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Characterization of I-*Ppo*, an Intron-Encoded Endonuclease That Mediates Homing of a Group I Intron in the Ribosomal DNA of *Physarum polycephalum*

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A novel and only recently recognized class of enzymes is composed of the site-specific endonucleases encoded by some group I introns. We have characterized several aspects of I-*Ppo*, the endonuclease that mediates the mobility of intron 3 in the ribosomal DNA of *Physarum polycephalum*. This intron is unique among mobile group I introns in that it is located in nuclear DNA. We found that I-*Ppo* is encoded by an open reading frame in the 5′ half of intron 3, upstream of the sequences required for self-splicing of group I introns. Either of two AUG initiation codons could start this reading frame, one near the beginning of the intron and the other in the upstream exon, leading to predicted polypeptides of 138 and 160 amino acid residues. The longer polypeptide was the major form translated in vitro in a reticulocyte extract. From nuclease assays of proteins synthesized in vitro with partially deleted DNAs, we conclude that both polypeptides possess endonuclease activity. We also have expressed I-*Ppo* in *Escherichia coli*, using a bacteriophage T7 RNA polymerase expression system. The longer polypeptide also was the predominant form made in this system. It showed enzymatic activity in bacteria in vivo, as demonstrated by the cleavage of a plasmid carrying the target site. Like several other intron-encoded endonucleases, I-*Ppo* makes a four-base staggered cut in its ribosomal DNA target sequence, very near the site where intron 3 becomes integrated in crosses of intron 3-containing and intron 3-lacking *Physarum* strains.

Group I introns are defined by the presence of conserved sequences and structural elements (reviewed in reference 2). Many group I introns can undergo autocatalytic splicing (self-splicing) in vitro, as first described for the prototypic intron of *Tetrahymena* cells (5). Of the more than 60 group I introns that have been identified to date (4), most are present in the DNA of mitochondria or chloroplasts or in the DNA of T-even bacteriophage. In only three organisms have group I introns been observed in nuclear genes, and in each of these they are located in the DNA coding for ribosomal RNA (rDNA). *Pneumocystis carinii* contains an intron in the rDNA encoding the small-subunit rRNA (18), but neither it nor the rDNA of this organism has been extensively characterized. Several but not all *Tetrahymena* species and strains contain the well-known self-splicing intron in extrachromosomal rDNA coding for the large-subunit rRNA (22, 23, 38). Depending on the strain, *Physarum polycephalum* contains two or three group I introns in the extrachromosomal rDNA coding for the large-subunit rRNA (28, 30, 31). Some group I introns have been found to be mobile elements. They rapidly and efficiently spread in vivo from a locus that contains the intron (I^+) to the same locus in a homologous gene that lacks the intron (I^-). This process, which has been termed intron homing (15, 16), is initiated by a double-strand break in the I^- locus that is introduced by a site-specific endonuclease encoded by the intron itself. The actual homing of the intron appears to be mediated by a double-strand break-and-repair mechanism in which the I^+ strand serves as a template for the repair of the I^- strand. This mechanism was proposed to explain the unidirectional meiotic gene conversion in yeast cells (36) and has been invoked to explain mating-type switching (29), which also is mediated by a site-specific endonuclease.

Several intron-encoded endonucleases have been identified. The first such enzyme, I-*Sce*I (for nomenclature conventions, see reference 16), was found for the omega intron of the mitochondrial rDNA of *Saccharomyces cerevisiae* (9, 21, 26, 42). More recently, endonucleases have been reported for mobile introns in the nuclear rDNA of *P. polycephalum* (I-*Ppo*; 28), in the *coxI* gene of yeast mitochondria (I-*Sce*II; 11, 37), and in the *td* and *sunY* genes of T-even bacteriophage (I-*Tev*I and I-*Tev*II; 34). It seems likely that the mobility of other group I introns, for example in the chloroplast rDNA of *Chlamydomonas eugametos*, will be explained by similar endonucleases (24, 25). Some group I introns, as well as group II introns, also encode maturases (proteins involved in the splicing of the intron RNA in vivo), and at least in the case of I-*Sce*II, an intron-encoded endonuclease can function as a maturase under special circumstances (14, 20).

We have previously described the unusual properties of a group I intron, designated intron 3, in the extrachromosomal nuclear rDNA of the acellular slime mold *P. polycephalum* (28). The 3′ half of intron 3 shows a very high degree of sequence homology with the *Tetrahymena* intron, and thus it would appear that the two introns are closely related in evolution. Consistent with this notion, both are inserted at exactly the same site in extrachromosomal rDNA. Both introns are also optional, i.e., are not present in all strains of their respective organisms. Intron 3 is mobile, as evidenced by the following observations. When haploid amoebae of an I^+ *Physarum* strain are mated with amoebae of an I^- strain, intron 3 colonizes all of the rDNA molecules in the resulting diploid plasmodium. A double-strand break in the recipient, I^- molecules can be detected in some crosses. This break

