Inteins of *Thermococcus fumicolans* DNA Polymerase Are Endonucleases with Distinct Enzymatic Behaviors*

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Isabelle Saves‡, Valérie Ozanne§§, Jacques Dietrich**, and Jean-Michel Masson‡ ‡‡ §§

*From the ‡Institut de Pharmacologie et Biologie Structurale, IPBS/CNRS, 205 Route de Narbonne, F-31077 Toulouse Cedex, §Appligene-Oncor, Parc d’innovation, BP 72, F-67402 Illkirch Cedex, ||fremer, Laboratoire de Biotechnologie, Centre de Brest, BP 70, F-29280 Plouzané, and ‡‡Institut National des Sciences Appliquées, Complexe Scientifique de Rangueil, F-31077 Toulouse Cedex, France*

The DNA polymerase gene of *Thermococcus fumicolans* harbors two intein genes. Both inteins have been produced in *Escherichia coli* and purified either as naturally spliced products from the expression of the complete DNA polymerase gene or directly from the cloned intein genes. Both recombinant inteins exhibit endonuclease activity, with an optimal temperature of 70 °C. The *Tfu* pol-1 intein, which belongs to the *Psp* KOD pol-1 allelic family, recognizes and cleaves a minimal sequence of 16 base pairs (bp) on supercoiled DNA with either Mn<sup>2+</sup> or Mg<sup>2+</sup> as cofactor. It cleaves linear DNA only with Mn<sup>2+</sup> and requires a 19-bp minimal recognition sequence. The *Tfu* pol-2 intein, which belongs to the *Tli* pol-2 allelic family, is a highly active homing endonuclease using Mg<sup>2+</sup> as cofactor. Its minimal recognition and cleavage site is 21 bp long either on linear or circular DNA substrates. Its endonuclease activity is strongly inhibited by the 3′ digestion product, which remains bound to the enzyme after the cleavage reaction. According to current nomenclature, these endonucleases were named PI-*Tfu*I and PI-*Tfu*II. These two inteins thus exhibit different requirements for metal cofactor and substrate topology as well as different mechanism of action.

Since the first report of a protein splicing element as an in-frame insertion in the VMA gene of *Saccharomyces cerevisiae* (1, 2), over 80 putative inteins have been identified.¹ Inteins are widely distributed in a variety of eucarya, eubacteria, and archaea genes and tend to invade highly conserved and functionally important regions of their host genes. More than 30 different host proteins have been reported, including DNA and RNA polymerases, helicases, gyrases, RecA recombinase, ribonuclease reductase, and metabolic enzymes.

Protein splicing is a rapid autocatalytic process occurring at the peptide level. The intein coding sequence is transcribed and translated in the proper reading frame with flanking regions of the extein to produce a large precursor peptide. The intein is then autocatalytically excised while the N and C extein fragments are ligated, yielding a native and functional host protein. The molecular mechanism of splicing has been thoroughly investigated (3–7). The excised intein is also a stable protein. Like some group I intron open reading frames, some intein genes have been found to encode double-strand DNA endonucleases, which cleave the inteinless allele of the host gene. Moreover, Gimble and Thorner (8) demonstrated that the VMA1-derived endonuclease (VDE or P1-SceI) of *Saccharomyces cerevisiae* introduces a staggered double-strand break, repair of which results in the inversion of the intein coding sequence in the VMA1 gene *in vivo*. Thus, even if this homing event still remains to be demonstrated in eubacteria and archaea, it is clear that the endonuclease activity potentially confers highly specific mobility to its own coding sequence (9).

