

Properties of I-Ppo I: A rare-cutting intron-encoded endonuclease

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I-Ppo I is a novel intron-encoded endonuclease which cuts with high specificity at a 15-base pair recognition sequence (1,2). The enzyme exhibits several advantages for digestion of genomic DNA, including stability and catalytic efficiency comparable to bacterial restriction enzymes, and insensitivity to cytosine methylation. The use of I-Ppo I for digestion of yeast and human genomic DNA is demonstrated.

Introduction

To construct a eukaryotic genomic map, large overlapping segments of chromosomal DNA must be identified and oriented relative to one another. Currently, independent research efforts focus on limited regions of human chromosomes, where prior investigation has made certain loci tractable. To join these vastly separated chromosomal segments into an overlapping map, however, new techniques will be needed to cleave and recover segments of chromosomal DNA in the megabase size range.

While technology such as pulsed field gel electrophoresis can resolve DNA fragments greater than 10 megabases in size (3), bacterial restriction enzymes cut too frequently to produce such large fragments. Though several approaches to overcome this limitation are under development, no single-step procedure has yet emerged to generate specific DNA fragments in the 2-20 megabase size range.

The recently discovered intron-encoded endonucleases may provide such a capability due to their specificity for cleavage at very large recognition sequences (4). One such endonuclease, I-Ppo I from *Physarum polycephalum*, has recently been overexpressed and purified at Promega. In this article, we present some of the properties of this novel enzyme and present data showing how the enzyme can be useful for large-scale mapping efforts.

Intron-encoded endonucleases

Group I introns are a recently discovered class of mobile genetic elements whose insertion is mediated by an endonuclease encoded within the intron (4). The hallmark of these intron-encoded endonucleases is the large size of their recognition sequences, which range from 15 to 39 base pairs (4). In some respects, the enzymes appear to function very similarly to bacterial type II restriction enzymes, yielding overlapping ends which can be ligated to regenerate the cut site.

Seven intron-encoded endonucleases have been cloned and shown to have activity *in vivo* or *in vitro*. These include I-Sce I (5) and I-Sce II (6) from yeast mitochondrial DNA, I-Tev I and II (7) from phage T4, I-Ceu I (8) and I-Cre I (9) from *Chlamydomonas* chloroplast DNA, and I-Ppo I from the rDNA of *Physarum polycephalum* (1). Expression of these endonucleases in *E. coli* has been problematic,

possibly because some of the endonucleases cleave the bacterial chromosome (6,10,11). However, I-*Sce* I (10), I-*Tev* I (7), I-*Ceu* I (12) and I-*Ppo* I have been produced successfully in *E. coli*. I-*Sce* II has been produced in a yeast mitochondrial expression system (13). The yeast enzymes and I-*Ppo* I have been characterized in detail (1,10,13,14).

Comparison of intron-encoded endonuclease properties

From the primary sequence comparisons and enzymological data generated thus far, significant differences have been recognized among the intron-encoded endonucleases. All of the mitochondrial and chloroplast intron-encoded endonucleases contain a consensus peptide, LAGLIDADG, which is not present in I-*Ppo* I or in the phage enzymes (4). The chloroplast and mitochondrial enzymes and I-*Ppo* I cleave near the center of maximal 18-base pair recognition sequences (4), while the phage enzymes cleave only 2 base pairs from the end of a 39-base pair recognition sequence (15).

The degree of specificity also is variable; I-*Sce* I shows little tolerance for base substitution within the cognate cut site (14,16), whereas I-*Sce* II (17) and I-*Tev* I (15) both tolerate greater than 50% degeneracy. A consequence of this tolerance for degeneracy is that I-*Sce* II cuts relatively frequently (approximately every 100-150kb) and thus has limited utility for mapping genomic DNA (18).

Properties of I-*Ppo* I

I-*Ppo* I has several physical and mechanistic similarities to type II restriction enzymes. The enzyme is a dimer in solution (2), and migrates as a single band with the 19.7kDa standard on SDS-PAGE, close to the monomer molecular weight of 18,000 predicted from the I-*Ppo* I gene sequence (1). I-*Ppo* I cuts very specifically, both *in vivo* and *in vitro*, within a 15-base pair recognition sequence (Figure 1) (2), leaving 4-base 3'-hydroxyl overhangs and 5'-phosphate recessed ends which can be ligated with T4 DNA Ligase (Figure 2) (1). DNA digested by I-*Ppo* I needs no special treatment before being used in standard molecular biological procedures.

