuclear genomes (1–6). The hallmark of homing endonucleases is their ability to recognize and cleave extended asymmetric sequences (14–40 bp) that are generally centered on the intein insertion site in inteinless alleles (1, 7). Homing endonucleases can be classified into four families based on the presence of conserved motifs: LAGLIDADG, GIY-YIG, His-Cys box, and H-N-H (1, 5, 6, 8). Among these, the LAGLIDADG family is the largest, widespread and much studied class of homing endonucleases. Structural and biochemical studies have demonstrated that homing endonucleases with one LAGLIDADG motif act as homodimers, whereas enzymes with two such motifs function as monomers during catalysis (9–12). Under standard assay conditions *in vitro*, these enzymes are extremely specific for their recognition sites. However, recent evidence suggests that self-splicing group II introns transpose into ectopic DNA sites that resemble their natural homing sites (13, 14). The transposition of group II introns involves reverse splicing of the intron into the inteinless allele, which are then reverse transcribed to give complementary DNA. Following the synthesis of the second strand, the intron is incorporated into the genomic DNA by homologous recombination (1–6). But equivalent information is not available for any intein endonuclease. The exact sequence of events that lead to recognition and cleavage of DNA by homing endonucleases is poorly understood. In addition, in no case the molecular mechanism underlying cleavage of ectopic DNA sites by an intein endonuclease has been elucidated.

Mycobacterium tuberculosis RecA intein (PI-*Mtu*I)[1] is a member of the LAGLIDADG superfamily of homing endonucleases (15–18). In previous studies, we showed that PI-*Mtu*I is a novel homing endonuclease, which inflicted a staggered double-strand break 24 bp upstream of the intein insertion site in the inteinless *rec*A allele (henceforth called cognate site) (18). Typically, Mg^{2+} is the preferred metal ion and the only cofactor required for cleavage of inteinless alleles by the LAGLIDADG family of homing endonucleases. In contrast, PI-*Mtu*I required both Mn^{2+} and ATP for cleavage within the inteinless *rec*A allele (18). In this report, we extend our focus on the mode of action of PI-*Mtu*I and define some of the basic conditions essential for optimal cleavage of ectopic DNA sites. Mutational analyses of presumptive metal ion-binding ligands and immunoprecipitation assays provided compelling evidence that Mn^{2+} and ATP as well as Mg^{2+}-dependent endonuclease activities are intrinsic to PI-*Mtu*I. Thus, PI-*Mtu*I is the first example of an intein endonuclease demonstrated to have the ability to recognize and cleave cognate as well as ectopic DNA sites in the presence of alternative cofactors. Thus, these data implicate a possible role for PI-*Mtu*I in its lateral transfer in natural populations.

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[1] The abbreviations used are: PI-*Mtu*I, RecA intein endonuclease of *M. tuberculosis*; form I DNA, negatively supercoiled DNA; form II DNA, nicked circular double-stranded DNA; form III DNA, linear double-stranded DNA; ATPγS, adenosine 5′-*O*-(thiotriphosphate).

TABLE I
List of oligonucleotides used to generate PI-MtuI variants by overlap PCR extension method

Nucleotide sequences that are bold and underlined indicate the restriction sites for appropriate enzymes.

Sequence (5′ to 3′)	Variants
TACCTGATCG**CATATG**GCAGGGAT	D122Y
CTGTTCGAA**AGTACT**GGGTGGGTGAGC	D222T
CACCCGTC**GCTAGC**GAACAGG	E220A
GGTTGGGTGGG**AGGCCT**CACTCCGATC	K131L
CGGAATCGT**GAATTC**CCACGCGAG	K195F
CAACCGCACCCGGGTCAA	Forward primer
GACGGGGGCGGGCAGGAC	Reverse primer

MATERIALS AND METHODS

Reagents, Proteins, and DNA—All the chemicals used in this study were of analytical grade. Buffers were prepared using deionized water. Restriction enzymes, nylon N^+ membrane, and T4 DNA ligase were obtained from Amersham Biosciences. Vent DNA polymerase was obtained from New England Biolabs. Phage T4 polynucleotide kinase and the synthetic oligonucleotides were purchased from Invitrogen. Circular single-stranded and form I DNA from wild-type bacteriophage M13 were prepared as described (19). DNA was dissolved in 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and the concentrations were expressed in moles of nucleotide residues.

Bacterial Strains and Media—Escherichia coli strain DH5α was used for plasmid manipulations and grown in liquid or solid agar LB media supplemented with appropriate antibiotics. *E. coli* strain DH5α bearing the plasmid pGRI was used for protein overexpression as described (18). PI-*Mtu*I was purified to homogeneity and its concentration was determined as described (18). The same purification method was used to purify the variants of PI-*Mtu*I.

PI-MtuI Cleavage Assay—Endonuclease assays were performed as described (18). Briefly, reaction mixtures (25 μl) contained 25 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 0.4 mM dithiothreitol, 16 μM form I M13 DNA and PI-*Mtu*I at the concentrations indicated in the legend to the figures. After incubation at 37 °C, reactions were stopped by the addition of SDS to a final concentration of 0.1%, and the samples were deproteinized by incubation with proteinase K (0.2 mg/ml) for 15 min at 37 °C. Three μl of gel loading buffer (20% glycerol containing 0.12% (w/v) each of bromphenol blue and xylene cyanol) was added to each sample and separated on a 0.8% agarose gel in 89 mM Tris borate buffer (pH 8.3) containing 2 mM EDTA at 3 V/cm. The gel was stained with ethidium bromide (0.5 μg/ml) and DNA was visualized by UV illumination. Subsequently, DNA was transferred to nylon N^+ membrane and visualized by Southern hybridization (21). The bands were quantified in a UVI-Tech gel documentation station using UVI-BandMap software version 99 and plotted using Graphpad Prism version 2.0.

Immunological Techniques—Polyclonal antibodies against PI-*Mtu*I were generated in rabbits and characterized as described (18). Immunoprecipitation reactions were performed by incubating 20 μg of PI-*Mtu*I in the endonuclease assay buffer with preimmune IgG or anti-PI-*Mtu*I IgG tethered to protein A-Sepharose for 2 h with continuous stirring at 4 °C. The slurry was centrifuged at 6000 rpm at 4 °C for 2 min, and the supernatant was assayed for Mg^{2+}-dependent PI-*Mtu*I endonuclease activity as described above. Wild-type and variant forms of PI-*Mtu*I were electroblotted onto a nitrocellulose membrane after SDS-PAGE. The membrane was stained with anti-PI-*Mtu*I antibodies and visualized by chemiluminescence as described (22, 23).

Site-directed Mutagenesis—PI-*Mtu*I variants were generated by site-directed mutagenesis by the overlap extension PCR-based method (20). The primers used for this purpose are listed in Table I. Single-site mutations of the conserved amino acid residues Asp^{122}, Asp^{222}, Glu^{220}, Lys^{131}, and Lys^{195} were generated using the plasmid, pEJ135 (Ref. 5), as a template. PCR products were digested by *Sma*I and *Acc*I, and the reaction mixture was separated by electrophoresis on a 0.8% agarose gel. The 851-bp fragment isolated from the gel was ligated to pGRI, which had been digested by the same set of enzymes. The variants were screened by digestion with an appropriate restriction endonuclease (21). The mutant sequences were verified by sequencing in ABI PRISM DNA sequencer (PerkinElmer Life Sciences). The recombinant plasmids were maintained and overexpressed in *E. coli* strain DH5α. The expression levels of all the PI-*Mtu*I variants were similar to the wild-type enzyme.