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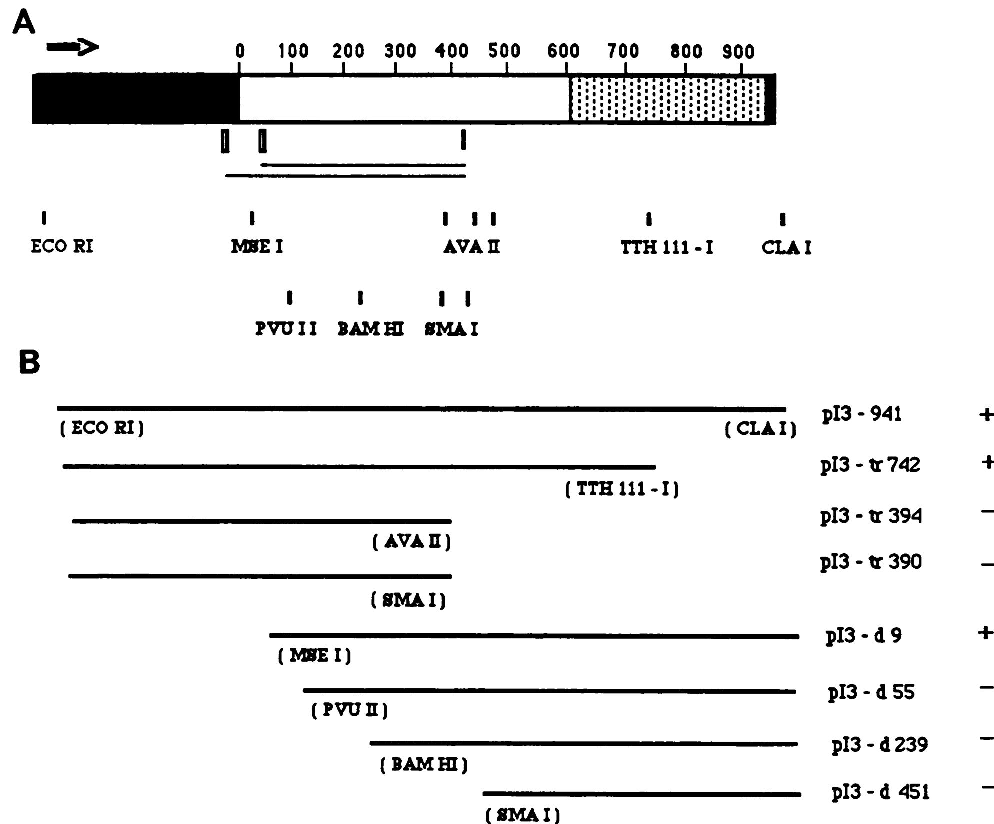

FIG. 1. (A) Partial restriction map of clone pI3-941. This plasmid contains the entire 941 bp of intron 3 flanked by 376 nucleotides of 26S sequence upstream and 27 bp of sequence downstream (■). The two possible methionines used for initiation of translation (open vertical bars) and the site for translation termination (solid vertical bar) are shown. Lines under the map correspond to the full-length protein and partially deleted protein made from pI3-d9. The stippled region corresponds to the region of homology of intron 3 to the *Tetrahymena* intron. Intron sequences are numbered above the map. Arrow indicates the direction of transcription from the T7 promoter of the Bluescript vector. (B) Plasmid constructs. Plasmids designated "tr" are 3′ truncations; those designated "d" are 5′ deletions. The numbers indicate the corresponding nucleotide in the intron shown above. The restriction site used for each construct is shown in parentheses. To the right of each construct is the name of the plasmid and a summary of the results indicating the ability of each plasmid to encode an active (+) or inactive (−) endonuclease.

also can be induced in the rDNA of heterokaryons generated by the fusion of two diploid plasmodia, one containing and one lacking intron 3. We have shown that when intron 3-containing RNA synthesized in vitro is translated in a reticulocyte extract, a protein is produced that is able to cleave the appropriate target sequence of cloned rDNA. Together, these data demonstrate that intron 3 encodes a site-specific endonuclease, which has been designated I-*Ppo* (16). Intron 3 is unique among mobile introns in that it is transcribed in a compartment, the nucleolus, where translation cannot occur; all other known mobile introns occur in systems where transcription and translation are coupled. Thus, an mRNA generated from an RNA polymerase I transcript must be transported to the cytoplasm. The nature of this message is unknown.

In this report, we describe further characterization of I-*Ppo* and its gene. We show that I-*Ppo* makes a four-base staggered cut at the site of intron insertion and that it is encoded by an open reading frame located in the 5′ half of intron 3 and extending a short distance into the upstream exon. The major polypeptide translated in vitro is initiated at an AUG in the exon, but a shorter and also enzymatically active polypeptide can be translated from an AUG at the beginning of the intron. We also show that I-*Ppo* can be expressed in *Escherichia coli*, where it functions as a site-specific endonuclease.

MATERIALS AND METHODS

Construction of plasmids and sequencing. All restriction sites used in construction of the plasmids described below are shown in Fig. 1. Plasmid pI3-941 was derived from pPHR 33 (28). It contains the entire 941-nucleotide-long intron plus 376 nucleotides of upstream exon, 24 nucleotides of downstream exon, and 3 nucleotides of the neighboring intron, cloned into the *Eco*RI-*Cla*I sites of Bluescript (Stratagene). Plasmid pI3-tr742 was constructed by deleting sequences in pI3-941 from the *Tth*111I site at nucleotide 742 in the intron to the *Sal*I site in the multiple cloning region downstream of the insert. Plasmid pI3-tr394 was constructed by isolating the *Eco*RI-*Ava*II fragment from pI3-941 and cloning it into the *Eco*RI-*Eco*RV site in Bluescript. The 3′ truncation pI3-tr390 was constructed by isolating a *Sma*I fragment from the Bluescript multiple cloning site to the intron-*Sma*I site and ligating it into the *Sma*I site of Bluescript. The 5′ deletion clones pI3-d451 and pI3-d239 were made by deleting a *Sma*I and a *Bam*HI fragment, respectively, from these restriction sites in the upstream region of the multiple cloning site to the

same sites within the intron. Plasmid pI3-d9 was made by subcloning the *Mse*I-*Bam*HI fragment from pI3-941 into the same plasmid in which all of the sequences upstream from the *Bam*HI site in the intron up to the *Xba*I site in Bluescript were deleted. The *Mse*I and *Xba*I sites were blunt-end ligated. Similarly, the *Pvu*II-*Bam*HI fragment was cloned into the *Xba*I-*Bam*HI site for the construction of pI3-d55.