The majority of known inteins contain two conserved sequence motifs characteristic of the LAGLIDADG endonuclease family, called the dodecapeptide motifs, in their central region. In addition, two other motifs found in homing endonucleases and four motifs involved in splicing are generally observed in these inteins (10–13). While sequence alignment analyses tend to show that inteins containing dodecapeptide motifs probably exhibit endonuclease activity, only six inteins, Sce VMA, *Tli* pol-1, *Tli* pol-2, *Psp* GBD pol-1, *Psp* KOD pol-1, and *Psp* KOD pol-2, have been shown to be specific endonucleases and named PI-Sce I, PI-*Tli* II, PI-*Tli* I, PI-*Psp* I, PI-*Pko* I, and PI-*Pko* II, respectively, according to the current nomenclature (8, 12, 14, 15).

The coding sequence of the *Tfu* DNA polymerase gene from *Thermococcus fumicolans* (GenBank accession no. Z69882)² exhibits two in-frame insertions of 1080 and 1167 bp³ at positions 1218 and 2700, respectively (Fig. 1a), corresponding to expected inteins of 41.4 kDa (*Tfu* pol-1) and 44.8 kDa (*Tfu* pol-2). While both *Tfu* polymerase inteins display the conserved motifs determined by sequence alignment of homing endonucleases, there is a marked divergence between their sequences (11). The *Tfu* pol-1 intein belongs to the allelic family of *Psp* KOD pol-1, while *Tfu* pol-2 intein is a *Tli* pol-2 allele. Thus, to compare the splicing and endonuclease activity of both *Tfu* DNA pol inteins, their coding sequences and the entire gene of the DNA polymerase were cloned in expression vectors. After the production and the purification of recombinant and spliced inteins, their putative endonuclease activities were investigated and characterized.

**EXPERIMENTAL PROCEDURES**

*Production and Purification of the Tfu Polymerase Inteins—The coding sequences of *Tfu* pol-1 and *Tfu* pol-2 inteins were amplified by PCR

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¶ Present address: Valigné, Tour Neptune, 92086 Paris La Défense, France.
** Supported by the Conseil Régional de Bretagne.
§§ Recipient of an incitative Ifremer grant. To whom correspondence should be addressed. Tel.: 33-5-61-17-54-76; E-mail: masson@ipbs.fr.

¹ This information is available via the World Wide Web (S. Pietrokovski’s intein site).

² M. Cambon and J. Quéréllou, unpublished data.

³ The abbreviations used are: bp, base pair(s); pol, polymerase; PCR, polymerase chain reaction.
from *Tfu* genomic DNA with oligonucleotides *Tfu* pol-1-ATG (5′-tagttatagactaggccagct-3′), *Tfu* pol-1-TAG (5′-gagtctcaggacatgtgtgctcg-3′), *Tfu* pol-2-ATG (5′-ctgtagctagagttcaggggaagc-3′), and *Tfu* pol-2-TAG (5′-agttgctcaggagttcaggggagtc-3′), respectively. These PCR products were a gift from J. Quérélo (Inremer). They were digested by NdeI and SalI and cloned into a NdeI-SalI-digested pET28b vector (Novagen). The resulting constructs (pET26-ctcgag-3′) coding sequences of inteins and exteins is by Cambon and Que´rellou (GenBank accession HS000001). The resulting plasmids were either linearized by *Sal*I and *Xba*I, respectively (Fig. 1b), and purified or the supercoiled forms of the DNA substrates were digested linear and circular forms of these substrates were diluted in water to a concentration of 100 ng/µl. Endonuclease activity assays were performed in a final volume of 10 µl in various reaction buffers and temperatures ranging from 37 °C to 80 °C (see “Results”). The reaction mixtures were analyzed on a 1% agarose gel in TBE buffer. The amount of undigested substrates and products were quantified with the ImageQUANT program (Molecular Dynamics Inc.).

One unit of PI-7fUI or PI-7fUII endonuclease was required to digest 1 µg of *SacI*-linearized DNA substrate 1 or 2, respectively, in 1 h at 70 °C, in optimal buffers. Specific activities of PI-7fUI and PI-7fUII were measured by incubating known amounts of linear DNA substrates with known amounts of purified endonucleases.