Mapping of the PI-MtuI Cleavage Sites in M13 DNA—To map ectopic DNA sites in the M13 genome, form I DNA was digested with PI-*Mtu*I to generate P1 (3.5 kb) and P2 (2.9 kb) fragments (Fig. 1). The fragments isolated from the gel were digested with appropriate restriction enzymes. The restriction map thus constructed indicated the presence of two PI-*Mtu*I cleavage sites in the wild-type M13 genome: one in the *Hin*fI-*Hin*fI fragment (2498 to 2845 bp), and the second in the *Nde*I-*Acc*I fragment (6003–6090 bp). Accordingly, we prepared fragments containing the ectopic DNA sites by digestion with appropriate restriction enzymes. The fragments were labeled with [γ-^{32}P]ATP and polynucleotide kinase. To label at 5′- or 3′-end, the 2498–2845-bp fragment was digested by *Hae*III, which cleaves once at 2556 bp, thereby generating a 58-bp fragment with label at the 5′-end of the upper strand. Similarly, the 2247–2556-bp *Hae*III fragment was digested by *Hin*fI, which cleaves once at 2498 bp, generating a 58-bp fragment with label at the 5′-end of the lower strand. After electrophoresis on a 8% polyacrylamide gel, the bands corresponding to these fragments were excised from the gel, cut into small pieces, and DNA was eluted with 10 mM Tris-HCl (pH 7.5) buffer containing 1 mM EDTA. DNA was precipitated by ethanol, collected by centrifugation, vacuum-dried, and dissolved in 10 mM Tris-HCl (pH 7.5) buffer containing 1 mM EDTA.

To map the second ectopic DNA site, the 2723–6003-bp *Nde*I fragment was labeled as described above, digested by *Acc*I to generate the 87-bp fragment with the label at the 5′-end of the upper strand. Similarly, form I DNA cut with *Acc*I was end labeled, and digested by *Nde*I to generate the 87-bp fragment with the label at 5′-end of the lower strand. The bands corresponding to these fragments were isolated from acrylamide gels as described above.

Reactions were performed in the cleavage assay buffer containing wild-type or variant forms of PI-*Mtu*I (100 pmol) and the specified DNA fragment with label at the 5′-end (100 ng), and stopped as described above. Reaction mixtures were deproteinized by extraction with phenol: chloroform:isoamyl alcohol solution (25:24:1), and the DNA was precipitated by ethanol. Control reactions were subjected to a Maxam-Gilbert chemical sequencing reaction except that the DNA substrates were not incubated with PI-*Mtu*I. Samples were run side by side on 12 or 14% polyacrylamide gels in the presence of 8 M urea at 1800 V for 2.5 h (24). The gel was dried onto a Whatman 3MM filter paper and visualized as described (18).

RESULTS

Purification of Wild-type and Variants of PI-MtuI—Wild-type and variants of PI-*Mtu*I were expressed in *E. coli* and purified to homogeneity as described (18). Gel filtration on Superdex 75 column in a buffer containing 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, and 10% glycerol showed that PI-*Mtu*I coeluted with molecular mass corresponding to 47 kDa, indicating that the quaternary structure of PI-*Mtu*I is that of a monomer (data not shown). The data are consistent with that of a monomer, provided that the protein behaves as an average globular protein in solution. The identity of the purified protein was verified by sequencing 10 amino acid residues at the N-terminal end. Purified PI-*Mtu*I and its variants were devoid of both 5′ to 3′ and 3′ to 5′ exonuclease activities.

PI-MtuI Binds Topologically Different Forms of DNA—In light of the evidence that negatively supercoiled DNA influences the activities of DNA-binding proteins, we explored the ability of PI-*Mtu*I to interact with topologically different forms of M13 DNA. We observed that PI-*Mtu*I bound form I, form II, or circular single-stranded DNA in a sequence nonspecific manner, and with similar affinities (data not shown). Further evidence in favor of sequence nonspecific binding of PI-*Mtu*I emerged from DNase I protection assays. In this assay, a 16 μM single-stranded, form I or linear duplex [^{3}H]DNA was first incubated with varying amounts of PI-*Mtu*I (0.5–7.5 μM), and then digested by DNase I (10 μg/ml) for 10 min. Under the conditions used in this assay, the amount of protection rendered by PI-*Mtu*I at $\geq$5 μM was >75% in all cases, indicating that binding is independent of single- or double-stranded nature of DNA or its topological state (data not shown).

PI-MtuI Cleaves Ectopic DNA Sites in the Presence of Mg^{2+}—To explore whether interaction of PI-*Mtu*I with ectopic DNA elicits endonuclease activity, we used form I M13 DNA as the substrate. The rationale for using form I DNA rather than

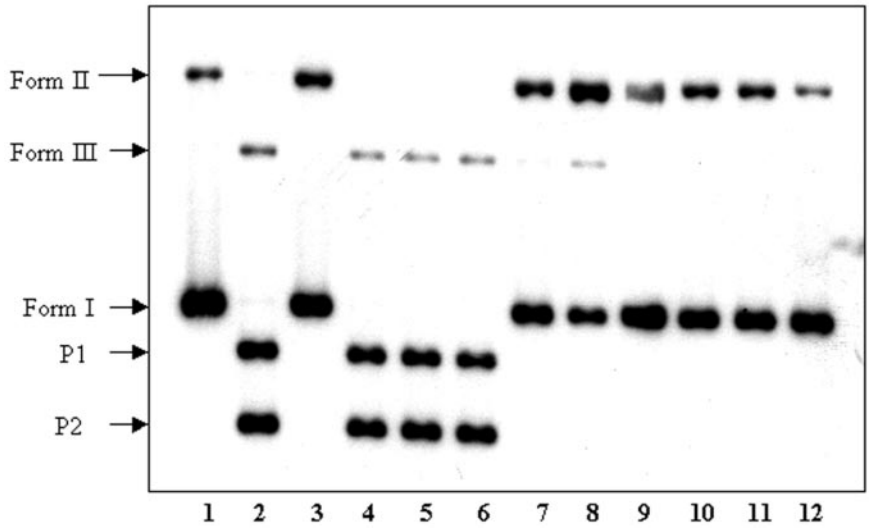

FIG. 1. **PI-*Mtu*I displays Mg^{2+}-dependent endonuclease activity on ectopic DNA sites.** Reactions were performed as described under "Materials and Methods." *Lane 1*, DNA lacking PI-*Mtu*I. The remaining samples were incubated with 16 μM M13 DNA (form I and form II, 90:10) and 1 μM PI-*Mtu*I in the presence of 5 mM Mg^{2+} (*lane 2*), 1.5 mM ATP (*lane 3*), 5 mM Mg^{2+} + 1.5 mM ATP (*lane 4*), 5 mM Mg^{2+} + 1.5 mM ATPγS (*lane 5*), 5 mM Mg^{2+} + 1.5 mM dATP (*lane 6*), 3 mM Mn^{2+} (*lane 7*), 3 mM Mn^{2+} + 1.5 mM ATP (*lane 8*), 3 mM Zn^{2+} (*lane 9*), 3 mM Ca^{2+} (*lane 10*), 5 mM Mg^{2+} + 10 mM EDTA (*lane 11*), and 0.1% SDS (*lane 12*). Reaction mixtures were deproteinized, and products were separated on a 0.8% agarose gel and visualized as described under "Materials and Methods." The positions of substrates and products are indicated on the *left*.