Plasmid pPHR 17, which was constructed by Patrick Ferris, was used as the target for cleavage in the *E. coli* experiments and has been described previously (28). It contains a 2.7-kilobase (kb) fragment of rDNA from an I^- strain of *P. polycephalum*.

To construct the plasmid used in mapping the cleavage site, p42, a single-stranded oligonucleotide was synthesized that contains 38 nucleotides corresponding to the rDNA sequence surrounding the insertion site for intron 3 plus 4 nucleotides that are complementary to the restriction site for *Pst*I. The oligonucleotide was ligated into the *Pst*I site of Bluescript which had been digested with *Pst*I plus *Eco*RV. The plasmid DNA then served as a primer for synthesis of the second strand of the oligomer, which was subsequently ligated at the *Eco*RV site (10).

The nucleotide sequence of the open reading frame was determined by using the pI3-941, pI3-d9, and pI3-d239 constructs. Both strands were sequenced by using the -20 and T7 primers of the Bluescript vector for one strand and oligonucleotides ending at intron positions 285, 419, and 605 for the other. Homology searches for the derived amino acid sequence were performed by using GenBank and NBRF data bases (13).

In vitro transcription, translation, and cleavage reactions. Plasmids were transcribed in vitro as follows: plasmid DNA was linearized in the multiple cloning region downstream of the insert, using either *Cla*I or *Xho*I. Each reaction mix contained 40 mM Tris hydrochloride (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 2.5 mM each ATP, GTP, CTP, and UTP, 10 mM dithiothreitol, 5 μg of linearized template DNA, 5 U of T7 RNA polymerase (United States Biochemical Corp.), and 1 U of RNasin (Promega Biotec) per ml in a total reaction volume of 50 μl. Reactions were incubated at 37°C for 1 h. The RNA was then precipitated with ethanol and dissolved in diethyl pyrocarbonate-treated water.

For the in vitro translations, approximately 5 to 10 μg of RNA was added to a rabbit reticulocyte lysate translation mix (Promega Biotec) lacking cysteine in the presence of 50 μCi of [^{35}S]cysteine (10 μCi/μl; Amersham Corp.) in a reaction volume of 50 μl. The reaction mixes were incubated at 30°C for 1 h. A sample (2.5 μl) was subjected to electrophoresis in a 17.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Fluorography was performed by standard procedures (6).

The in vitro cleavage assay was performed as previously described (28). Briefly, 10 ng of the end-labeled 476-base-pair (bp) *Eco*RI-*Cla*I fragment that contains the target site for cleavage by I-*Ppo* was mixed with 2.5 μl of the appropriate translation mix in 10 mM $MgCl_2$–50 mM Tris hydrochloride (pH 8.0)–100 mM NaCl in a total volume of 25 μl. The reaction mixes were incubated at 30°C for 1 h. (The incubation time was reduced to 10 min for the activity assay presented in Table 1.) The reactions were stopped by the addition of SDS to 1%. The mixture was extracted with phenol-chloroform and precipitated with ethanol. The DNA was electrophoresed in a 2% agarose gel, which was then dried and subjected to autoradiography.

Mapping of the cleavage site for I-*Ppo*. DNA sequencing was carried out on 1 μg of p42, using Sequenase and either the reverse primer or the M13 −20 primer for the Bluescript vector. A sample representing 2.5% of the DNA from each sequencing reaction was subjected to cleavage by I-*Ppo*; 4 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol) was added to the remainder. Cleavage was carried out in a 30-μl reaction mix containing 50 mM Tris hydrochloride (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 10 μg of sonicated salmon sperm DNA, and 2.5 μl of the programmed reticulocyte lysate. The samples were incubated for 1 h at 30°C, with further additions of 2.5 μl of lysate at 20 and 40 min. The cleavage reaction was stopped by the addition of 200 μl of 10 mM EDTA (pH 8.0)–1% SDS. Each sample was subsequently treated with 25 μg of proteinase K at 50°C for 1 h and purified by extractions with phenol and chloroform. The samples were treated with 50 μg of RNase A at 37°C for 1 h, and the organic extractions were repeated. The DNA was precipitated with ethanol and suspended in loading buffer. The sequencing reactions that were subjected to cleavage by I-*Ppo*, as well as those that were not, were denatured with heat and electrophoresed in a 7% polyacrylamide gel containing 7 M urea.

Expression of I-*Ppo* in *E. coli*. All of the *E. coli* experiments were performed with strain C600 (λ-*thr-1 leu-6 thi-1 supE44 lacY1 ton A21*), which was supplied by Jeffrey Roberts. Transcription of the intron RNA from the T7 promoter of Bluescript was induced using a lambda phage (CE6) from William Studier (35) that contains the gene for T7 RNA polymerase under the control of the lambda p_L and p_I promoters. The induction experiments were performed as follows. A 1-ml sample of an overnight culture of the appropriate bacterial strain was inoculated into 40 ml of complete medium supplemented with the appropriate antibiotics. The cultures were grown at 37°C until they reached an optical density at 550 nm of approximately 1. The cells were pelleted and suspended in 1 ml of 10 mM $MgSO_4$–25 ml of original culture. An equal volume from a stock of lambda CE6 (3×10^{10} phage per ml) was added to the bacteria. The phage were preadsorbed for 15 min at 37°C.

