

Digestion of I-PpoI Recognition Sites in Unfavorable Sequence Contexts Achieved by Changing the Reaction Conditions

Christina B. McCarthy¹ and Victor Romanowski^{1,2,3}

Received 9 March 2005—Final 19 July 2005

Published online: 29 April 2006

INTRODUCTION

I-PpoI is an intron-encoded endonuclease that cuts with high specificity at a 15 base pair (bp) recognition sequence. It belongs to a diverse family of proteins designated as homing endonucleases, found in unicellular eukaryotes, *Archaea* and *Eubacteria*. They are encoded by open reading frames nested within mobile introns or inteins, and they recognize lengthy DNA target sites (14–40 bp). Group I intron homing endonucleases have been classified in four distinct families on the basis of the following conserved sequence motifs: LAGLIDADG, His-Cys Box, GIY-YIG, and HNH. I-PpoI was the first identified member of the His-Cys Box homing endonuclease family and is encoded by an open reading frame in the third intron of the nuclear, extrachromosomal 26S rRNA gene of the slime mold *Physarum polycephalum* (Muscarella and Vogt, 1989; Muscarella *et al.*, 1990). I-PpoI is a homodimer that cleaves a partially symmetric 15 bp homing site (5'-CTCTCTTAA↓GGTAGC-3') generating 5'-TTAA-3' cohesive overhangs (Lowery *et al.*, 1992; Ellison and Vogt, 1993; Wittmayer and Raines, 1996).

Unlike restriction sites, homing sites may vary in sequence at many of their individual nucleotide positions while still being recognized and cleaved. I-PpoI requires at least 14 bp of its 15 bp recognition sequence for efficient binding and cleavage and protects up to 4 bp to either side of its homing site (here considered as the sequence context) in footprint experiments (Argast *et al.*, 1998; Flick *et al.*, 1998). Wittmayer *et al.* (1998) examined the specificity of the I-PpoI endonuclease. They tested the catalytic activity of I-PpoI with DNA recognition

¹IBBM, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina.

²Dto. Ciencia y Tecnología, Universidad Nacional de Quilmes, Bernal, Argentina.

³To whom correspondence should be addressed; e-mail: victor@biol.unlp.edu.ar.

sites containing mutations that spanned the entire 23 bp footprint. They found that a degree of degeneracy is tolerated within the recognition site of I-*PpoI*. In contrast to substitutions within the I-*PpoI* recognition site, single deletions or insertions are detrimental to catalysis, suggesting that proper spacing of contacts along the protein–DNA interface is critical. They also found that bases flanking the 15 bp recognition site could affect the activity of I-*PpoI*. Single substitutions within the flanking regions did not affect catalysis by I-*PpoI*. Multiple substitutions within the flanking regions only, however, had variable effects on catalysis by I-*PpoI*. Their results revealed that substitutions outside the 15 bp recognition site could modulate catalysis by I-*PpoI*. Nevertheless, they did not report a sequence that thwarted I-*PpoI*'s catalysis. In contrast to the findings of Wittmayer *et al.* (1998), we found that a certain combination of multiple substitutions in the sequence context decreased the cleavability of the I-*PpoI* site dramatically.

METHODS AND RESULTS

We explored different alternatives to improve I-*PpoI*'s deficient performance due to a highly unfavorable sequence context and found an experimental condition that allowed efficient cutting by I-*PpoI*.

Strategies to Improve I-*PpoI*'s Performance

Construction of Two Plasmids

We explored two different approaches to improve I-*PpoI*'s deficient performance due to an adverse sequence context. We used a previously constructed plasmid (Arana *et al.*, 2001) in which we inserted I-*PpoI*'s restriction site in an unfavorable sequence context (pAg-*IPpoI*), to find an experimental condition in which the enzyme worked satisfactorily. For comparative assays, we also built a different plasmid (pZ-*IPpoI*) with an I-*PpoI* site in a favorable sequence context, according to the information provided by Wittmayer *et al.* (1998) (see Fig. 1).