linear double-stranded DNA would be to permit detection of even the cleavage of single strands of the substrate by PI-*Mtu*I. After incubation, the reaction mixtures were analyzed as described under "Materials and Methods." In the presence of Mg^{2+} (Fig. 1, *lane 2*), incubation of form I DNA with PI-*Mtu*I produced form III DNA and two products of size 3.5 and 2.9 kb (Fig. 1, marked P1 and P2). The combined sizes of P1 and P2 products equal the size of form III DNA, indicating that the latter was further cleaved to produce two products. Thus, form I M13 DNA contained two cleavage sites for PI-*Mtu*I. Interestingly, cleavage proceeded to a similar extent in the presence of ATP or its analogues (Fig. 1, *lanes 4–6*), but not in the absence of $MgCl_2$ (Fig. 1, *lane 3*). In the presence of alternative divalent cations, PI-*Mtu*I catalyzed the cleavage of single strands of the substrate (*i.e.* "nick" the DNA), but failed to produce form III DNA, and two final cleavage products (Fig. 1, *lanes 7*, *9*, and *10*). However, in the presence of Mn^{2+} and ATP, PI-*Mtu*I generated a significant amount of form II and a trace amount of form III DNA (Fig. 1, *lane 8*). Addition of EDTA to the reaction mixture abolished cleavage, indicating that Mg^{2+} is essential for the catalytic activity of PI-*Mtu*I (Fig. 1, *lane 11*). Also, addition of SDS to the reaction mixture abolished endonuclease activity of PI-*Mtu*I (Fig. 1, *lane 12*). Optimal temperature for cleavage was found to be 37 °C under standard assay conditions containing 5 mM $MgCl_2$ (data not shown).

In previous studies, we showed that PI-*Mtu*I required both Mn^{2+} and ATP for optimal cleavage of the inteinless *rec*A allele (18). Thus, PI-*Mtu*I shows dual target specificity depending, first, on whether the substrate contains a cognate or ectopic site and, second, on whether the cofactor is Mg^{2+} or Mn^{2+} and ATP. One possible reason for this dual target specificity could be because of a contaminating endonuclease in the purified PI-*Mtu*I preparation. Although the identity of PI-*Mtu*I was established by sequencing 10 amino acid residues at the N-terminal end, it is necessary to validate the dual target specificity of PI-*Mtu*I. Accordingly, immunoprecipitation assay was performed using polyclonal antibodies raised against PI-*Mtu*I and the resulting immunosupernatant was assayed for endonuclease activity. As shown in Fig. 2, cleavage assay performed with immunosupernatant from the preimmune serum displayed Mg^{2+}-dependent endonuclease activity. However, the same from the reaction carried out with anti-PI-*Mtu*I failed to cleave M13 DNA, indicating that anti-PI-*Mtu*I antibodies had precipitated Mg^{2+}-dependent endonuclease activity. In gel filtration chromatography, both Mg^{2+}- and Mn^{2+} and ATP-dependent endonuclease activities were coincident with that of

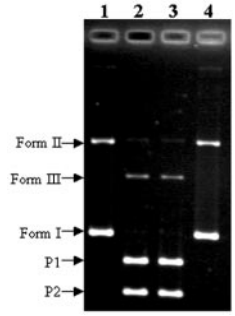

FIG. 2. **Immunoprecipitation of PI-*Mtu*I with anti-PI-*Mtu*I antibodies inhibited Mg^{2+}-dependent endonuclease activity.** Reaction mixtures contained 5 mM Mg^{2+}, PI-*Mtu*I (1 μM), and a mixture of form I and II DNA (16 μM), in the absence of PI-*Mtu*I or IgG (*lane 1*), in the absence of anti-PI-*Mtu*I (*lane 2*), in presence of preimmune IgG (*lane 3*), or in presence of anti-PI-*Mtu*I (*lane 4*). Positions of form I, form II, form III, and P1 and P2 products are indicated on the *left*.

PI-*Mtu*I elution profile (data not shown). Together, these observations suggest that both Mg^{2+}- and Mn^{2+} and ATP-dependent activities are intrinsic to PI-*Mtu*I.

Specificity of DNA Cleavage by PI-MtuI—The finding that PI-*Mtu*I bound various topologically different forms of M13 DNA with equal affinity raised the pertinent question of whether PI-*Mtu*I can distinguish these substrates during catalysis. To this end, we incubated the same molar concentrations of topologically different forms of M13 DNA separately with increasing concentrations of PI-*Mtu*I in the presence of Mg^{2+}. After incubation, reaction mixtures were deproteinized and the samples were analyzed by agarose gel electrophoresis. The results show that PI-*Mtu*I failed to cleave single-stranded DNA (Fig. 3, *lanes 2–5*), whereas form I and form II (Fig. 3, *lanes 7–10*), or form III DNA (Fig. 3, *lanes 12–15*) were converted to P1 and P2 products. Although PI-*Mtu*I bound form III as well as single-stranded DNA with similar binding pattern and affinity, it was able to distinguish between single- and double-stranded DNA during catalysis.

Effect of Ionic Strength on Cleavage of Ectopic DNA Sites by PI-MtuI—It has been reported that altering the ionic strength in the assay buffer influences the ability of homing endonucleases to recognize and cleave cognate DNA (1–6). In accord, we found that 50 mM NaCl or potassium glutamate was sufficient to abolish both cleavage and nicking of cognate DNA by PI-*Mtu*I (18). Interestingly, ectopic DNA was cleaved equally well in the absence or presence of 50 mM salt. However, cleavage of

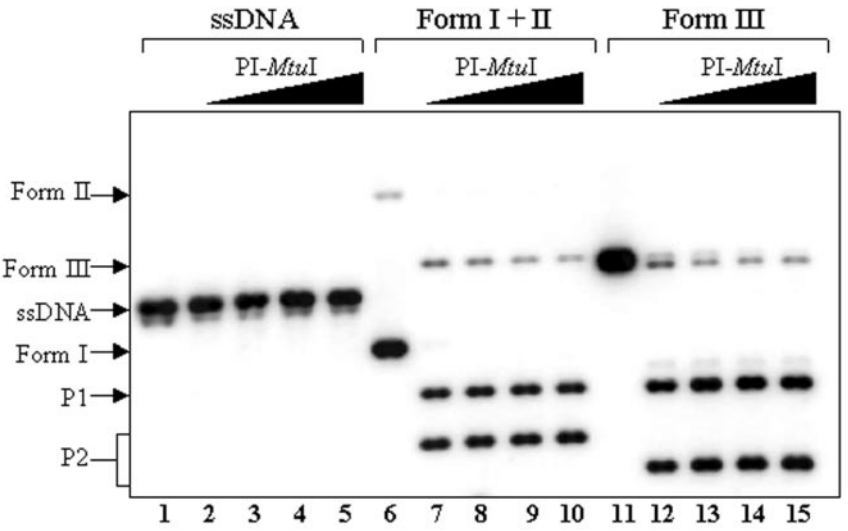

FIG. 3. **Specificity of DNA cleavage by PI-*Mtu*I.** Reaction mixtures contained M13 DNA (16 μM) and increasing concentrations of PI-*Mtu*I with 5 mM Mg^{2+}. Reactions were terminated and analyzed as described in the legend to Fig. 1. *Lanes 1*, *6*, and *11*, substrate DNA lacking PI-*Mtu*I. To the remaining lanes (*lanes 2–5*, *7–10*, and *12–15*) PI-*Mtu*I was added to concentrations of 1, 2, 3, and 5 μM, respectively. In reactions containing form III DNA (form I digested with *Bam*HI) as the substrate, P2 migrates faster because cleavage by PI-*Mtu*I generated 309 bp from P2, which ran out of the gel. The positions of substrates and products are indicated on the *left*.

ectopic DNA decreased progressively whereas nicking increased across a range of NaCl concentrations from 50 to 250 mM (data not shown). In contrast, cleavage of ectopic DNA by PI-*Mtu*I was not attenuated even in the presence of 250 mM potassium glutamate (data not shown). The basis for these differences in relative salt profiles is unclear.

