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-MtuI to cleave double-stranded DNA at ectopic sites. In the absence of Mg$^{2+}$, PI-MtuI formed complexes with topologically different forms of DNA containing ectopic recognition sequences with equal affinity but failed to cleave DNA. We observed that PI-MtuI 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-MtuI. The kinetic mechanism of PI-MtuI 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-MtuI, 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 intronless 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-MtuI)$^3$ is a member of the LAGLIDADG superfamily of homing endonucleases (15–18). In previous studies, we showed that PI-MtuI is a novel homing endonuclease, which inflicted a staggered double-strand break 24 bp upstream of the intein insertion site in the inteinless recA 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-MtuI required both Mn$^{2+}$ and ATP for cleavage within the inteinless recA allele (18). In this report, we extend our focus on the mode of action of PI-MtuI 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-MtuI. Thus, PI-MtuI 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-MtuI in its lateral transfer in natural populations.

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$^3$The abbreviations used are: PI-MtuI, 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; ATP$\gamma$S, adenosine 5’-O-(thiotriphosphate).
Table I

| Sequence (5’ to 3’) | Variants |
|---------------------|----------|
| TACCTGATCCATGGCAGGAGT | D122Y |
| CTGGCGAAGATCTGGTGGTGAGCC | D222T |
| CACCCCCTCTGAGCAACAGG | E220A |
| GTTGGTGGAGGAGCTACTCCGCGT | K131L |
| CGGAATCGTGAATCCAGCGGAG | K159F |
| GACGGGGGGCGGCAAGAG | Reverse primer |

Materials and Methods

Reagents, Proteins, and DNA—All the chemicals used in this study were of analytical grade. Buffers were prepared by deionized water. Restriction enzymes, nylon N² 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 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-MtuI was purified to homogeneity and its concentration was determined as described (18). The same purification method was used to purify the variants of PI-MtuI.

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 MgCl₂, 0.4 mM dithiothreitol, 16 mM form I M13 DNA and PI-MtuI 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) of each of bromphenol blue and xylene cyanol) was added to each sample and separated on a 8% acrylamide 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 buffer (pH 7.5) containing 1 mM EDTA.

To map the second ectopic DNA site, the 2723–6003-bp NdeI fragment was labeled as described above, digested by AccI to generate the 87-bp fragment with the label at the 5’-end of the upper strand. Similarly, form I DNA cut with AccI was end-labeled, and digested by NcoI to generate the 87-bp fragment with the label at the 5’-end of the lower strand. After electrophoresis on a 8% polyacrylamide gel, the bands corresponding to these fragments were isolated from acrylamide gels as described above.

Results

Purification of Wild-type and Variants of PI-MtuI—Wild-type and variants of PI-MtuI 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-MtuI coeluted with molecular mass corresponding to 47 kDa, indicating that the quaternary structure of PI-MtuI 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-MtuI 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-MtuI to interact with topologically different forms of M13 DNA. We observed that PI-MtuI 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-MtuI emerged from DNase I protection assays. In this assay, a 16 μM single-stranded, form I, or linear duplex [3H]DNA was first incubated with varying amounts of PI-MtuI (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-MtuI at ≥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).
linear double-stranded DNA would be to permit detection of even the cleavage of single strands of the substrate by PI-MtuI. 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-MtuI 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-MtuI. 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-MtuI 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-MtuI 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-MtuI (Fig. 1, lane 11). Also, addition of SDS to the reaction mixture abolished endonuclease activity of PI-MtuI (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-MtuI required both Mn\(^{2+}\) and ATP for optimal cleavage of the inteinless recA allele (18). Thus, PI-MtuI 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-MtuI preparation. Although the identity of PI-MtuI was established by sequencing 10 amino acid residues at the N-terminal end, it is necessary to validate the dual target specificity of PI-MtuI. Accordingly, immunoprecipitation assay was performed using polyclonal antibodies raised against PI-MtuI 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-MtuI failed to cleave M13 DNA, indicating that anti-PI-MtuI 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. 1. PI-MtuI displays Mg\(^{2+}\)-dependent endonuclease activity on ectopic DNA sites.** Reactions were performed as described under “Materials and Methods.” Lane 1, DNA lacking PI-MtuI. The remaining samples were incubated with 16 µM M13 DNA (form I and form II, 90:10) and 1 µM PI-MtuI 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 ATP + 1.5 mM dATP (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.