Endonuclease Assays Using pAg-IPpoI as Substrate (Unfavorable Sequence Context). Published reports indicate that I-*PpoI* is optimally active at a basic pH (pH 10; Lowery *et al.*, 1992) and that it is slightly less active at a more neutral pH (pH 7.5; Wittmayer and Raines, 1996). To find an experimental condition in which the enzyme worked satisfactorily, we assayed I-*PpoI*'s capacity to digest pAg-*IPpoI* DNA at different pHs and ionic strengths. We found that the enzyme worked optimally in buffer E with a 1 × composition of 6 mM Tris-HCl, 6 mM MgCl₂, 100 mM NaCl, 1 mM DTT, pH 7.5 (hereafter referred to as the new buffer conditions), as opposed to the commercial buffer provided by the manufacturer with a 1 × composition of 25 mM CHES, 25 mM CAPS, 2 mM MgCl₂, 1 mM DTT, and pH 10.0 (here referred to as the prescribed buffer

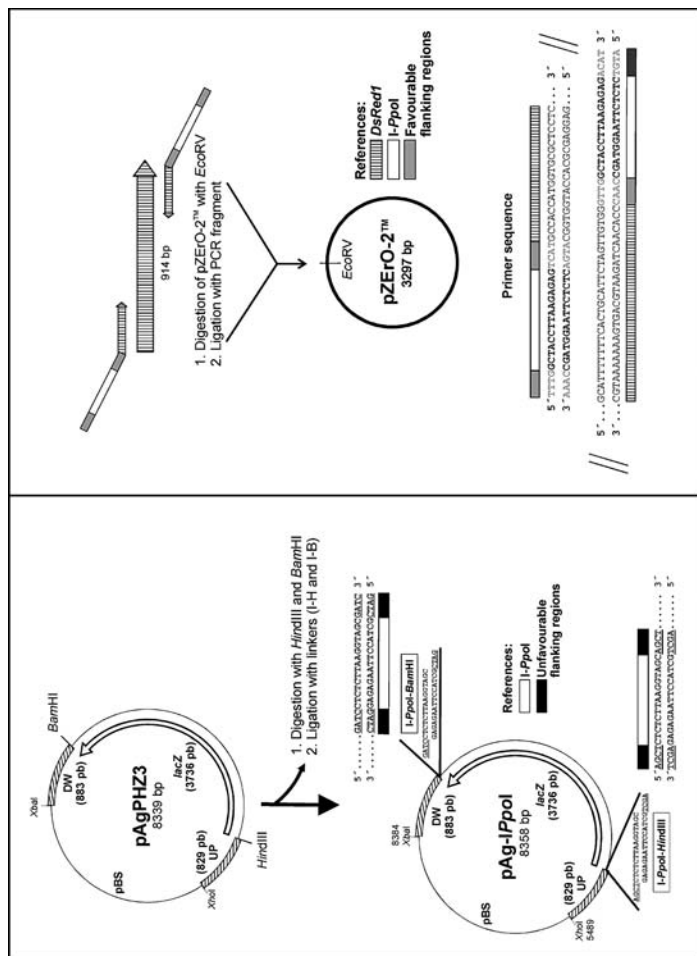


Fig. 1. Construction of two plasmids (pZ-*I-Ppol* and pAg-*I-Ppol*) comprising favorable and unfavorable sequence contexts flanking *I-Ppol* restriction sites. *Left:* Construction of pAg-*I-Ppol*. Synthetic oligonucleotides that included the desired *I-Ppol* restriction site (*white solid blocks*) and flanking *HindIII* or *BamHI* sequences (*black solid blocks*) indicate unfavorable sequence contexts were generated. They were annealed and used as linkers after sequentially digesting pAgPHZ3 (Arama *et al.*, 2001) with the appropriate enzymes (first *HindIII* and later *BamHI*). Positive clones were confirmed by PCR, enzymatic digestion, and sequencing (data not shown). *Right:* Construction of pZ-*I-Ppol*. Primers that included favorable flanking sequences (*gray solid blocks*) as well as *I-Ppol* restriction sites (*white solid blocks*) were generated to amplify a gene of interest (*DsRed1*, Clontech; hatched blocks represent sequences homologous to this gene), which was subsequently cloned in pZExr-2 (Invitrogen). Positive clones were confirmed by PCR, enzymatic digestion, and sequencing (data not shown). DNA preparations and cloning were performed according to Sambrook *et al.* (1989).

conditions). The enzyme did not present any star activity under the new buffer conditions.