Mechanism of Cleavage of Ectopic DNA by PI-MtuI—We next investigated the mechanism of Mg^{2+}-dependent cleavage of ectopic DNA by PI-*Mtu*I using form I DNA. Given form I DNA containing the recognition sequence, PI-*Mtu*I can linearize the substrate by two alternative mechanisms. In a sequential mechanism, nicking of form I DNA would lead to the formation of form II, and cutting the second strand in the vicinity of the first would generate form III DNA. In the concerted mechanism, form I DNA is directly converted to form III by cutting both strands simultaneously. The kinetics of the cleavage reaction showed a relatively slow phase during the first 10 min, at the same time the amount of form I DNA progressively decreased with a gradual increase in the levels of form II, followed by form III DNA (Fig. 4*A*). Quantification of cleavage products suggest that all of form I DNA was converted to form III and P1 and P2 products by 60 min (Fig. 4*B*). Together, these results indicate that Mg^{2+}-dependent cleavage of ectopic DNA by PI-*Mtu*I proceeds through a sequential mechanism. Whereas this mechanism is not unique, it is one of the simplest mechanisms that can fit the data presented in this report.

To further characterize the kinetics of cleavage, initial velocities of cleavage reactions catalyzed by PI-*Mtu*I were measured using form I DNA in the presence of Mg^{2+}. The velocities were determined from the initial linear phase of the cleavage reaction across a range of DNA concentrations from 2.5 to 30 μM. A plot representing velocity *versus* substrate concentration displayed a linear increase at lower substrate concentrations, and then plateaued at higher concentrations. The reaction velocities followed a conventional Michaelis-Menten curve when plotted against substrate concentration (Fig. 5*A*). The linear phase of the curve was fitted into Lineweaver-Burk plot (Fig. 5*B*) to yield the empirical kinetic constants, K_m, k_{cat} and V_{max} values to be 5.79 μM, 0.103 min^{-1}, and 0.31 μmol min^{-1} $liter^{-1}$, respectively.

PI-MtuI Distinguishes Cognate Versus Ectopic DNA in Mixed Reactions—To examine whether PI-*Mtu*I shows a preference for cognate over ectopic DNA during catalysis, reactions were performed in the presence of all the obligatory cofactors: Mn^{2+}, ATP, and Mg^{2+}. Because pEJ244 DNA bearing the inteinless *rec*A allele lacks the ectopic DNA site, it was possible to monitor the cleavage of each of the DNA substrates by simply observing the conversion of form I DNA to their respective nicked circular and linear DNA fragments. Under the conditions of this assay, PI-*Mtu*I showed important differences in the extent of cleavage. In particular, PI-*Mtu*I displayed robust Mg^{2+}-dependent cleavage of ectopic DNA sites over Mn^{2+} and ATP-dependent cleavage of cognate DNA (Fig. 6). The prominent products in the Mg^{2+}-dependent reaction were two expected cleavage products (marked P1 and P2) resulting from cleavage at two ectopic sites. As for the Mn^{2+} and ATP-dependent cleavage of cognate DNA, the rate of product formation was much slower (Fig. 6*B*). The kinetics of cleavage of ectopic DNA

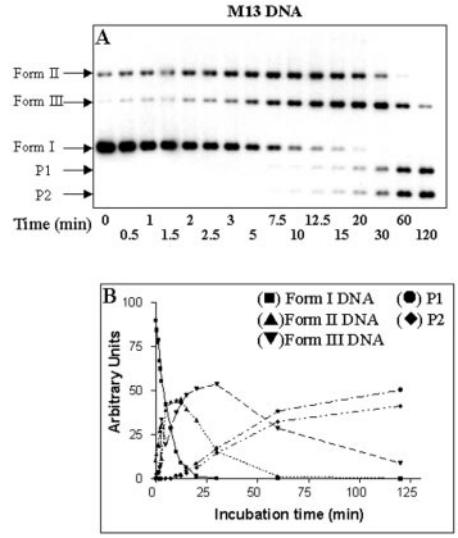

FIG. 4. **Kinetics of cleavage of ectopic DNA by PI-*Mtu*I.** Reactions were performed using form I DNA (16 μM) and PI-*Mtu*I (1 μM) in the presence of 5 mM Mg^{2+}. *Panel A*, at specified time intervals as indicated *below* each lane aliquots were removed, and the reaction was stopped immediately by the addition of SDS and proteinase K, followed by incubation for 15 min at 37 °C. The products were analyzed on an agarose gel as described in the legend to Fig. 1. The positions of substrates and products are indicated on the *left*. *Panel B*, the amount of products generated during the reaction were determined by quantification of the gel shown in *A*. The bands were quantified by scanning in a UVI-Tech gel documentation station using UVI-BandMap software version 99 and plotted using Graphpad Prism version 2.0. The extent of the formation of products (in arbitrary units) was plotted *versus* incubation time. The final cleavage products are labeled as P1 and P2. Quantification of the product generated as a function of time is an average of three independent experiments.

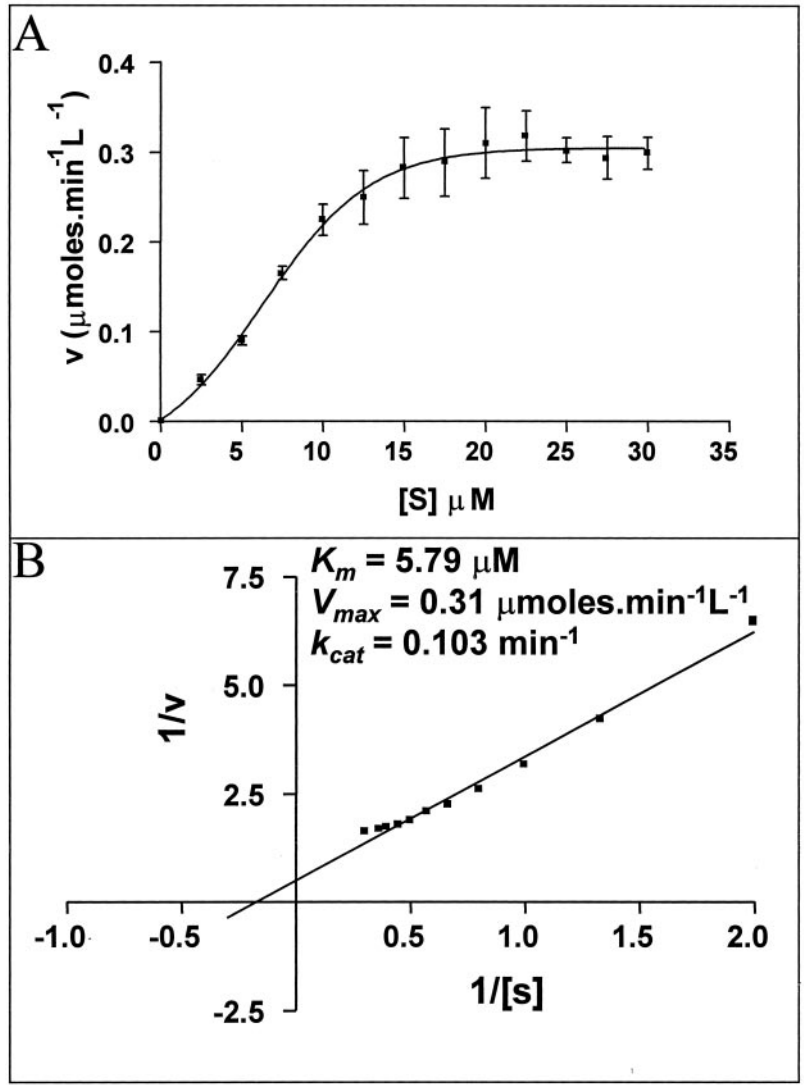

FIG. 5. **Cleavage of double-stranded DNA at ectopic sites by PI-*Mtu*I follows Michaelis-Menten kinetics.** Reactions were performed at different concentrations of the substrate with a fixed concentration of PI-*Mtu*I in the presence of 5 mM Mg^{2+}. The reaction products were separated on an agarose gel, and developed by Southern hybridization. The products were quantified by scanning in a UVI-Tech gel documentation station using UVI-BandMap software version 99 and plotted as a function of time. The rate of the reaction was calculated from the slopes of such plots. *Panel A*, plot of velocity *versus* substrate concentration using Graphpad Prism version 2. The data is the mean of three experiments. *Panel B*, plot of 1/*v versus* 1/S in the form of Lineweaver-Burk plot, and the K_m, V_{max}, and k_{cat} values were calculated from the intercepts.