**Definition of the Minimal Recognition and Cleavage Site of Endonucleases**—The endonuclease recognition sites were determined by a primer extension method as described by Wenzlau et al. (17). NaOH-denatured plasmids S1 and S2 were sequenced in both orientations using the T7 polymerase sequencing kit (Amerham Pharmacia Biotech) and universal primers SeqPuc (5′-gtaacgccagggttttcc-3′) and M13Rev (5′-gaaagcatgcaagacg-3′). After chain termination reaction, the samples were split in two. The first half of the sample was overdigest with 100 units of PI-7fUI or PI-7fUIII for 1 h at 70 °C, in a 50 µl Tris acetate, pH 8, buffer containing 100 µM NH₄OAc and 25 µM MnSO₄ or 75 µM Mg(OAc)₂, respectively. The cleavage reactions and the undigested half of the sequence reactions were ethanol-purified and resuspended in 5 µl of water. 4 µl of stop solution were added, and the samples were subjected to electrophoresis in a standard 6% denaturing polyacrylamide gel.

**Inhibition of Cleavage by Reaction Products**—4 µg of *SacI*-linearized substrate 2 were overdigest in PI-7fUII in optimal conditions by successive additions of enzyme. Both cleavage reaction products (940 and 1790 bp) were separated and purified from each other from a 1% agarose gel in TBE buffer. These two DNA fragments were then used as competitors in standard cleavage assays of substrate 2.

**Electrophoretic Mobility Shift Assay**—A 119-bp DNA probe containing the 43-bp recognition and cleavage site of PI-7fUII was synthesized by PCR. The universal M13Rev oligonucleotide was first phosphorylated with [32P]ATP (NEB Life Science Products) (18) and then used together with the pUC5-Xba oligonucleotide (5′-agctcggtaccgcgtaccgctg-3′) and 26 ng of substrate 2 DNA as matrix in a standard PCR amplification reaction. The 119-bp 32P-labeled fragment was purified and diluted in water to a concentration of 25 nm. 7.5 fmol of this DNA probe was incubated in a 20 µl Tris acetate, pH 8, buffer containing 20 µM Mg(OAc)₂, 20 µM NH₄OAc, 0.1% Triton X-100, and 16% glycerol, either with or without PI-7fUII, in a final volume of 20 µl, for 15 min at 37 °C or 70 °C. These reaction mixtures were then submitted to electrophoresis in a 7.5% non-denaturing polyacrylamide (30:1) gel in 0.25× TBE buffer at 220 V for 2 h. After electrophoresis, the gel was dried and radioactive bands were detected using a PhosphorImager (Molecular Dynamics Inc.). The specificity of the DNA-protein interaction was controlled using 7.5 pmol of herring sperm DNA as a nonspecific competitor in the binding reactions (data not shown).
RESULTS

Tfu Polymerase Inteins Were Spliced in E. coli—The complete Tfu polymerase gene was expressed in E. coli, at 25 °C. The SDS-polyacrylamide gel electrophoresis separation of the crude extract shows two bands of approximately 41 and 45 kDa, which comigrated with Tfu pol inteins (Fig. 2). At this temperature, a partial splicing reaction was observed since the DNA polymerase was not detected after staining the gel with Coomassie Blue, and we consistently obtained more Tfu pol-2 intein than Tfu pol-1.

Incubating this bacterial extract at 70 °C increased the yield of spliced proteins, the DNA polymerase being detected after heating for 30 min (Fig. 2). Even then, although no protein bands corresponding to the polymerase precursors were detected on the gel, splicing was probably not complete after 1 h of incubation at 70 °C because of the different amounts of each of the spliced products, i.e. the polymerase and both inteins.

Purified Tfu DNA pol is active in standard PCR reactions (Appligène-Oncor). Moreover, purified spliced Tfu pol-2 has the same specific endonuclease activity as the recombinant protein. In contrast, we could never detect any endonuclease activity for the purified spliced Tfu pol-1, unlike the recombinant protein (see below). We used the recombinant proteins to investigate further the endonuclease activity of both inteins.