Endonuclease Assays Using pZ-IPpoI as Substrate (Favorable Sequence Context). We covered two aspects in the assays we designed for pZ-IPpoI. The first aimed at confirming that changing an unfavorable sequence context to a favorable one via mutation would improve the catalytic efficiency in the prescribed buffer conditions. The results were as expected. The second aimed at analyzing the enzyme's performance using pZ-IPpoI as substrate in the new and prescribed buffer conditions (buffers E and I) and changing other variables. We found that even though its performance was satisfactory in both buffers, it was always higher in buffer E.

Assays Using pAg-IPpoI and pZ-IPpoI as Substrates

Modification of Time, Enzyme Concentration, and DNA Mass Variables

To confirm that the results we observed in both pAg-IPpoI and pZ-IPpoI were not due to the effect of different factors like time, mass, and enzyme concentration, we performed assays to evaluate each one (Fig. 2). We assayed I-PpoI's cleavage efficiency using pAg-IPpoI and pZ-IPpoI as substrates in the following experimental conditions.

Buffer Conditions. To confirm that I-PpoI digested more efficiently in buffer E (panels 1 through 4 in Fig. 2), the cleavage assays were performed using 300 ng of each of the plasmids incubated with 18 enzyme units at 37°C in buffers E (6 mM Tris-HCl, 6 mM MgCl₂, 100 mM NaCl, 1 mM DTT, pH 7.5) and I (25 mM CAPS-CHES pH 10, 2 mM MgCl₂, 1 mM DTT) in a final volume of 10 μ L, for different periods of time (30 min, 2 h, 5 h, and ON).

Incubation Times. An activity vs. time course was established to observe how the latter influenced cleavage efficiency (panels 1 through 4 in Fig. 2). The cleavage assays were performed using 300 ng of each of the plasmids incubated with 18 enzyme units for different periods of time (30 min, 2 h, 5 h, and ON) at 37°C in buffers E (6 mM Tris-HCl, 6 mM MgCl₂, 100 mM NaCl, 1 mM DTT, pH 7.5) and I (25 mM CAPS-CHES pH 10, 2 mM MgCl₂, 1 mM DTT) in a final volume of 10 μ L.

Enzyme Concentrations. To ensure that cleavage efficiency was not due to an excess of enzyme (panels 5 and 6 in Fig. 2), cleavage assays were performed using 300 ng of each of the plasmids incubated ON at 37°C in buffer E (6 mM Tris-HCl, 6 mM MgCl₂, 100 mM NaCl, 1 mM DTT, pH 7.5), with different enzyme concentrations (1, 3, 9, and 18 units) in a final volume of 10 μ L.

DNA Amounts. To ensure that I-PpoI's cleavage efficiency in the new buffer conditions was independent of DNA mass (panels 7 and 8 in Fig. 2), cleavage