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

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

**Specificity of DNA Cleavage by PI-MtuI—**The finding that PI-MtuI bound various topologically different forms of M13 DNA with equal affinity raised the pertinent question of whether PI-MtuI 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-MtuI 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-MtuI 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-MtuI 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-MtuI (18). Interestingly, ectopic DNA was cleaved equally well in the absence or presence of 50 mM salt. However, cleavage of
Mechanisms that can fit the data presented in this report. Whereas this mechanism is not unique, it is one of the simplest described in the legend to Fig. 1.

Reactions were terminated and analyzed as follows. PI-MtuI was added to concentrations of 1, 2, 3, and 5 μM, respectively. In reactions containing form III DNA (form I digested with BamHI) as the substrate, P2 migrates faster because cleavage by PI-MtuI 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-MtuI 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-MtuI using form I DNA. Given form I DNA containing the recognition sequence, PI-MtuI 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. 4A). 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. 4B). Together, these results indicate that Mg\(^{2+}\)-dependent cleavage of ectopic DNA by PI-MtuI 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-MtuI 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. 5A). The linear phase of the curve was fitted into Lineweaver-Burk plot (Fig. 5B) 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-MtuI 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 recA 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-MtuI showed important differences in the extent of cleavage. In particular, PI-MtuI 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. 6B). The kinetics of cleavage of ectopic DNA...
Mutation of Acidic Residues Alter the Catalytic Activity of PI-MtuI—Based on the crystal structure of PI-SceI, and multiple sequence alignments of the LAGLIDADG family of homing endonucleases, we identified residues Asp122, Asp222, Glu220, and Lys195 in PI-MtuI as important for the recognition and cleavage of DNA (1, 25, 26). Asp122, Asp222, and Glu220 are highly conserved ligands probably required for binding of a divalent cation and hence catalysis. In addition to Lys 195, Lys131 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. 7A). 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-MtuI variants were further confirmed by Western blot analysis using antibodies raised against the wild-type PI-MtuI (data not shown).

We examined the ability of PI-MtuI variants to cleave ectopic DNA in the presence of Mg2+ (Fig. 7B). 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, Asp122, is dispensable, and the second LAGLIDADG motif in PI-MtuI is necessary for Mg2+-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-MtuI. Restriction mapping revealed that PI-MtuI 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. Asp122 is a highly conserved residue, therefore, likely to be critical for metal binding and catalysis. However, with ectopic DNA as the substrate, the Mg2+-dependent cleavage activity of the D122Y variant was similar to that of the wild-type enzyme (Fig. 7B).
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 $\sim$3 mM similar to that seen previously for the wild-type enzyme (Fig. 8, lower panels). Surprisingly, unlike the wild-type enzyme (Fig. 8A, upper panel), the D122Y variant was most active

Fig. 6. PI-MtuI 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-MtuI (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-MtuI.

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on the cognate substrate in the presence of MgCl₂ (Fig. 8, upper panel). The optimal Mg²⁺ concentration for cleavage activity was ~18 mM, and gradually decreased at higher Mg²⁺ concentrations. In this regard, the D122Y mutation in PI-MtuI resulted in a gain of function effect by acquiring the ability to catalyze cleavage of cognate DNA in the presence of Mg²⁺. The metal-mediated inhibition of cleavage activity at high Mg²⁺ has been noted for several Mg²⁺-dependent nucleases (6). What is the mechanism of metal ion-dependent cleavage of DNA? It is known that Asp can coordinate with only Mn²⁺, whereas Tyr can coordinate with oxophilic Mg²⁺, as well as thiophilic Mn²⁺, during catalysis. This partly explains the altered specificity of the D122Y variant to act on cognate DNA in the presence of Mg²⁺.