(this study) or cognate DNA (18) in separate reactions were not significantly different from that of mixed reactions.

Mutation of Acidic Residues Alter the Catalytic Activity of PI-MtuI—Based on the crystal structure of PI-*Sce*I, and multiple sequence alignments of the LAGLIDADG family of homing endonucleases, we identified residues Asp^{122}, Asp^{222}, Glu^{220}, and Lys^{195} in PI-*Mtu*I as important for the recognition and cleavage of DNA (1, 25, 26). Asp^{122}, Asp^{222}, and Glu^{220} are highly conserved ligands probably required for binding of a divalent cation and hence catalysis. In addition to Lys^{195}, Lys^{131} was chosen to investigate its role in catalysis. These residues were subjected to site-directed mutagenesis, and the variants were overexpressed in *E. coli* and purified to homogeneity (Fig. 7*A*). Each variant protein exhibited the same purification behavior as the wild-type enzyme, indicating proper folding of proteins (data not shown). The identity of PI-*Mtu*I variants were further confirmed by Western blot analysis using antibodies raised against the wild-type PI-*Mtu*I (data not shown).

We examined the ability of PI-*Mtu*I variants to cleave ectopic DNA in the presence of Mg^{2+} (Fig. 7*B*). In comparison with the endonuclease activity of the wild-type enzyme, the variant enzymes can be classified into three categories. First, the pattern and the extent of cleavage products generated by the D122Y variant was quite similar to the wild-type enzyme. Second, the E220A and K195F variant enzymes produced a significant amount of form II DNA together with a trace amount of form III DNA. Third, D222T and K131L variant enzymes generated nearly equivalent amounts of form II and form III DNA, but failed to convert the latter to P1 and P2 products. Although nicking activity was displayed by all the variant enzymes, nicking was not at the same positions as created by the wild-type enzyme (see below). These results indicate that the penultimate residue in the first LAGLIDADG motif, Asp^{122}, is dispensable, and the second LAGLIDADG motif in PI-*Mtu*I is necessary for Mg^{2+}-dependent cleavage of ectopic DNA sites.

In a parallel set of experiments, we sought to relate the site of cleavage by restriction analysis. In particular, we wished to determine which of the two ectopic sites in M13 DNA was first cleaved by PI-*Mtu*I. Restriction mapping revealed that PI-*Mtu*I inflicted a double-strand break first at 2529 bp resulting in linearization of circular DNA, followed by a second double-strand break at 6042 bp to generate P1 and P2 products (data not shown).

D122Y Variant of PI-MtuI Displays Gain of Function Effect—The above results identified presumptive metal-binding ligands that are necessary for catalysis. Asp^{122} is a highly conserved residue, therefore, likely to be critical for metal binding and catalysis. However, with ectopic DNA as the substrate, the Mg^{2+}-dependent cleavage activity of the D122Y variant was similar to that of the wild-type enzyme (Fig. 7*B*).

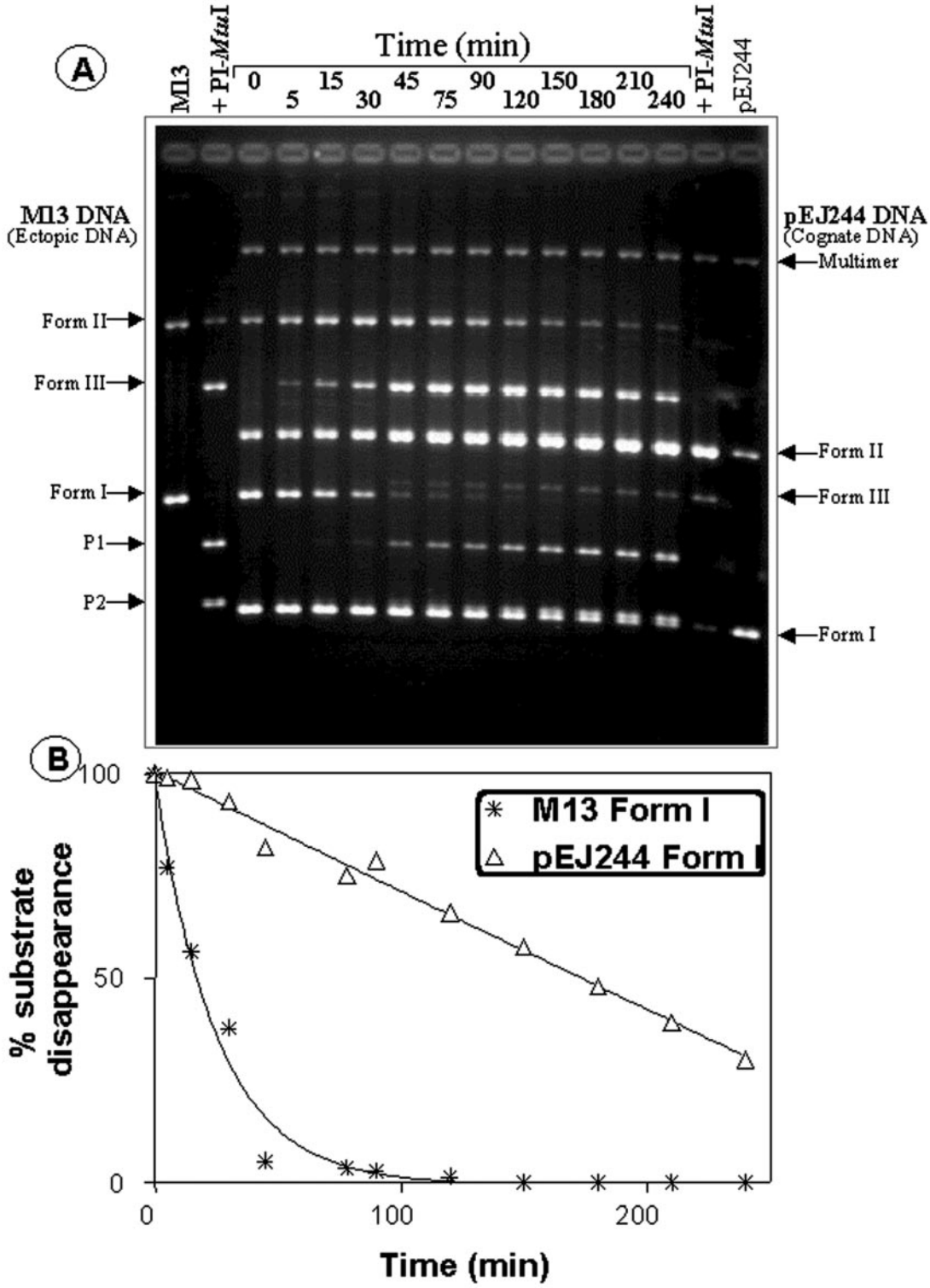

FIG. 6. **PI-*Mtu*I can effectively distinguish cognate and ectopic DNA sites during catalysis.** *Panel A*, cleavage reactions containing cognate (16 μM form I pEJ244 DNA) and ectopic DNA (16 μM form I M13 DNA) and PI-*Mtu*I (1 μM) in the presence of 3 mM Mn^{2+}, 1.5 mM ATP, and 5 mM Mg^{2+} were incubated as described in the legend to Fig. 1. At the specified time intervals as indicated *above* each lane, aliquots were withdrawn and the reaction was immediately stopped by the addition of SDS followed by proteinase K. Samples were analyzed on an agarose gel by electrophoresis. *Panel B*, the amount of form I DNA cleaved during the reaction was determined by quantitation of the gel shown in *A*, and represented as a function of time. In *panel A*, separation of DNA fragment P2 and form I DNA (last band) was not complete and migrate as a single band at the *bottom* in this gel system. However, loading them separately indicated that they are closely spaced bands. First and last lanes represent M13 or pEJ244 DNA in the absence of PI-*Mtu*I.