For ^{35}S labeling, samples of 200 μl were added to 1 ml of prewarmed medium. After 5 min of incubation, rifampin was added to a final concentration of 250 μg/ml. Incubation continued for various lengths of time, as indicated in Results. Cells were then pelleted and suspended in 500 μl of minimal medium M9 (27) containing 50 μCi of [^{35}S]methionine (10^3 Ci/μl; ICN trans-label) or [^{35}S]cysteine. The cells were labeled for 5 min, pelleted, and immediately frozen on dry ice. The cells were lysed in 250 μl of 1% SDS, and samples of 10 μl were electrophoresed in a 17.5% SDS-polyacrylamide gel.

For the cleavage assay in *E. coli*, bacteria were infected as described above. However, after the preadsorption period, the total volume of cells was inoculated into 40 ml (i.e., the original volume) of prewarmed medium. After incubation for the periods of time indicated in Results, 1.5-ml samples were removed, and the cells were pelleted, suspended in 150 μl of 50 mM Tris hydrochloride (pH 7.5)–25% sucrose, and immediately frozen on dry ice. DNA was prepared by using a gentle lysis procedure (39) as follows. Cells were digested with lysozyme (50 μg per sample) for 10 min on ice. EDTA was then added to a final concentration of 150 mM, followed by the addition of an equal volume of a solution consisting of 0.1% Triton X-100, 50 mM EDTA, and 50 mM Tris hydrochloride (pH 8.0). The lysate was incubated on ice for an additional 10 min, after which NaCl and SDS were added to

CAGTGCTCTGGATGTTAAA **ATG** GCG AAA TCC AAC CAA GCT CGG GTA
met ala lys ser asn gln ala arg val

(1)
AAC GGC GGG AGT AAC TAT GAC TCT CTc acc ccc tta aat **atg** (16)
asn gly gly ser asn tyr asp ser leu thr pro leu asn met

gcg ctc acc aat gct caa atc ttg gct gtg att gac agc tgg (58)
ala leu thr asn ala gln ile leu ala val ile asp ser trp

gaa gaa aca gtc ggt cag ttt cca gtg ata acg cac cat gta (100)
glu glu thr val gly gln phe pro val ile thr his his val

cca tta ggt ggc ggt ctg caa gga acg ctc cat tgt tac gag (142)
pro leu gly gly gly leu gln gly thr leu his cys tyr glu

atc ccc cta gca gct cct tat ggg gtt ggc ttt gct aag aat (184)
ile pro leu ala ala pro tyr gly val gly phe ala lys asn

ggg cct acc cgc tgg caa tac aaa cgg aca atc aat caa gtc (226)
gly pro thr arg trp gln tyr lys arg thr ile asn gln val

gtc cac aga tgg gga tcc cac aca gtc cct ttt cta tta gaa (268)
val his arg trp gly ser his thr val pro phe leu leu glu

ccg gat aac atc aac ggc aaa acc tgc aca gca tcg cac cta (310)
pro asp asn ile asn gly lys thr cys thr ala ser his leu

tgt cat aat act cga tgc cac aat ccc ttg cac ttg tgc tgg (352)
cys his asn thr arg cys his asn pro leu his leu cys trp

gag tca cta gac gac aac aaa ggc aga aac tgg tgc ccg ggt (394)
glu ser leu asp asp asn lys gly arg asn trp cys pro gly

cca acg ggg gat gtg tcc atg cgg tgg ttt gtt taa (430)
pro thr gly asp val ser met arg trp phe val OCH

FIG. 2. Nucleotide and deduced amino acid sequences of the open reading frame for intron 3. Capital letters correspond to upstream exon sequences; lowercase letters correspond to intron sequences. The two possible amino-terminal methionines are in boldface, and a potential ribosome-binding site in the upstream exon is underlined.

final concentrations of 0.1 M and 1%, respectively. Proteinase K (50 μg) was then added, and the samples were incubated for 2 h at 50°C. The DNA was further purified by phenol-chloroform extraction and precipitated with ethanol. The DNA was electrophoresed in a 2% agarose gel, transferred to Zeta-probe blotting membrane (Bio-Rad Laboratories), and hybridized as directed by the manufacturer.

RESULTS

Mapping of the coding region for I-*Ppo* endonuclease synthesized in vitro. Intron 3 of *P. polycephalum* is similar in several respects to the much smaller *Tetrahymena* intron. In addition to self-splicing in vitro, it is integrated at the same nucleotide in rDNA and contains a stretch of high sequence homology. This region of homology is approximately the 3′ half of intron 3, which includes the sequence elements that are highly conserved for all group I introns and are required for self-splicing (2) (Fig. 1A). On the basis of this structure and analogies with mitochondrial introns encoding maturases (3, 9, 12), it seemed likely that the intron 3-encoded endonuclease would be derived from the 5′ half of the intron. Sequence analysis revealed an open reading frame beginning in the upstream exon and extending to nucleotide 427 (Fig. 1 and 2). Two methionine codons, at nucleotide +14 in the intron and at nucleotide −53 upstream in the exon, potentially could serve to initiate polypeptides 138 and 160 amino acid residues in size, respectively.