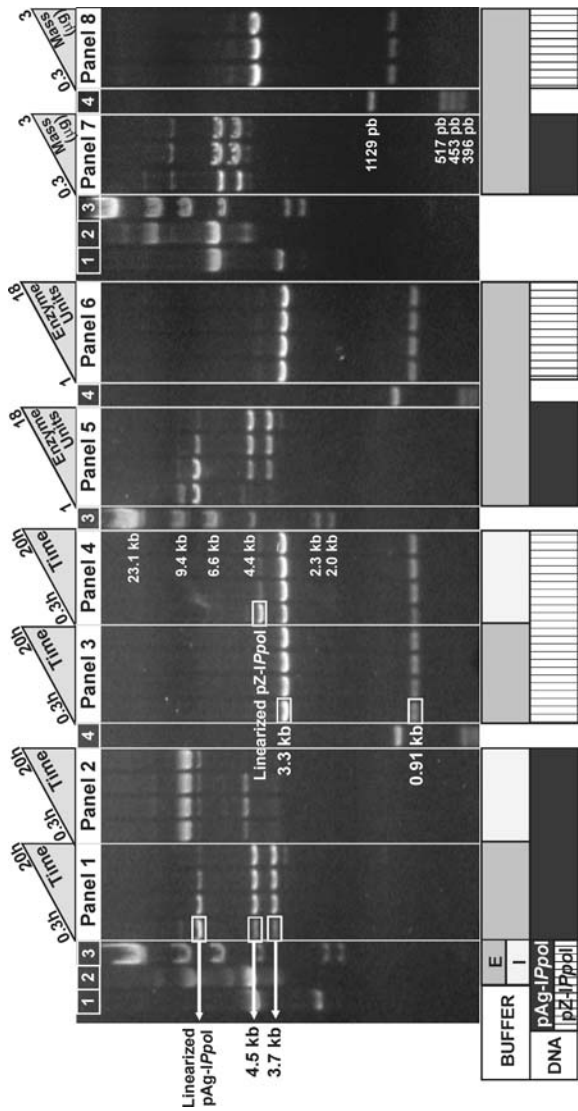


Fig. 2. Comparative analysis of I-PpoI's cleavage efficiency in different assays in which time, enzyme concentration, and DNA mass were changed, using pAg-IPpoI and pZ-IPpoI as substrates. Lane 1: undigested pZ-IPpoI; lane 2: undigested pAg-IPpoI; lane 3: HindIII digested λ DNA; lane 4: pcDNAII digested with HindIII + HinfI. Substrates and buffers are indicated as different blocks in the bottom part of the photograph. Black blocks indicate the reactions in which pAg-IPpoI was used as substrate; hatched blocks indicate the reactions in which pZ-IPpoI was used as substrate. All the reactions carried out in the new buffer conditions (buffer E) are indicated with darker gray blocks, and those that were carried out in the prescribed buffer conditions (buffer I) are indicated with lighter gray blocks. The gray triangles above the gel indicate variations in incubation time, enzyme units, or DNA mass. Even though the quantity of DNA mass varied in the different reactions in which it was a variable (panels 7 and 8), equivalent amounts were loaded in the agarose gel (300 ng). I-PpoI restriction enzyme (Promega) is provided at a concentration of 180 u/uL. pAg-IPpoI digested with I-PpoI yields two fragments of 3.7 kb (*lacZ*) and 4.5 kb (rest of the plasmid). pZ-IPpoI digested with I-PpoI yields two fragments of 0.91 kb (*DsRed1*, Clontech) and 3.3 kb (pZErO-2, Invitrogen).

assays were performed using different masses of each of the plasmids (300 ng, 1 μ g, and 3 μ g), incubated ON at 37°C with 18 enzyme units in buffer E (6 mM Tris-HCl, 6 mM MgCl₂, 100 mM NaCl, 1 mM DTT, pH 7.5) in a final volume of 10 μ L.

The agarose gel electrophoresis of the processed samples was stained with ethidium bromide and photographed under UV illumination. These assays confirmed our initial results.

DISCUSSION

In this study, we found that multiple substitutions in the sequence context can be extremely detrimental to *I-PpoI*'s catalytic activity. Nevertheless, we circumvented this limitation by simply changing the reaction conditions. We also found that *I-PpoI* always digested more efficiently in more neutral pH conditions (pH 7.5) than those recommended as optimal (pH 10), regardless of the sequence context. When we varied the reaction time and buffer conditions for both plasmids (pAg-*IPpoI* and pZ-*IPpoI*), we found that, for pAg-*IPpoI*, an extended reaction improved the cutting capacity in buffer E, but this same increase in time did not have any effect in buffer I; i.e., in the prescribed buffer conditions, an increase in the reaction time was not sufficient to overcome the unfavorable sequence context. In the case of pZ-*IPpoI*, the enzyme's performance was optimal in buffer E, and in these buffer conditions, an increment in time did not have any effect over the enzyme's performance; i.e., in a favorable sequence context the new buffer conditions were sufficient to ensure the enzyme's optimal performance, and a further extension of the reaction did not have any visible effect. Nevertheless, when pZ-*IPpoI* was digested in buffer I, the enzyme's performance was not optimal, and an increase in time did improve its cutting efficiency; i.e., in a favorable sequence context and the prescribed buffer conditions, an increase in time was necessary to optimize the enzyme's performance.