Endonuclease Activity of Tfu Inteins—Inteins with known endonuclease activity recognize and cut a DNA sequence spanning the junction site created when the intein coding region is deleted from the host gene (homing site). We thus constructed substrate 1 for Tfu pol-1 and substrate 2 for Tfu pol-2 by cloning synthetic homing sites of 43 and 41 bp, respectively (Fig. 1). The supercoiled and ScaI-linearized forms of these DNA substrates were purified and used to assay the endonucleasic digestion of the supercoiled DNA (Fig. 3).

DNA polymerase in E. coli. 10% SDS-polyacrylamide gel electrophoresis of crude extract of Tfu DNA pol unheated (lane A) or treated at 70 °C for 30 min (lane B) and 1 h (lane C), of purified Tfu DNA pol (lane D) and recombinant Tfu pol-1 and Tfu pol-2 inteins (lanes E and F, respectively).

Fig. 2. Expression and splicing of Tfu DNA polymerase in E. coli. 10% SDS-polyacrylamide gel electrophoresis of crude extract of Tfu DNA pol unheated (lane A) or treated at 70 °C for 30 min (lane B) and 1 h (lane C), of purified Tfu DNA pol (lane D) and recombinant Tfu pol-1 and Tfu pol-2 inteins (lanes E and F, respectively).

Fig. 3. Standard cleavage assays for PI-TfuI and PI-TfuII. a, cleavage assay for PI-TfuI on circular substrate 1. 100 ng of purified supercoiled substrate 1 (Fig. 2) were incubated either with (+) or without (−) 40 ng of PI-TfuI, for 10 min at 70 °C, in a 50 mM Tris acetate, pH 8, buffer containing 100 mM NH₄OAc, and 50 mM Mg(OAc)₂ (Mg++) or 25 mM MnSO₄ (Mn++)). Supercoiled (sc), open circular (oc), and linear (lin) forms of DNA were separated on a 1% agarose gel in TBE buffer. b, cleavage assay for PI-TfuI on linear substrate 1. 100 ng of ScaI-linearized substrate 1 were incubated either with (+) or without (−) 40 ng of PI-TfuI, for 10 min at 70 °C, in a 50 mM Tris acetate, pH 8, buffer containing 100 mM NH₄OAc, and 50 mM Mg(OAc)₂ (Mg++) or 25 mM MnSO₄ (Mn++)). Substrate (S, 2730 bp) and products (P, 940 and 1790 bp) were separated on a 1% agarose gel in TBE buffer. c, standard cleavage assay for PI-TfuII. 100 ng of linear or circular substrate 2 (Fig. 2) were incubated with (+) or without (−) 0.1 ng of PI-TfuII in a 50 mM Tris acetate, pH 8, buffer containing 100 mM NH₄OAc, 75 mM Mg(OAc)₂, and 10% glycerol, for 10 min at 70 °C. Linear (lin) and linear (lin) forms were separated on a 1% agarose gel in TBE buffer.
3c). The effects of DNA topology and divalent ions used as cofactor on endonuclease activity of PI-TfuI and PI-TfuII are summarized in Table I.

**Minimal Recognition Sequences**—A mixture of potential double-strand DNA substrates for PI-TfuI and PI-TfuII were generated by primer extension using circular substrate 1 or substrate 2 as templates, respectively. The extension products including all the bases needed for cleavage were digested and thus disappeared from the sequence reaction, while those lacking one or more bases of the minimal recognition site were not digested. Hence, the comparison of digested and undigested sequence reactions allowed the determination of the bases necessary for cleavage (Fig. 4a). This method yielded a 16-bp non-palindromic site for PI-TfuI and a 21-bp non-palindromic site for PI-TfuII. In both cases, cleavage generated non-identical 3’ overhangs of 4 bases (Fig. 4b). Both cleaved substrates could be religated with T4 DNA ligase. Thus, cleavage by PI-TfuI or PI-TfuII generated 5’-phosphate and 3’-hydroxyl ends. To confirm the results obtained by primer extension, plasmids containing the 21-bp site for PI-TfuII (substrate 3) or the 16-bp site for PI-TfuI (substrate 4, Fig. 1b) were constructed and submitted to cleavage assays either as linear or supercoiled DNA.