To delineate which sequences actually encode the endonuclease, we tested the ability of plasmids containing various 3′ truncations or 5′ deletions of intron 3 to generate polypeptides by in vitro translation (Fig. 1B). The enzymatic activities of the proteins derived from these plasmids (see below) are summarized in Fig. 1B. When a plasmid containing the entire sequence of intron 3 (pI3-941) was subjected to transcription in vitro by using bacteriophage T7 RNA poly-

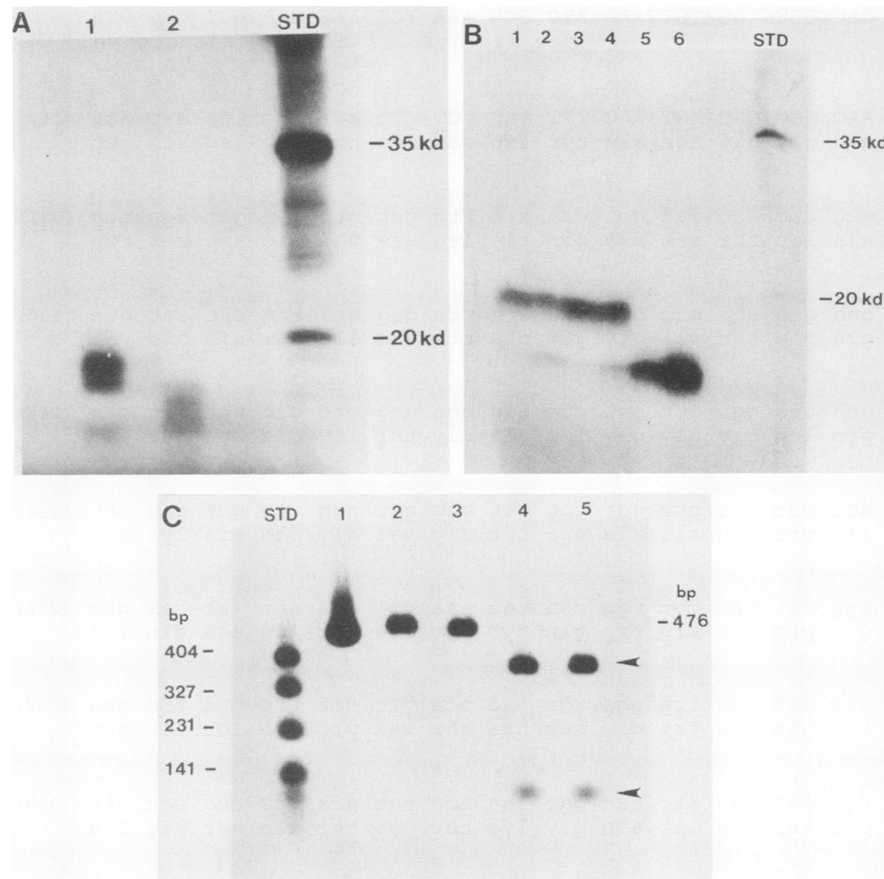

FIG. 3. In vitro expression of I-*Ppo*. The various constructs were linearized in the multiple cloning site downstream of the insert and subjected to in vitro transcription and translation as described in Materials and Methods. Proteins were labeled with [^{35}S]cysteine. A sample of the translation mix was electrophoresed on a 17.5% SDS-polyacrylamide gel and then subjected to fluorography. (A) I-*Ppo* synthesized from pI3-941 (lane 1) and the truncated protein synthesized from pI3-tr394 (lane 2). Lane STD, Size standards. kd, Kilodaltons. (B) Comparison of proteins produced by plasmid pI3-941 (lanes 1 and 2), 3′ truncation pI3-tr742 (lanes 3 and 4), and 5′ deletion pI3-d9 (lanes 5 and 6). Each lane represents an independent transcription-translation reaction. Size standards (lane STD) were derived by translation of brome mosaic virus RNA. kd, Kilodaltons. (C) Example of in vitro cleavage assay. A sample of the translation mix was added to a fragment of cloned rDNA that had been end labeled. Cleavage was carried out as described in Materials and Methods. After cleavage, the DNA was electrophoresed on a 2% agarose gel along with the end-labeled fragments of *Taq*I-digested φX174 DNA (lane STD). Input DNA fragment (lane 1) was incubated with unprogrammed translation mix (lane 2) or translation mixes of RNA derived from plasmids pI3-tr394 (lane 3), pI3-tr742 (lane 4), and pI3-941 (lane 5). Arrows indicate cleavage products whose expected sizes are 376 and 100 bp.

merase, followed by translation of RNA with use of a standard rabbit reticulocyte lysate, a major ^{35}S-labeled polypeptide of approximately 17 to 18 kilodaltons (kDa) was produced (Fig. 3A, lane 1; Fig. 3B, lanes 1 and 2). Transcription and translation of a plasmid containing a truncation of the intron at nucleotide 742 (pI3-tr742) also resulted in the production of this full-length polypeptide (Fig. 3B, lanes 3 and 4). Since this truncation deletes sequences that are required for self-splicing of the intron RNA, it is unlikely that splicing is involved in the generation of the message for these polypeptides, at least in vitro. Transcription and translation of a plasmid containing a truncation of the intron further upstream, at nucleotide 394 (pI3-tr394), resulted in the production of a smaller polypeptide (Fig. 3A, lane 2), the size of which is consistent with the assignment of the codon at nucleotide 427 as the termination site for the full-length polypeptide. This result was confirmed by using a second construct, pI3-tr390 (not shown).