All assays in which the enzyme concentration and DNA mass varied were performed in the new buffer conditions (buffer E). In the case of pAg-*IPpoI*, when we varied the enzyme concentration, *I-PpoI*'s catalytic efficiency was progressively higher as the enzyme units increased; i.e., in an unfavorable sequence context more enzyme units were necessary for *I-PpoI* to reach a higher performance. In the case of pZ-*IPpoI*, the enzyme's performance was always optimal; i.e., in a favorable sequence context the enzyme's optimal performance was independent of the number of enzyme units. We also varied the amount of DNA without exceeding the mass that was expected to be completely digested by the amount of enzyme added, according to the enzyme unit definition. In both cases, i.e., in favorable (pZ-*IPpoI*) and unfavorable sequence contexts (pAg-*IPpoI*) *I-PpoI*'s high performance was not affected.

CONCLUSIONS

These results indicate that the pH and NaCl concentration of buffer E probably affect the structure of the enzyme in such a way that the protein–DNA interactions, critical for recognition and cleavage, are restricted to the core 15-nucleotide I-*Ppo*I site. Conversely, the high pH–low salt I-*Ppo*I buffer would virtually extend the 15-nucleotide target including additional flanking sequences as a requirement for efficient cutting. These results would be in accordance with those published by Wittmayer and Raines (1996), who found that the DNA/I-*Ppo*I interaction is weakened in the presence of increasing concentrations of monovalent cations.

The assays in which we varied time, enzyme concentration, and DNA mass allowed us to draw some general conclusions. When the target site is in an unfavorable sequence context, an increase in the reaction time is not sufficient to improve I-*Ppo*I's performance in the prescribed buffer conditions (buffer I). In contrast, in the new buffer (buffer E), an increase in the reaction time and in the number of enzyme units improved I-*Ppo*I's performance on the same target. When in a favorable sequence context and the prescribed buffer conditions, an increase in time improved the cutting of I-*Ppo*I, whereas in the new buffer, the enzyme's performance was always optimal, and consequently, extension of the reaction in time and an increase in the number of enzyme units had no visible effect on it.

ACKNOWLEDGMENTS

This research was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, Argentina) and Universidad Nacional de La Plata (UNLP, Argentina) to VR, who is a member of the research career at CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina).

REFERENCES

- Arana, E. I., Albariño, C. G., O'Reilly, D. R., Ghiringhelli, P. D., and Romanowski, V. (2001). Generation of a recombinant *Anticarsia gemmatilis* multi capsid nucleopolyhedrovirus expressing a foreign gene under the control of a very late promoter. *Virus Genes* **22**:363–372.
- Argast, G. M., Stephens, K. M., Emond, M. J., and Monnat, R. J. J. (1998). I-*Ppo*I and I-*Crel* homing site sequence degeneracy determined by random mutagenesis and sequential *in vitro* enrichment. *J. Mol. Biol.* **280**:345–353.
- Ellison, E. L., and Vogt, V. M. (1993). Interaction of the intron-encoded mobility endonuclease I-*Ppo*I with its target site. *Mol. Cell. Biol.* **13**:7531–7539.
- Flick, K. E., Jurica, M. S., Monnat, R. J., and Stoddard, B. L. (1998). DNA binding and cleavage by the nuclear intron-encoded homing endonuclease I-*Ppo*I. *Nature* **394**:96–101.
- Lowery, R., Hung, L., Knoche, K., and Bandziulis, R. (1992). Properties of I-*Ppo*I: A rare-cutting intron-encoded endonuclease. *Promega Notes* **38**:8–12.

- Muscarella, D. E., Ellison, E. L., Ruoff, B. M., and Vogt, V. M. (1990). Characterization of I-PpoI, an intron-encoded endonuclease that mediates homing of a group I intron in the ribosomal DNA of *Physarum polycephalum*. *Mol. Cell. Biol.* **10**:3386–3396.
- Muscarella, D. E., and Vogt, V. M. (1989). A mobile group I intron in the nuclear rDNA of *Physarum polycephalum*. *Cell* **56**:443–454.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, CO.
- Wittmayer, P. K., McKenzie, J. L., and Raines, R. T. (1998). Degenerate DNA recognition by I-PpoI endonuclease. *Gene* **206**:11–21.
- Wittmayer, P. K., and Raines, R. T. (1996). Substrate binding and turnover by the highly specific I-PpoI endonuclease. *Biochemistry* **35**:1076–1083.