Standard cleavage assays on linear and circular DNA substrates 3 and 4, under optimal conditions for each enzyme, highlight the different catalytic behaviors of the endonuclease inteins (Table I). While PI-TfuII cleaved with the same efficiency linear and circular substrates 2 and 3 in the magnesium-containing buffer, PI-TfuI cleaved linear substrate 1 but did not cleave linear substrate 4 even in presence of manganese (Fig. 5a). On the other hand, PI-TfuI cleaved with the same efficiency supercoiled substrates 1 and 4 (Fig. 5b). Cleavage assays on various linear substrates for PI-TfuI (data not shown), containing synthetic homing sites of different lengths, led to a minimal linear site of 19 bp (Fig. 1b), which is homologous to the one described for PI-PkoI (15).

Thus, PI-TfuII cleaved its homing site and made no difference between linear and supercoiled DNA containing the minimal or a larger recognition site. In contrast, PI-TfuI cleaved supercoiled DNA harboring its 16-bp recognition sequence but required a longer recognition sequence (>19 bp) and manganese as cofactor to cleave linear DNA. Moreover, under these conditions, linear DNA was cleaved as efficiently as circular DNA (Table I).

**Product Inhibition of PI-TfuII**—While incubation of linear substrate 1 with PI-TfuI led to its complete cleavage, prolonged incubation of substrate 2 with PI-TfuII resulted in only partial cleavage (Fig. 6a). The same results were obtained with circular DNA substrates (data not shown). After 10 min, 80% of the cleavage was performed and the reaction stopped. Addition of fresh PI-TfuII allowed the reaction to proceed, but a loss of activity rapidly followed (data not shown). As the enzyme was stable at 70 °C, this loss of activity could result from an inhibition of PI-TfuII by one of its reaction products. To test this hypothesis, the two DNA fragments resulting from the cleavage of ScaI-linearized substrate 2 were purified and added as potential inhibitors in cleavage reactions. Under the conditions used, 50 ng of substrate 2 were digested by PI-TfuII, when no inhibitor was added to the reaction. Fig. 6b shows that the 940-bp fragment, which includes the homing site 5’ end, inhibited weakly the cleavage reaction. On the other hand, the 1790-bp fragment, which includes the homing site 3’ end, was an efficient inhibitor of the reaction, a 1:1 ratio between substrate and DNA fragment resulting in 80% inhibition. Electro-

![Table I](image)

**Relative Activities of PI-TfuI and PI-TfuII**

Relative activities of PI-TfuI and PI-TfuII on substrates 1 and 4, or 2 and 3, respectively. The cleavages of linear or circular forms of substrates are compared, and Mn²⁺ and Mg²⁺ are used as cofactors. ++ indicates that the cleavage is optimal; + indicate a 5–10-fold loss in cleavage efficiency; − indicates that no activity is detected.

|                          | Linear DNA | Circular DNA |
|--------------------------|------------|--------------|
|                          | Mn²⁺ | Mg²⁺ | Mn²⁺ | Mg²⁺ |
| PI-TfuI Substrate 1 (43 bp) | ++   | −   | +++  | +   |
| PI-TfuI Substrate 4 (16 bp) | −   | −   | +++  | +   |
| PI-TfuII Substrate 2 (41 bp) | +   | +++ | +   | +++ |
| PI-TfuII Substrate 3 (21 bp) | +   | +++ | +   | +++ |

![Diagram](image)