To re-evaluate the role of Asp^{122}, we compared the cleavage activity of the D122Y variant using cognate and ectopic DNA substrates at varying concentrations of divalent metal ions. The cleavage activity of the D122Y variant increased gradually with increasing concentrations of $MnCl_2$ to reach its peak at ~3 mM similar to that seen previously for the wild-type enzyme (Fig. 8, *lower panels*). Surprisingly, unlike the wild-type enzyme (Fig. 8*A*, *upper panel*), the D122Y variant was most active

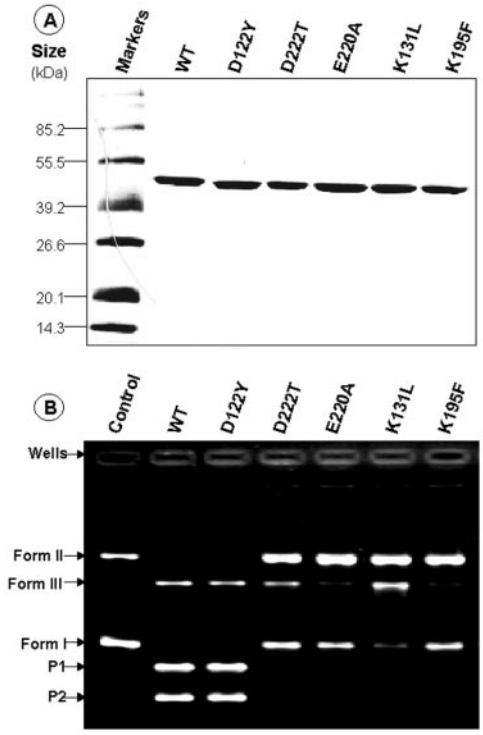

FIG. 7. **Analysis of PI-*Mtu*I variant enzymes.** *A*, SDS-PAGE analysis of purified wild-type and variant enzymes of PI-*Mtu*I. Approximately 10 μg of protein was separated by 10% SDS-PAGE and visualized by staining with Coomassie Blue. *Lane 1*, molecular mass markers (Combithek, Roche Molecular Biochemicals); *lanes 2–7* represent the identity of wild-type (*WT*) and variants of PI-*Mtu*I. *B*, endonuclease activity of wild-type and variants of PI-*Mtu*I. The assay was performed with a fixed concentration of wild-type or variants of PI-*Mtu*I (1 μM), M13 form I DNA (16 μM), and 5 mM Mg^{2+}. The reaction products were separated on an agarose gel and visualized as described in the legend to Fig. 1. The position of substrate and products generated are indicated on the *left*.

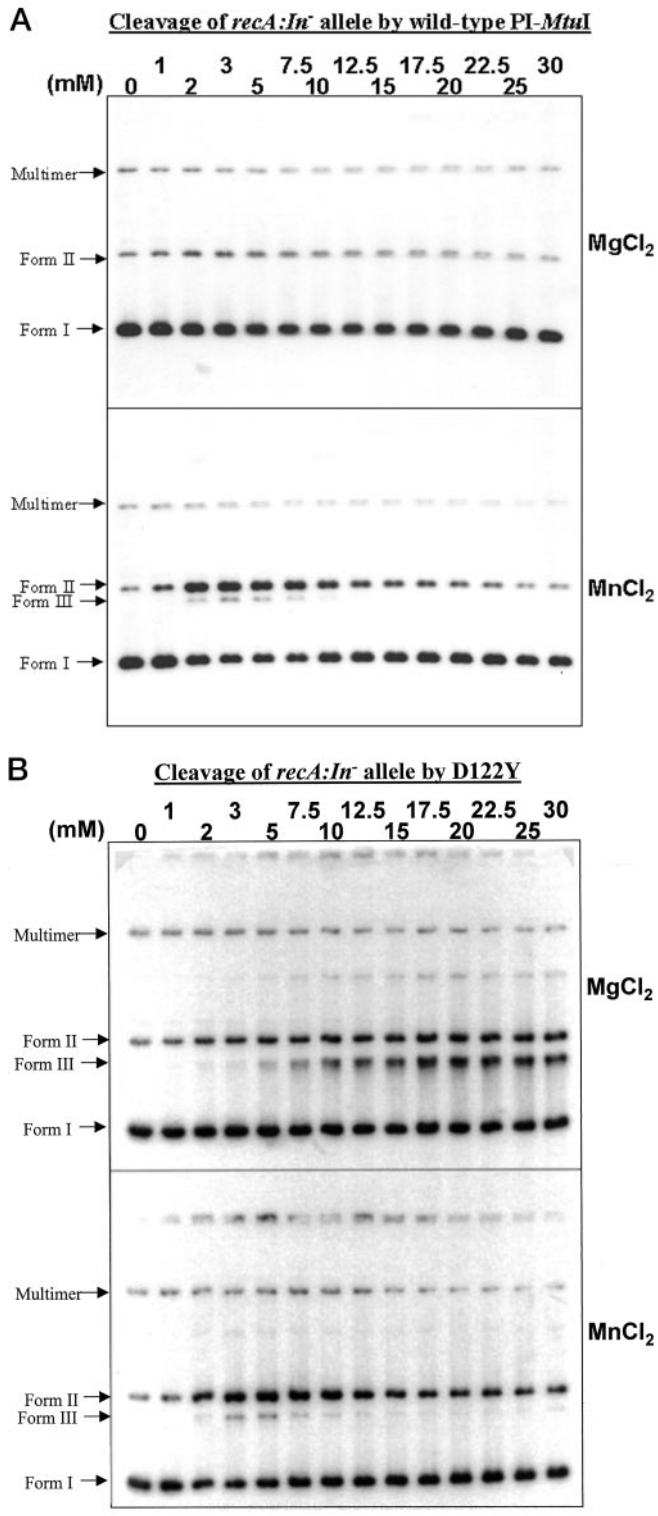

FIG. 8. **D122Y variant enzyme of PI-*Mtu*I promotes cleavage of the cognate site in the presence of Mg^{2+}.** Reaction mixtures contained 16 μM pEJ244 DNA, 1 μM PI-*Mtu*I (*A*) or D122Y (*B*) with the indicated concentrations of $MgCl_2$ (*upper panels*) or $MnCl_2$ + 1.5 mM ATP (*lower panels*). Reactions were performed and analyzed as described in the legend to Fig. 1.

on the cognate substrate in the presence of $MgCl_2$ (Fig. 8*B*, *upper panel*). The optimal Mg^{2+} concentration for cleavage activity was ~18 mM, and gradually decreased at higher Mg^{2+} concentrations. In this regard, the D122Y mutation in PI-*Mtu*I resulted in a gain of function effect by acquiring the ability to catalyze cleavage of cognate DNA in the presence of Mg^{2+}. The metal-mediated inhibition of cleavage activity at high Mg^{2+} has been noted for several Mg^{2+}-dependent nucleases (6). What is the mechanism of metal ion-dependent cleavage of DNA? It is known that Asp can coordinate with only Mn^{2+}, whereas Tyr can coordinate with oxophilic Mg^{2+}, as well as thiophilic Mn^{2+}, during catalysis. This partly explains the altered specificity of the D122Y variant to act on cognate DNA in the presence of Mg^{2+}.