At which of the two possible AUG codons is translation initiated? To address this question, we made two constructs with 5′ deletions. Plasmid pI3-d9 contains a deletion that eliminates the entire 5′ exon and the first eight nucleotides of the intron but leaves the intron AUG at nucleotide 14. Plasmid pI3-d55 contains a deletion that extends beyond this codon, 55 nucleotides into the intron. Translation of pI3-d9 led to a polypeptide of reduced size, approximately 15 to 16 kDa (Fig. 3B, lanes 5 and 6), which corresponded in mobility to a minor product that also was detected when the full-length intron constructs were used (compare with lanes 1 to 4). The construct that was deleted beyond the intron-encoded methionine, pI3-d55, failed to produce a detectable polypeptide (not shown). From these results, we conclude that the major initiation site for the radioactive polypeptide synthesized in vitro from the full-length construct is the AUG in the upstream exon.

We tested the ability of the truncated and deleted constructs to produce catalytically active endonuclease. Previous results had shown that the protein made in vitro is able to cleave the appropriate target sequence in a fragment of cloned rDNA (28). An example of the cleavage reaction used

TABLE 1. Relative cleavage activity in vitro of full-length versus 5′-deleted I-*Ppo*[a]

Dilution	% Cleaved[b]	
	pI3-941	pI3-d9
1×	100	100
1:10	45	56
1:50	7	16
1:100	NC	NC

[a] Programmed translation mixes containing equivalent amounts of protein as determined by ^{35}S labeling were diluted as indicated, and a sample of each dilution was incubated with the 476-bp target rDNA fragment for 10 min at 30°C.

[b] Percent of the total amount of input fragment that was cleaved, as determined by densitometry of the autoradiograph. NC, No detectable cleavage.

in these experiments is shown in Fig. 3C. An end-labeled 476-bp target fragment (i.e., fragment of I$^-$ rDNA) was incubated with samples of translation mix derived from the various constructs described above. Cleavage of the target near the site of intron integration resulted in the generation of fragments of 376 and 100 bp. Cleavage yielding fragments of this size was seen for full-length (Fig. 3C, lane 5), truncation tr742 (lane 4), and deletion d9 (see below) constructs. No cleavage was seen with truncation tr394 (lane 3) or deletion d55 (not shown). We conclude that at least part of the stretch of 12 C-terminal amino acids of the open reading frame is essential for activity and that protein initiated at the AUG in the intron is enzymatically active.

The conditions normally used for the in vitro endonuclease assay do not allow quantitative estimates of enzymatic activity, and therefore it is not possible to conclude rigorously that proteins initiating at the exon-encoded and the intron-encoded methionine possess equivalent activity. Indeed, it seemed possible that the smaller, 15- to 16-kDa protein seen in minor amounts in the translation of pI3-941 and pI3-tr742 could be the only enzymatically active species. To address this problem, we sought to compare the endonuclease activities of the two proteins initiated at the exon AUG and the intron AUG, respectively. Table 1 shows the results obtained when samples of translation mixes from pI3-941 and from pI3-d9, each containing approximately the same amount of in vitro-translated protein, were subjected to a series of dilutions and then allowed to cleave target DNA for a short period of time. The results are expressed as the percent of total input fragment that was cleaved, as determined by densitometry of the autoradiograph. At all dilutions, protein derived from the two constructs gave similar amounts of cleavage. Since the 15- to 16-kDa protein is only a very minor product in translation of full-length intron, comprising no more than about 5% of the larger radioactive protein, it is unlikely that this product is solely responsible for the endonuclease activity. We favor the alternative that the proteins initiated at the exon AUG and at the intron AUG have similar specific activities in vitro.

Expression of I-*Ppo* in *E. coli*. Many intron-encoded endonucleases are known to be lethal in *E. coli* (for example, see reference 11), presumably because of the presence of cleavable sequences in the bacterial chromosome. To express I-*Ppo* in *E. coli*, we used a system that is particularly useful for expressing potentially deleterious proteins (35). A strain of bacteriophage lambda that contains the gene for bacteriophage T7 RNA polymerase (strain CE6) was used to infect cultures of *E. coli* containing the various plasmid constructs described above. After infection, the cultures were pulse-labeled, and lysates of the cells were electrophoresed on a polyacrylamide gel. In initial experiments (data not shown), several labeled protein bands were observed, but no differences were seen between samples from cells carrying the intron and cells carrying only the Bluescript vector. These bands presumably represent *E. coli* and lambda proteins. However, we were able to identify a distinct protein when rifampin, an inhibitor of *E. coli* RNA polymerase but not of T7 RNA polymerase, was used to suppress transcription of host cell and lambda phage genes (Fig. 4A). In this experiment, rifampin was added to each culture 5 min after infection by CE6. Cells were incubated with the drug from 5 to 30 min, after which [^{35}S]methionine was added and incubation continued for 5 min before harvesting of the cultures. All of the radioactive proteins in the control (vector alone; lanes 7 to 12) decreased in intensity as a result of increased times of exposure to rifampin. Similar sensitivity to rifampin was present in the cultures containing pI3-941 (lanes 1 to 6). However, the latter culture showed an additional radioactive band of 17 to 18 kDa (see also Fig. 6). We interpret this band to be the same polypeptide as that translated in vitro, i.e., I-*Ppo*.