**Fig. 4.** Minimal recognition site of PI-TfuI and PI-TfuII, determined using the primer extension procedure. a, autoradiogram of the sequencing gel for PI-TfuII site determination. The sequencing reactions were performed in direct (SeqPuc) or reverse (M13Rev) orientations. PI-TfuII-digested (+ lanes) and undigested (− lanes) reactions were loaded side by side on a 6% denaturing polyacrylamide gel. A large arrow indicates the cleavage site on each DNA strand. Boxes represent bases belonging to the minimal site, in each direction. b, minimal nucleotide sequence necessary for recognition and cleavage by PI-TfuI and PI-TfuII. The arrows indicate the homing sites (HS) of the inteins in the DNA polymerase gene. The dashed boxes indicate the minimal recognition sequences.
phoresis mobility shift assays confirmed the strong interaction between the 3′ cleavage fragment and PI-TfuII. Fig. 7 shows that this digestion product remained bound to the enzyme after cleavage was performed at 70 °C. Under these experimental conditions (large excess of enzyme over substrate), cleavage could also be observed at 37 °C but there was surprisingly no mobility shift for the resulting fragment.

**DISCUSSION**

Most DNA polymerase genes from archaeabacteria sequenced to date harbor one to three intein genes. These inteins are in-frame insertions at precisely the same locations, in the conserved motifs II, III, or I of the class B DNA polymerases (pol α). Thus, three allelic families of inteins have been defined, corresponding to these three different locations (Ref. 12; available via the World Wide Web from InBase, the New England Biolabs intein data base). Among the seven known intein-harboring polymerases, the Tfu polymerase is the only one that does not have a large intein within the conserved motif III. Its two inteins, Tfu pol-1 in motif II and Tfu pol-2 in motif I, belong to the Psp KOD pol-1 and Tli pol-2 allelic families, respectively. Both inteins exhibit endonuclease activity with an optimal temperature of 70 °C, which is in line with the thermophilic nature of *T. fumicolans*. Splicing of the inteins does occur in *E. coli*, but it is very inefficient. It is greatly enhanced by a prolonged incubation at 70 °C. Thus, unlike the splicing of PI-SceI, splicing of the Tfu pol inteins seems quite temperature-dependent. One puzzling result was the fact that no endonuclease activity could be detected in vitro for the spliced PI-TfuI, while the recombinant protein exhibits enzymatic activity. This suggests that, when produced in *E. coli*, the precursor protein fold is not fully correct, resulting in hindered splicing as well as inactive PI-TfuI. Indeed, splicing of PI-TfuII is much more efficient that splicing of PI-TfuI and the spliced PI-TfuII is fully active. When produced separately, the recombinant inteins and the DNA polymerase are active. High salt buffers are needed for optimal cleavage activity of both inteins. This feature could be linked to the fact that *T. fumicolans* is a marine organism, optimal growth of which is obtained with 30–40 g/liter salt (19). Indeed, the DNA polymerase activity is also stimulated by salts, while DNA polymerases from freshwater thermophilic bacteria, such as the Taq enzyme, do not need high salt concentrations.

Tfu pol-1 (PI-TfuI) is 75.5% identical to Psp KOD pol-1, also known as homing endonuclease PI-PkoI (15). PI-TfuI cleaves supercoiled DNA with magnesium or manganese as cofactor.
but requires manganese and a longer recognition site to cleave linear DNA. A difference in size requirement for minimal recognition sequence between linear and circular DNA has already been described for PI-SceI (20). The 16-bp minimal recognition sequence for PI-TfuI is 3 bp shorter than the one determined for PI-PkoI (15). In fact, on linear DNA, PI-TfuI and PI-PkoI both require a 19-bp sequence for binding and cleavage. The main difference between the two enzymes then seems to be metal requirements. Within the conserved sequence motifs that constitute the endonuclease signature, there are few differences between the two inteins PI-TfuI and PI-PkoI. The two acidic residues that are part of the catalytic triad (Glu-125 and Asp-225) are conserved, but the third residue, Lys-203, is replaced by an arginine in PI-TfuI. Such a substitution in PI-SceI (K301R) led to a 15-fold loss of activity and to a larger increase of activity when substituting magnesium with manganese (21). The equivalent substitution K92R totally inactivated EcoRV (22). On the other hand, an arginine is also found in the catalytic triad of PI-TII, which requires Mg$^{2+}$ as cofactor (12), as does PI-PkoI. This change in residue therefore cannot account for the specific metal requirement of PI-TfuI.