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 ³²P-labeled DNA substrates were incubated with wild-type or variant enzymes of PI-MtuI in the presence of Mg²⁺ 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

![Figure 7: Analysis of PI-MtuI variant enzymes](image)

**Fig. 7.** Analysis of PI-MtuI variant enzymes. A, SDS-PAGE analysis of purified wild-type and variant enzymes of PI-MtuI. 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-MtuI. B, endonuclease activity of wild-type and variants of PI-MtuI. The assay was performed with a fixed concentration of wild-type or variants of PI-MtuI (1 μM), M13 form I DNA (16 μM), and 5 mM MgCl₂. 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.

![Figure 8: Cleavage of the cognate site in the presence of MgCl₂](image)

**Fig. 8.** D122Y variant enzyme of PI-MtuI promotes cleavage of the cognate site in the presence of Mg²⁺. Reaction mixtures contained 16 μM pEJ244 DNA, 1 μM PI-MtuI (A) or D122Y (B) with the indicated concentrations of MgCl₂ (upper panels) or MnCl₂ + 1.5 mM ATP (lower panels). Reactions were performed and analyzed as described in the legend to Fig. 1.
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 hexamer sequence in the recognition site of PI-MtuI is similar to the cleavage site determined for Clai (27). We searched sequence databases for sites in phage H9261, pBR322, and pET11d, and the potential Clai recognition sites were identified. Comparison of the profiles of reaction products generated by PI-MtuI with that of Clai indicated that the former cleaved at canonical Clai 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), Clai generated 5′-ended termini, whereas PI-MtuI 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-MtuI possessed 5′-phosphate and 3′-hydroxyl groups (data not shown).

DISCUSSION

In previous studies, we showed that PI-MtuI is a novel Mn2+ and ATP-dependent homing endonuclease, which was able to cleave the inteinless recA 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-MtuI possesses an intrinsic ability to cleave ectopic DNA sites within the sequence 5′-ATCGAT-3′, in the presence of Mg2+, resulting either in a blunt end or 1–2-nucleotide 3′-hydroxyl overhangs. However, the reaction promoted by PI-MtuI with these two substrates are seemingly different. PI-MtuI 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-MtuI 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-MtuI could recognize and cleave two different sequences using alternative cofactors. Although the crystal structure of PI-MtuI has not yet been determined, the crystal structure of PI-SceI, a LAGLIDADG homing endonuclease, showed that it contains two active sites per
monomer (4). If so, each LAGLIDADG motif of PI-MtuI might be involved in recognition and cleavage of cognate or ectopic DNA sites using alternative cofactors.

In the absence of divalent metal ions, PI-MtuI 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 forms of M13 DNA under physiological ionic strength. How do these mutations inhibit DNA cleavage? One possibility is that they might promote a particular inefficient conformation in PI-MtuI. The results of our mutational analysis of the active site residues of PI-MtuI closely mimic those observed for PI-SceI. Furthermore, both mutational and crystal structure data implicate the corresponding residues (Asp218 and Asp223) of PI-SceI 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-MtuI.

Our understanding is less complete regarding the molecular mechanism of lateral transfer of intransis 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 trans- pose 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-MtuI 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. Introns 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-MtuI 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-MtuI 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-MtuI 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.