Mapping of PI-MtuI Ectopic Cleavage Sites—To map the cleavage sites to the nucleotide level, we used 58- or 87-bp double-stranded DNA derived from form I M13 DNA. The ^{32}P-labeled DNA substrates were incubated with wild-type or variant enzymes of PI-*Mtu*I in the presence of Mg^{2+} as described above. The cleavage products, together with the Maxam-Gilbert chemical sequencing ladder, were separated by electrophoresis on polyacrylamide gels in the presence of urea. Interestingly, wild-type and D122Y variant cleaved both 58- and 87-bp substrates at very similar positions (compare Fig. 9, *A* and *B versus C* and *D*). Overall, the efficiency of cleavage was comparable. On the other hand, D222T and K131L variant enzymes displayed residual amounts of nicking activity in both the upper and lower strands of the 87-bp DNA substrate at several positions upstream of the ectopic site. E220A and K195F variant enzymes showed very weak nicking activity with the 87-bp substrate on both strands. However, except D122Y, the variant enzymes had no observable nucleolytic activity on the 58-bp DNA substrate (Fig. 9, *A* and *B*). The base sequence encompassing the cleavage site corresponding to P1

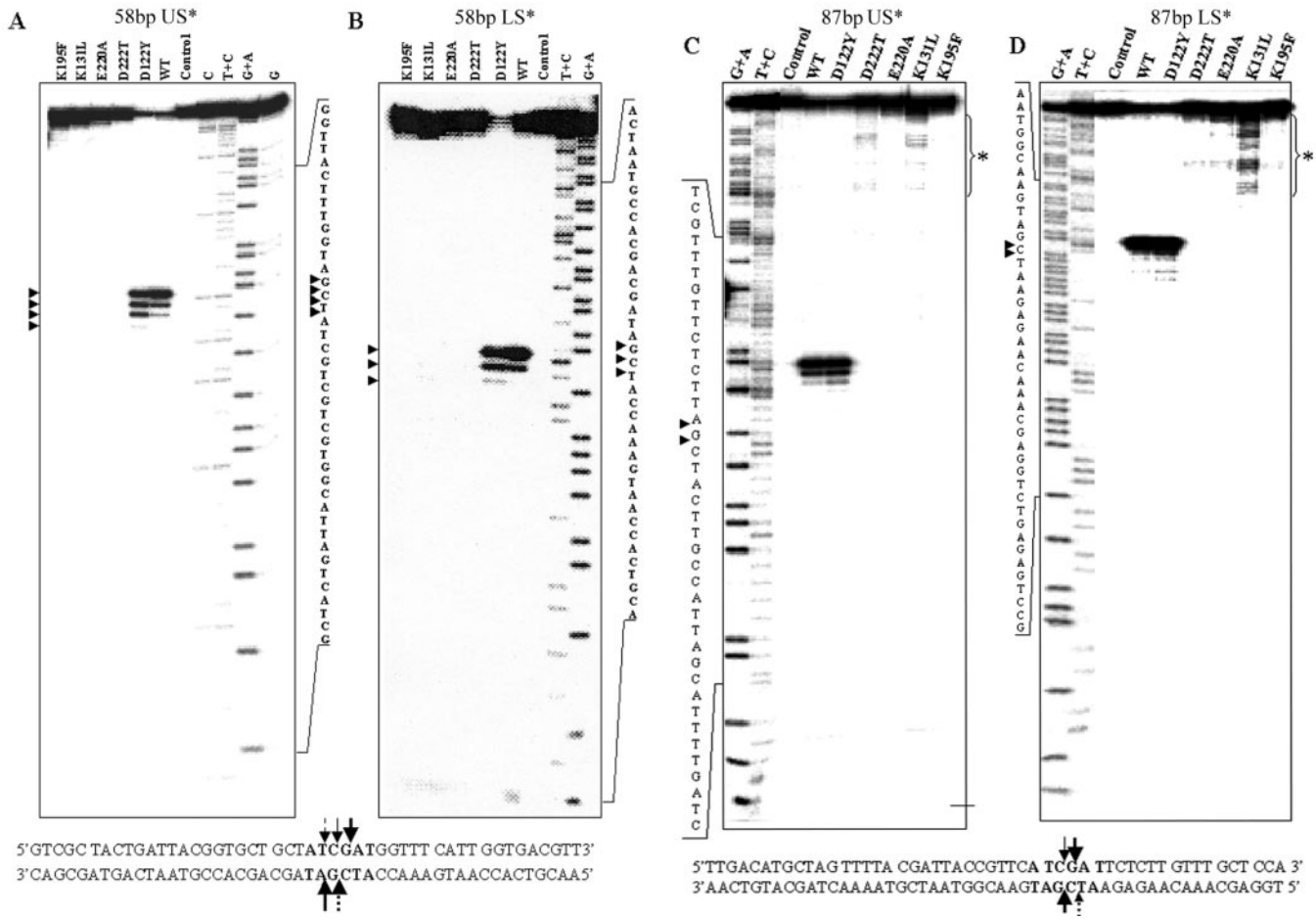

FIG. 9. **Mapping of the PI-*Mtu*I cleavage site on ectopic DNA.** 58- and 87-bp DNA substrates containing P1 and P2 ectopic sites, respectively, were separately labeled using [γ-^{32}P]ATP and polynucleotide kinase. Wild-type or variant enzymes of PI-*Mtu*I were incubated with labeled DNA as described under "Materials and Methods." *Panels A* and *B* show sequence around the ectopic site on the upper strand (*US*) and lower strand (*LS*) of the 58-bp substrate, respectively. *Panels C* and *D* show the sequence around the ectopic site on the upper and lower strands of the 87-bp substrate, respectively. The summary of cleavage site in individual DNA fragments is shown at the bottom of each pair of panels. The lanes marked *G* + *A* and *T* + *C* contain products generated by Maxam-Gilbert sequencing reactions. Similarly, wild-type (*WT*) and variants of PI-*Mtu*I used for cleavage reactions are indicated *above* each lane. The nucleotide sequences around the ectopic DNA sites are shown on the *right side* (for *panels A* and *B*) or left side (for *panels C* and *D*). The *arrowheads* indicate cleavage sites. The region marked with an *asterisk* denotes altered specificity of cleavage by the variants. The band intensities were quantified by PhosphorImager using Image Gauge version 3.0. *Solid* and *dotted vertical arrows* on the upper and lower strands denote the position of cleavage sites, and *thicker arrows* indicate the result of more intense cleavage. The sequence data shown is a summary of three independent experiments.

(Fig. 9, *A* and *B*) and P2 sites (Fig. 9, *C* and *D*) are summarized at the bottom of each pair of panels in Fig. 9.