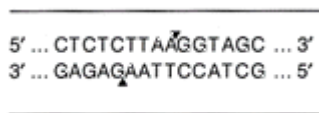


Figure 1. Fifteen base pair I-*Ppo* I recognition sequence.

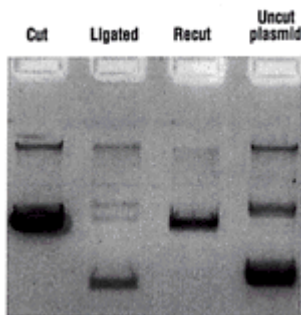


Figure 2. I-*Ppo* I cut/ligate/recut assays. Plasmid p42 (5µg) was digested with 40u of I-*Ppo* I for 20 minutes at 37°C (lane 1), purified and ligated with T4 DNA Ligase (3u/µg DNA) for 3 hours at 14°C (lane 2), and repurified and digested again with I-*Ppo* I as described above (lane 3). Lane 4 contains undigested p42 DNA. DNA samples were analyzed on a 1% agarose gel.

I-*Ppo* I cleavage specificity

The nominal size of the I-*Ppo* I recognition sequence was refined using deletion analysis and *in vitro* cleavage assays (1,2). These studies showed that the 15 bases shown in [Figure 1](#) are sufficient for cleavage rather than the 17 previously reported. Also, cutting was prevented by substitution of either of the two outermost bases or of one of the cytosines in the internal symmetrical hexamer. Because the known intron-encoded endonucleases vary in their tolerance of degeneracy within the recognition sequence, and thus in their specificity, we performed preliminary experiments to characterize this aspect of I-*Ppo* I activity. Three lines of evidence suggest that I-*Ppo* I cuts with very high specificity.

First, we incubated the enzyme with several genomes of varying, but relatively low, complexity and analyzed the digestion patterns using agarose gel electrophoresis. I-*Ppo* I cleaved p42 (a test plasmid which contains the 15-base pair cognate cut site) with high efficiency. Yet, even at greater than a 100-fold excess of enzyme, no detectable cleavage was observed of the other double-stranded DNA substrates tested, ranging in size from 2-49kb (pBR322, lambda, adenovirus 2, phiX174, phage T7).

Second, high level I-*Ppo* I expression can be induced in *E. coli* without any deleterious effects on the host and I-*Ppo* I has been shown to be active in *E. coli* (1). These observations indicate that the enzyme does not cut the *E. coli* chromosome. This further suggests that I-*Ppo* I has a low tolerance for degeneracy within the recognition sequence; the canonical recognition sequence in *E. coli* rDNA differs from the *P. polycephalum* recognition sequence at only 3 positions (19), yet I-*Ppo* I does not cut in *E. coli*.

Third, preliminary experiments using I-*Ppo* I for genomic DNA digestions ([Figures 4](#) and [5](#)), also showed that I-*Ppo* I cleaved with high specificity in a high background of complex DNA. I-*Ppo* I cleaved the 10 megabase yeast genome at a single recognition sequence, and did not cleave in yeast DNA containing only a single base substitution in the recognition sequence. We have now initiated a systematic analysis of the effect of base substitutions in the I-*Ppo* I recognition sequence.

Catalytic efficiency

High catalytic efficiency is an important factor for genomic mapping purposes because of the limited diffusion of macromolecules in the agarose plugs used for genomic DNA digestions. Using the supercoiled plasmid p42 with a single cut site as the substrate, a specific activity of 8.69×10^6 u/mg was obtained with purified I-*Ppo* I; this corresponds to a turnover of 2.6 min^{-1} for the dimer. The most intensively studied bacterial type II restriction enzyme, *EcoR* I, has a turnover number of 3.4 min^{-1} (20). I-*Sce* I and I-*Sce* II are much slower, with turnover numbers of 0.058 and 0.011 respectively (10,13) ([Table 1](#)). Thus, in overall catalytic efficiency, I-*Ppo* I is very similar to the bacterial type II restriction enzymes and is far superior to the other intron-encoded endonucleases that have been characterized.