To test for enzymatic activity of this polypeptide, we constructed strains of *E. coli* that along with pI3-941 also carry plasmid pPHR 17. This plasmid contains the target for I-*Ppo* in a fragment of *Physarum* rDNA derived from an I$^-$ strain of *P. polycephalum*. In addition, it contains the selectable tetracycline resistance marker. Cells carrying the target plasmid, plus either pI3-941 or the control plasmid pI3-d451 in which the entire open reading frame is deleted, were infected with CE6. Total DNA was isolated from each of these cultures at different times after infection. The DNA was electrophoresed on an agarose gel, transferred to blotting membrane, and hybridized with a fragment of rDNA from pPHR 17, so that only the target plasmid was visualized. For the control plasmid, at all time points tested only two forms of pPHR 17 were apparent, corresponding to supercoiled and relaxed circles (Fig. 4B, lanes 5 to 8). For the plasmid capable of expressing intact I-*Ppo*, a DNA species corresponding in size to linear pPHR 17 also was seen (lanes 1 to 4). The relative amount of the linear fragment increased with time after infection. By restriction mapping, the site of breakage for pPHR 17 was found to be indistinguishable from the target site for I-*Ppo* (data not shown). Plasmids not containing the I-*Ppo* target site, e.g., pI3-941, remained unchanged throughout the course of infection (data not shown).

A direct size comparison of I-*Ppo* made in vitro and in vivo is shown in Fig. 5. Full-length I-*Ppo* made in vitro (lane 2) and in vivo (lanes 3 and 4) from plasmid pI3-tr742 were identical in mobility in SDS-polyacrylamide gels. We conclude that the major initiation site for translation of I-*Ppo* in *E. coli* is the same AUG in the exon that is also used in the reticulocyte extract. The protein made in vitro from the deleted plasmid pI3-d9 (lane 1) comigrated with a minor species discernible above the background bands in the in vivo samples, suggesting that at a lower level, translation may also initiate at the intron AUG in *E. coli*.

Activity of deleted and truncated forms of I-*Ppo* in *E. coli*. We tested the ability of the 5′ deletions and 3′ truncations to direct synthesis of endonuclease in vivo, using the CE6 lambda bacteriophage system described above. The pattern of cleavage activity of the 3′ truncations measured as described in Fig. 4B was similar to that found in vitro: the plasmid encoding a full-length I-*Ppo*, pI3-tr742, produced an active endonuclease, whereas pI3-tr394 did not (data not

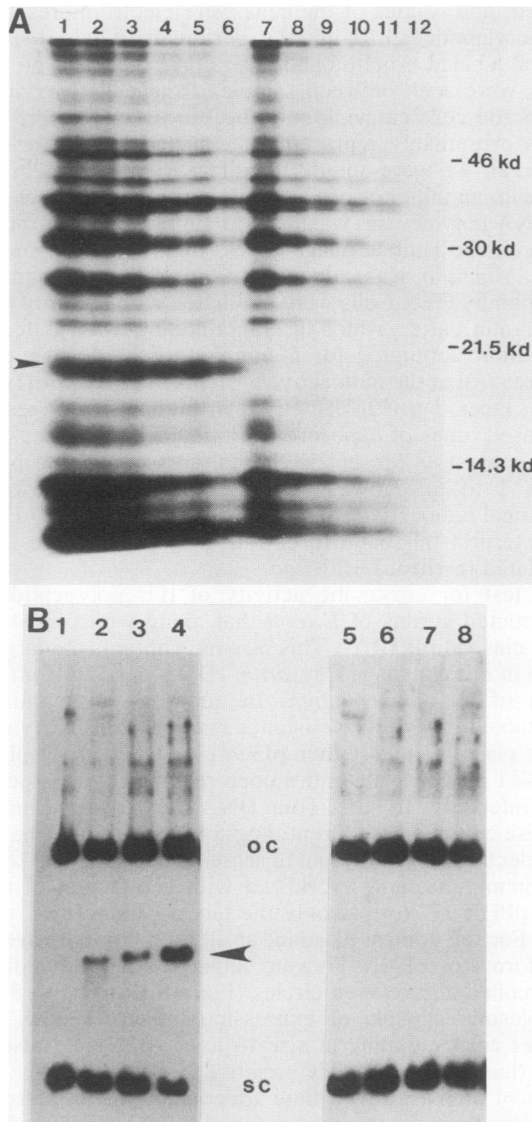

FIG. 4. (A) Expression of active I-*Ppo* in *E. coli*. *E. coli* harboring pI3-941 (lanes 1 to 6) or Bluescript control (lanes 7 to 12) was infected with lambda CE6 as described in Materials and Methods. Infection was allowed to proceed for 5 min. Rifampin was then added for the lengths of time indicated below, followed by a 5-min pulse-label as described in Materials and Methods. Cells were pelleted and lysed, and a sample was subjected to electrophoresis in a 17.5% SDS-polyacrylamide gel, followed by fluorography. The lengths of rifampin treatments were 5 min (lanes 1 and 7), 10 min (lanes 2 and 8), 15 min (lanes 3 and 9), 20 min (lanes 4 and 10), 25 min (lanes 5 and 11), and 30 min (lanes 6 and 12). Arrow indicates protein seen only in the cells harboring the I-*Ppo*-containing plasmid. Size standards shown at right were extrapolated from unlabeled rainbow markers (Amersham). kd, Kilodaltons. (B) Cleavage of target plasmid in vivo. Plasmid pPHR 17, which contains a 2.7-kb fragment of rDNA derived from an intron 3-lacking strain of *P. polycephalum*, was maintained along with either pI3-941 (lanes 1 to 4) or pI3-d451, a plasmid in which the entire open reading frame has been deleted (lanes 5 to 8). Cells were either uninfected (lanes 1 and 5) or infected with lambda CE6 and incubated for various lengths of time as follows: 15 min (lanes 2 and 6), 30 min (lanes 3 and 7), and 45 min (lanes 4 and 8). After incubation, the cells were pelleted, and DNA was prepared by the gentle lysis procedure described in Materials and Methods. Total *E. coli* DNA was electrophoresed in a 0.8% agarose gel, transferred to blotting membrane, and probed with a ^{32}P-labeled fragment from pPHR 17 so that only the target plasmid was visualized. Arrow indicates the 8-kb linear form of pPHR 17, as determined by size standards visualized on the ethidium bromide-stained gel (not shown). This linear form was seen only in the culture that contained the intact open reading frame for I-*Ppo* (lanes 1 to 4). The bands corresponding to supercoiled (sc) and open circular (oc) forms of the plasmid are indicated.