The data presented here show that although the flanking sequences around the ectopic sites are very different, the cleavage pattern generated by the wild-type enzyme and D122Y variant with both the substrates is strikingly similar. The influence of flanking sequences on the rate of cleavage of P1 and P2 sites remains to be elucidated. Intriguingly, the hexameric sequence in the recognition site of PI-*Mtu*I is similar to the cleavage site determined for *Cla*I (27). We searched sequence data bases for sites in phage λ, pBR322, and pET11d, and the potential *Cla*I recognition sites were identified. Comparison of the profiles of reaction products generated by PI-*Mtu*I with that of *Cla*I indicated that the former cleaved at canonical *Cla*I sites to varying degrees of efficiency, whereas the latter cleaved at equal efficiency (data not shown). As a further test for specificity of their action, we examined the ends generated by these enzymes. Consistent with the previous studies (27), *Cla*I generated 5′-ended termini, whereas PI-*Mtu*I cleaved within its recognition sequences to leave 1–2-bp 3′-hydroxyl overhangs. The DNA ends in the P1 and P2 products generated by PI-*Mtu*I possessed 5′-phosphate and 3′-hydroxyl groups (data not shown).

DISCUSSION

In previous studies, we showed that PI-*Mtu*I is a novel Mn^{2+} and ATP-dependent homing endonuclease, which was able to cleave the inteinless *rec*A allele at 24 and 33/43 bases upstream of the intein insertion site, in the upper and lower strands, respectively (18). Here, we show that PI-*Mtu*I possesses an intrinsic ability to cleave ectopic DNA sites within the sequence 5′-ATCGAT-3′, in the presence of Mg^{2+}, resulting either in a blunt end or 1–2-nucleotide 3′-hydroxyl overhangs. However, the reaction promoted by PI-*Mtu*I with these two substrates are seemingly different. PI-*Mtu*I cleaved only a fraction of cognate substrate even after prolonged incubation, whereas all the substrate was digested in the case of ectopic DNA. The rate at which PI-*Mtu*I cleaved the ectopic DNA sites was much faster than the cognate substrate (this study and Ref. 18). The cleavage reaction profile with ectopic DNA sites indicated a sequential mechanism similar to the substrate bearing the cognate site. It is intriguing how PI-*Mtu*I could recognize and cleave two different sequences using alternative cofactors. Although the crystal structure of PI-*Mtu*I has not yet been determined, the crystal structure of PI-*Sce*I, a LAGLIDADG homing endonuclease, showed that it contains two active sites per

monomer (4). If so, each LAGLIDADG motif of PI-*Mtu*I might be involved in recognition and cleavage of cognate or ectopic DNA sites using alternative cofactors.

In the absence of divalent metal ions, PI-*Mtu*I was able to form stable protein-DNA complexes with topologically different forms of M13 DNA under physiological ionic strength. However, binding to single-stranded DNA failed to elicit cleavage in the absence or presence of metal ion, indicating that the single-stranded DNA·PI-*Mtu*I complex is not in a favorable conformation for cleavage. The key to our findings is the sensitive assay with which we were also able to decipher the cleavage mechanism promoted by PI-*Mtu*I with ectopic DNA. There are other examples from the LAGLIDADG family of endonucleases, such as PI-*Sce*I (28), PI-*Pfu*I (29), and group II introns (13, 14), that promote cleavage of both cognate as well as ectopic DNA sites. Furthermore, group II introns have been shown to promote transposition events into ectopic DNA sites *in vitro* and *in vivo*. However, in all these examples the ectopic DNA site closely resembles the natural homing site. In contrast, the ectopic DNA site of PI-*Mtu*I is distinct in its nucleotide sequence, and its cleavage requires a different cofactor.

The observation that PI-*Mtu*I promotes DNA cleavage at ectopic sites in the presence of Mg^{2+} was unexpected. Consequently, it remained plausible that the display of such an activity could arise from a contaminating endonuclease. Although preincubation of PI-*Mtu*I with anti-PI-*Mtu*I antibodies prior to the endonuclease assay indicated that both the activities are intrinsic to the same polypeptide, the evidence was less direct. We therefore sought a mutagenesis approach to formally resolve this issue. Sequence comparison of PI-*Mtu*I with PI-*Sce*I revealed conserved residues at the active site region. In addition, the availability of the crystal structure of PI-*Sce*I (25) abetted mutagenesis experiments. Based on these analyses, we assumed that PI-*Mtu*I is similar to PI-*Sce*I with an analogous catalytic domain.

In the co-crystal structure of I-*Cre*I the two LAGLIDADG motifs are a part of two catalytic centers (10). Structural and mutational evidence suggests a role for this motif in DNA-binding and phosphodiester bond cleavage. The penultimate Asp residue of the first LAGLIDADG motif is one of the ligands for coordination of metal ion. The role(s) of conserved acidic amino acid residues in DNA cleavage has been ascertained from crystal structure and mutational analysis of I-*Cre*I (30), PI-*Sce*I (31), and PI-*Pfu*I (32). In PI-*Mtu*I, the first dodecapeptide motif bears the sequence: ^{115}LLGYLIGD*G^{123} and, the second, ^{214}LLFGLFE*SD*G^{223} (asterisks denote conserved acidic amino acid residues). We performed mutational analysis of these presumptive metal ion-binding ligands (D122Y, E220A, and D222T) in PI-*Mtu*I. In addition, K195F and K131L variant enzymes were also generated to test their influence on catalysis. We measured the catalytic activity of these variant enzymes in comparison with the wild-type enzyme in the presence of Mg^{2+}. The PI-*Mtu*I variant enzymes can be grouped according to the severity of the defect: E220A and K195F were inactive in DNA cleavage. Whereas D222T and K131L displayed partial activity by generating form II and form III DNA thus confirming the essential role of these residues in catalysis (Fig. 7*B*). There is, however, an exception, the D122Y mutation did not affect the cleavage activity thus indicating plasticity of the catalytic center. We further show that the D122Y mutation in PI-*Mtu*I resulted in a gain of function effect by acquiring the ability to catalyze cleavage of cognate DNA in the presence of Mg^{2+}. On the other hand, E220A and K195F variants were inactive in endonuclease activity implicating these residues in cleavage of ectopic sites in the presence of Mg^{2+}. Nevertheless, these data suggest that Asp^{222} and Glu^{220} are likely to be the ligands involved in the Mg^{2+}-dependent cleavage activity of PI-*Mtu*I. How do these mutations inhibit DNA cleavage? One possibility is that they might promote a particular inefficient conformation in PI-*Mtu*I. The results of our mutational analysis of the active site residues of PI-*Mtu*I closely mimic those observed for PI-*Sce*I. Furthermore, both mutational and crystal structure data implicate the corresponding residues (Asp^{218} and Asp^{326}) of PI-*Sce*I to be the residues involved in metal ion binding and hence nucleophile activation (31–33). Thus, these data provide compelling evidence that Mg^{2+}, as well as ATP and Mn^{2+}-dependent endonuclease activities, are intrinsic to PI-*Mtu*I.

Our understanding is less complete regarding the molecular mechanism of lateral transfer of inteins in natural populations. Equally, our knowledge is limited by the paucity of factors thus far shown to be involved in this process. Therefore, the recent identification that bacterial group II introns effectively transpose into ectopic DNA sites (13), and yeast group II intron into sites that resemble the natural homing sites (14) has generated much excitement. The finding that PI-*Mtu*I was able to cleave the ectopic DNA site efficiently has raised the possibility that it might use such an activity to spread through natural populations. Inteins are now seen to be widespread among mycobacteria where they might play an important evolutionary role in restructuring the genome. It is also possible that the ability of PI-*Mtu*I to cleave at ectopic sites may provide defense against intruding DNA molecules. Although the occurrence of such a phenomenon is unclear in regard to *M. tuberculosis*, it is interesting to note that some eubacteria have evolved to do just that. Because overproduction of PI-*Mtu*I in *E. coli* had no deleterious effect on growth, it is possible that the sites are masked by the interaction of sequence nonspecific proteins such as HU, IHF, and other DNA-binding proteins. However, it is also possible that activation of PI-*Mtu*I in the presence of Mg^{2+} under *in vitro* conditions might only reflect the potential, rather than the primary biological activity under *in vivo* conditions.

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