The pH optimum of the I-*Ppo* I cleavage reaction is 9.5-10.0. MgCl_2 is required for activity, and 50% inhibition occurs in the presence of 150mM NaCl or 25% glycerol ([Table 2](#)).

Table 1. Comparison of Restriction Enzyme Turnover Rates.

Enzyme	Source	Turnover Rate (min^{-1})
I- <i>Ppo</i> I	<i>Physarum polycephalum</i>	2.6

I-Sce I	<i>Saccharomyces cerevisiae</i>	0.058
I-Sce II	<i>Saccharomyces cerevisiae</i>	0.011
EcoR I	<i>Escherichia coli</i>	3.4

Table 2. Effect of Reaction Conditions on I-Ppo I Activity.

Agent	Effect
pH	optimum = 9.5-10.0
Temperature	optimum = 37°C; no activity loss after 60 min at 37°C
Mg ²⁺	optimum = 2mM
DTT	no effect
NaCl	50% inhibition at 150mM
Glycerol	50% inhibition at 25%
EDTA	100% inhibition at 2mM

Standard reaction conditions were: 25mM CAPS, 25mM CHES, pH 10.0, 1mM DTT, 2mM MgCl₂, 1µg of p42 and 1-100u of I-Ppo I in a total volume of 25µl. Reactions were incubated for 5-20 minutes at 37°C and quenched by the addition of 5µl of sample buffer (0.5% bromophenol blue, 100mM EDTA, 30% glycerol) prior to electrophoresis on a 1% agarose gel containing 1µg/ml ethidium bromide.

Stability

I-Ppo I is stable at the reaction temperature of 37°C. No activity loss was detected after preincubation of a dilute solution of enzyme (4.5µg/ml) in reaction buffer for 60 minutes, either in the presence or the absence of MgCl₂. This result contrasts sharply to that found with I-Sce I, which loses 80% of activity in the first two minutes of preincubation at 37°C with MgCl₂ present, and 50% activity in this time period in the absence of MgCl₂ (10). The stability of I-Ppo I at reaction temperature is especially important for genomic mapping due to the prolonged incubation times necessary for diffusion of enzymes into an agarose matrix.

Insensitivity to cytosine methylation

I-Ppo I is completely insensitive to the presence of methylated cytosines in the recognition sequence. This provides a significant advantage for digestion of mammalian DNA, which is highly methylated. The ability of I-Ppo I to cleave methylated DNA was tested by incubating the enzyme with a double-stranded oligonucleotide in which all seven of the cytosine residues within the recognition sequence were methylated. There was no detectable decrease in the ability of I-Ppo I to cleave the methylated oligo compared to the unmethylated substrate (Figure 3).

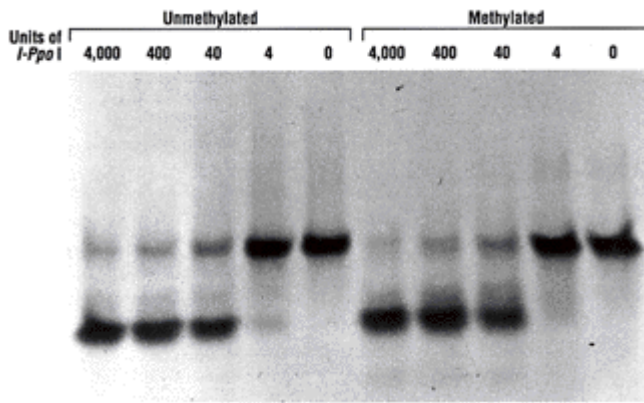


Figure 3. I-*Ppo* I cleavage of a methylated oligonucleotide. Two double-stranded 37bp oligonucleotides containing the I-*Ppo* I recognition site were prepared without or with methylation of all cytosines at the 5'-OH position. One μ g of each oligonucleotide was digested for 1 hour at 37°C with the indicated amount of I-*Ppo* I and analyzed on a 4-20% polyacrylamide gel.