Although manganese has been shown to stimulate the activity of magnesium requiring endonucleases, including PI-SceI (8, 21), it usually results in relaxed specificity, allowing cleavage of non-cognate sites. PI-TfuI is peculiar, for it has no detectable activity with magnesium on linear DNA while its activity with manganese as a cofactor is comparable to that of PI-PkoI. Thus, manganese relaxes the topological specificity of PI-TfuI, but not its requirement for cognate sequence, which is the same with either metal on supercoiled DNA. In this respect, it is reminiscent of the I91L mutant of EcoRV, where a single mutation within the active site of the enzyme results in a total shift in metal requirement while retaining specificity for the cognate sequence (23). The longer sequence needed for cleavage of linear DNA could translate the requirement of initial DNA bending for efficient substrate binding. On bent DNA, the enzyme takes a conformation that allows either magnesium or manganese to occupy the cofactor site and neutralize the phosphorus to trigger the cleavage of the phosphodiester bond. On linear DNA, the enzyme might take a slightly stretched conformation such that magnesium cannot occupy the cofactor site anymore while manganese, due to its 20% larger bonding radius, can still efficiently bind to its site on the protein and neutralize the phosphorus on DNA.

An open circular intermediate accumulates during the reaction with supercoiled DNA, suggesting a two-step mechanism. A slower reaction rate with magnesium translates into more open circular intermediate. In a similar way, an open circular intermediate also accumulates during the magnesium-catalyzed cleavage by EcoRV, but disappears with manganese (either with the wild type EcoRV or with the I91L mutant) due to the enhanced cleavage rate.

$Tfu$ pol-2 (PI-TfuII), which is 65% identical to $Tli$ pol-2 (PI-TII), is a very active endonuclease. It recognizes and cuts a 21-bp site on linear or circular DNA, leaving a 4-base 3'-OH overhang. This site is 15 bp shorter than the PI-SceI homing site (24) and slightly larger than the sites recently determined for the PI-PkoI, PI-PkoII (15), or PI-TfuI (this work) enzymes. It only differs by 3 bases from the 21-bp sequence central to the PI-TII recognition site. A more thorough comparison of these two enzymes is needed to determine if they truly have different cleavage sites. Unlike PI-TfuI, PI-TfuII requires Mg$^{2+}$ as a cofactor and is less active with Mn$^{2+}$ for cleavage of its cognate sequence.

PI-TfuII is subject to efficient product inhibition by one of the cleavage fragments. Our gel shift experiments indicate that the $K_I$ for the product should be of the same order of magnitude as the $K_m$ for the substrate. We also observe that binding of the substrate at 37°C is still efficient, while cleavage is almost abolished as well as product inhibition. This result is reminiscent of an inhibition mechanism involving the catalytic residues. Similar product inhibition has been reported for other endonucleases, including PI-SceI, although in this latter case inhibition appears more drastic (20). Thus, the DNA recognition and cleavage mechanism is probably homologous for both endonucleases, with a strong binding site 3' to the cleavage site.

$T. fumicolans$ DNA polymerase gene harbors two intein genes like many other archaea DNA polymerase genes, but it is the only one that does not have a $Tli$ pol-1 type intein allele. Its two intein genes code for active endonucleases that greatly differ in specific activity, cognate sequence, as well as metal requirement, cleavage site topology, and sensitivity to product inhibition. Such a situation has not been yet described and provides a contrasted model for the study of archaeabacterial endonuclease inteins. Site-directed mutagenesis of the active site of PI-TfuI will provide more information on the role of its residues and on its unusual topological and cofactor specificity.

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