**REFERENCES**

1. Belfort, M., and Roberts, R. J. (1997) *Nucleic Acids Res.* 25, 3379–3388
2. Liu, X. Q. (2000) *Annu. Rev. Genet.* 34, 61–76
3. Noren, C. J., Wang, J., and Perler, F. B. (2000) *Angew. Chem. Int. Ed. Engl.* 39, 450–469
4. Gimbé, P. S. (2000) *FEBS Microbiol. Lett.* 185, 99–107
5. Jurica, M. S., and Stoddard, B. L. (1999) *Cell. Mol. Life Sci.* 55, 1304–1326
6. Chevalier, B. S., and Stoddard, B. L. (2001) *Nucleic Acids Res.* 29, 5757–5774
7. Mueller, J. E., Bryk, M., Loizou, N., and Belfort, M. (1993) in _Nucleases_ (Linn, S. M., Lloyd, R. S., and Roberts, R. J., eds) pp. 111–144, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
8. Belfort, M., and Perlman, P. S. (1995) *J. Biol. Chem.* 270, 30237–30240
9. Wang, J., Kim, H. Y., Xu, A., and Herrin, D. L. (1997) *Nucleic Acids Res.* 25, 3767–3776
10. Jurica, M. S., Monnat, R. J., and Stoddard, B. L. (1998) *Mol. Cell* 2, 469–476
11. Dalgaard, J. Z., Garrett, R. A., and Belfort, M. (1994) *J. Biol. Chem.* 269, 28885–28892
12. Christ, P., Schoettler, S., Wende, W., Steuer, S., Pingoud, A., and Pingoud, V. (1999) *EMBO J.* 18, 6906–6916
13. Munoz, E., Villadas, P. J., and Toro, N. (2001) *Mol. Microbiol.* 41, 645–652
14. Duan, X., Huang, H.-R., Li, L., Matsuura, M., Lambowitz, A. M., and Perlman, P. S. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 13207–13212
15. Davies, E. O., Sedgwick, S. G., and Colston, M. J. (1991) *J. Bacteriol.* 173, 5653–5662
16. Davies, E. O., Thangaraj, H. S., Brooks, P. C., and Colston, M. J. (1994) *EMBO J.* 13, 699–703
17. Davies, E. O., Jenner, P. J., Brooks, P. C., Colston, M. J., and Sedgwick, S. G. (1992) *Cell* 71, 201–210
18. Guhan, N., and Muniyappa, K. (2002) *J. Biol. Chem.* 277, 16257–16264
19. Cunningham, R. P., DasGupta, C., Shibata, T., and Radding, C. M. (1986) *Cell* 45, 223–235
20. Aiyar, A., Xiang, Y., and Leis, J. (1996) *Methods Mol. Biol.* 57, 177–191
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual, 2nd Ed.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22. Laemmli, U. K. (1970) *Nature* 227, 680–685
23. Bakesi, R. P., Galande, S., Bali, P., Dighe, R., and Muniyappa, K. (2001) *J. Mol. Endocrinol.* 26, 153–156
24. Maxam, A. M., and Gilbert, W. (1980) *Methods Enzymol.* 65, 499–560
25. Duan, X., Gimble, P. S., and Quiocho, F. A. (1997) *Cell* 89, 555–564
26. Dalgaard, J. Z., Klar, A. J., Moser, M. J., Holley, W. R., Chatterjee, A., and Mian, I. S. (1997) *Nucleic Acids Res.* **25**, 4626–4638
27. Mayer, H., Grosschedl, R., Schutte, H., and Hobom, G. (1981) *Nucleic Acids Res.* **9**, 4833–4845
28. Gimble, F. S., and Thorner, J. (1992) *Nature* **357**, 301–306
29. Komori, K., Fujita, N., Ichiyanagi, K., Shinagawa, H., Morikawa, K., and Ishino, Y. (1999) *Nucleic Acids Res.* **27**, 4167–4174
30. Chevalier, B. S., Monnat, R. J., Jr., and Stoddard, B. L. (2001) *Nat. Struct. Biol.* **8**, 312–316
31. Schottlers, S., Wende, W., Pingoud, V., and Pingoud, A. (2000) *Biochemistry* **39**, 15995–15999
32. Gimble, F. S., and Stephens, B. W. (1995) *J. Biol. Chem.* **270**, 5849–5856
33. Komori, K., Ichiyanagi, K., Morikawa, K., and Ishino, Y. (1999) *Nucleic Acids Res.* **27**, 4175–4182