Many bacterial restriction enzymes will not cleave DNA if there are methylated bases in the recognition sequence (21). Since the DNA methylation pattern varies widely between individuals in any species (21), obtaining reproducible digestion patterns using bacterial restriction enzymes is problematic. The ability of I-*Ppo* I to cleave methylated DNA indicates that reproducible digestion patterns will be obtained from the DNA of individuals with varying methylation patterns, an important characteristic for mapping disease-linked alleles within a population.

Applications for cleavage of genomic DNA

Yeast chromosomal DNA

I-*Ppo* I can be used to cleave specific sites in both yeast and human genomic DNA. In *S. cerevisiae*, the rDNA genes (approximately 140 copies) are located near the center of chromosome XII (22). DNA sequence data from this region of chromosome XII has shown that the sequence of the I-*Ppo* I insertion site is identical to the canonical 15-base pair recognition sequence in *P. polycephalum* (2).

S. cerevisiae DNA was incubated with I-*Ppo* I in agarose plugs and then analyzed using pulsed field gel electrophoresis (Figure 4). The I-*Ppo* I cleavage sites in chromosome XII gave rise to the I-*Ppo* I digestion products indicated: the 600kb and 400kb "arms" of chromosome XII (on either side of the rDNA cleaved by I-*Ppo* I) and multiple copies of the repeated 9kb rDNA fragment (23).

In contrast, chromosomal DNA from an isogenic yeast strain which contains a single base pair within the 15bp I-*Ppo* I recognition site (2) is completely resistant to I-*Ppo* I cleavage, even with an 80-fold excess of enzyme (Figure 4). Thus, I-*Ppo* I recognizes only its specific recognition sequence and cleaves to completion within a background of approximately 10 megabases of genomic DNA.

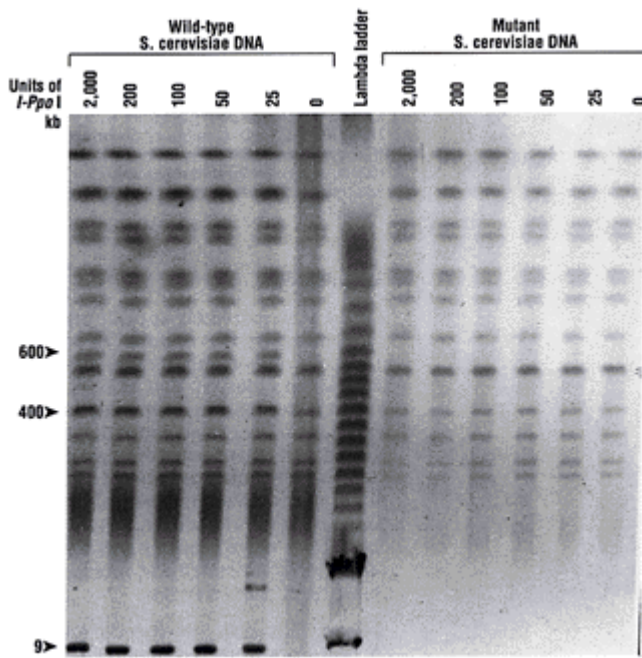


Figure 4. I-*Ppo* I digestion of *S. cerevisiae* chromosomal DNA (wild-type and rDNA mutant strains).

Wild type or mutant chromosomal DNA samples were digested with the indicated amounts of I-*Ppo* I and then resolved by pulsed field gel electrophoresis. Fragment sizes were determined by comparison to Promega-Markers® Lambda Ladders. Running conditions: 1% agarose gel, switch time 60 seconds for 15 hours, 90 seconds for 9 hours, at 200V, 0.5X TBE buffer, 12°C.

Human DNA

I-*Ppo* I cleavage sites also exist in human genomic DNA. A 17mer DNA probe homologous to the I-*Ppo* I recognition site was used to probe a Southern blot containing *Hae* III-digested human K562 genomic DNA (Figure 5, lane 1). A prominent signal was detected for a 0.2kb fragment. Using densitometry, this fragment was shown to be present in about 200 copies per genome (data not shown). The presence of the I-*Ppo* I site was verified by digestion and hybridization of the K562 DNA cut with *both* *Hae* III and I-*Ppo* I. As expected, this double digestion destroyed the I-*Ppo* I site and eliminated probe hybridization to the 0.2 kb band (Figure 5, lane 2).



Figure 5. Southern blot demonstrating an I-*Ppo* I cleavage site in human K562 genomic DNA. Human K562 DNA (2 μ g) was digested with *Hae* III (lane 1) or *Hae* III + I-*Ppo* I (lane 2) and resolved on a 1% agarose gel. The DNA fragments were transferred to a nylon membrane and hybridized with a 17mer LIGHTSMITHTM I AP-conjugated probe specific for the I-*Ppo* I recognition sequence. Southern blot detection was performed using Quantum YieldTM Reagents as described in Promega Technical Manual 229.

Discussion

I-*Ppo* I is an extremely specific and very efficient restriction endonuclease. Its turnover rate on supercoiled plasmid DNA is very close to that of *Eco*R I, and is much faster than that reported for any other intron-encoded endonuclease. The stability of I-*Ppo* I and its resistance to inhibition by other agents are greater than for many commercially available restriction enzymes. In addition, it appears to be a much more robust enzyme than the other intron-encoded endonucleases that have been characterized.

I-*Ppo* I cleavage is site-specific on *S. cerevisiae* chromosomal DNA, and is completely prevented by a single base pair insertion in the recognition sequence. In human K562 DNA, *Hae* III digestion defines a repeating unit which apparently contains all of the I-*Ppo* I recognition sequences. The I-*Ppo* I recognition sequence is completely conserved in human rDNA (24), and thus rDNA is probably the site of I-*Ppo* I cleavage. This site may be a useful marker for mapping, as there are an estimated 50-200 rDNA copies per haploid human genome, located on chromosomes 13, 14, 15, 21 and 22 (25).

I-*Ppo* I also may lend itself to applications other than mapping, such as highly specific excision of cloned sequences in cDNA or genomic libraries. We are currently investigating these applications.

References:

1. Muscarella, D.E., *et al.* (1990) *Molec. Cell. Biol.* **10**, 3386.
2. Ellison, E. and Vogt, V., personal communication.
3. Levine, J.D. and Cech, C.L. (1989) *Biotechnology* **7**, 1033.

4. Perlman, P.S. and Butow, R.A. (1989) *Science* **246**, 1106.
5. Colleaux, L., *et al.* (1986) *Cell* **44**, 521.
6. Delahodde, A., *et al.* (1989) *Cell* **56**, 431.
7. Bell-Pedersen, D., *et al.* (1990) *Nucl. Acids Res.* **18**, 3763.
8. Steinmetz, M., *et al.* (1983) *Mol. Gen. Genet.* **191**, 138.
9. Durrenberger, F., and Rochaix, J.-D., (1991) *EMBO J.* **10**, 3495.
10. Monteilhet, C., *et al.* (1990) *Nucl. Acids Res.* **18**, 1407.
11. Rochaix, J.-D., personal communication.
12. Gauthier, A., Turmel, M., and Lemieux, C. (1991) *Curr. Genet.* **19**, 43.
13. Wernette, C.M., *et al.* (1990) *J. Biol. Chem.* **265**, 18976.
14. Thierry, A., *et al.* (1991) *Nucl. Acids Res.* **19**, 189.
15. Chu, F.K., *et al.* (1991) *Nucl. Acids Res.* **24**, 2863.
16. Colleaux, L. *et al.* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6022.
17. Wernette, C. *et al.* (1992) *Molec. Cell Biol.* **12**, 617.
18. Butow, R.A., personal communication.
19. Egebjerg, J., Larsen, N., and Garret, R.A. (1990) In: *The Ribosome: Structure, Function and Evolution*, Ed., Hill, W.E., American Society of Microbiology, Washington, D.C.
20. Modrich, P. and Zabel, D. (1976) *J. Biol. Chem.* **251**, 5866.
21. McClelland, M. and Nelson, M. (1988) *Gene* **74**, 291.
22. Petes, T.D. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 410.
23. Link, A.J. and Olson, M.V. (1991) *Genetics* **127**, 681.
24. Gonzalez, I.L., *et al.* (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7666.
25. Long, E.O. and Dawid, I.B. (1980) *Ann. Rev. Biochem.* **49**, 727.

Ordering Information

Product	Size	Cat.#

I-Ppo I Intron-Encoded Endonuclease	10,000u	R7031
	50,000u (5 x 10,000u)	R7032

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