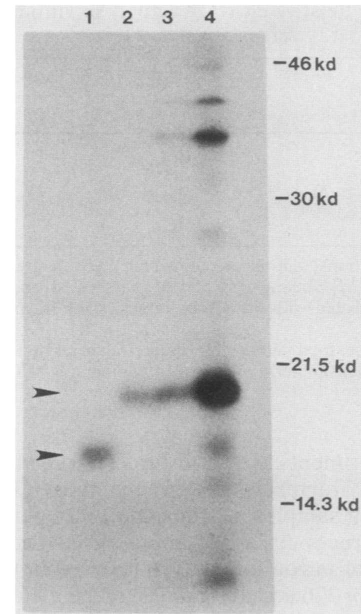

FIG. 5. Comparison of in vitro- and in vivo-synthesized I-*Ppo*. I-*Ppo* made in vitro (as described for Fig. 3) from deleted plasmid pI3-d9 (lane 1) or full-length I-*Ppo* made from plasmid pI3-tr742 (lane 2) was run on a 17.5% SDS-polyacrylamide gel along with full-length I-*Ppo* that was made from pI3-tr742 in vivo (lanes 3 and 4). (Lane 4 contains 10-fold more sample than lane 3 in order to show the minor bands.) The upper arrowhead indicates the full-length protein that initiates in the upstream exon; the lower arrowhead indicates the shorter protein that initiates within the intron. Size standards (shown in kilodaltons [kd] on the right) were extrapolated from rainbow markers as described previously.

shown). However, the in vivo results for expression of the 5′ truncations differed from those obtained in vitro. Figure 6A shows the profiles of radioactive polypeptides from cells expressing the 5′ deletion plasmids. Neither pI3-d55 (lanes 10 to 12) nor pI3-d9 (lanes 7 to 9) yielded a detectable rifampin-resistant band. For comparison, the expression of I-*Ppo* from parallel cultures carrying pI3-941 (lanes 1 to 3) and pI3-tr742 (lanes 4 to 6) also is shown. The result for pI3-d9 initially was unexpected, since RNA from this plasmid is readily translated in vitro into an enzymatically active species of 15 to 16 kDa. The most likely explanation for lack of translation in *E. coli* is absence of an efficient ribosome-binding site. Neither in pI3-d9 nor in the parent plasmid pI3-941 is there a good consensus match of the commonly used bacterial ribosome-binding sequences upstream of the intron AUG. By contrast, the exon AUG is in a good context for translation. If the absence of a radioactive band in pI3-d9 is due to a poor ribosome-binding site, we reasoned that a more sensitive assay based on enzymatic activity might still be able to give evidence for synthesis of the shorter form I-*Ppo*. The results from an experiment to measure in vivo

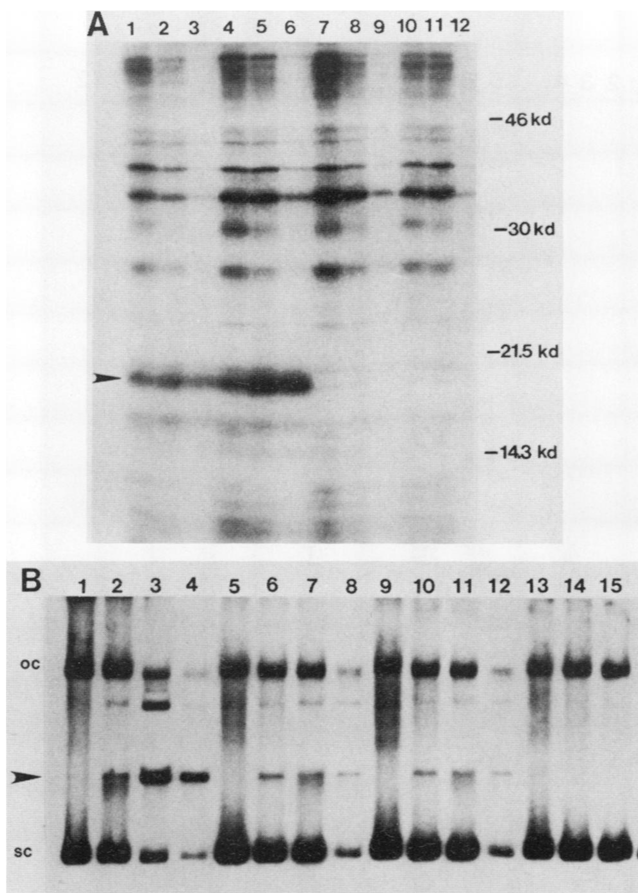

FIG. 6. Expression of deleted plasmids in *E. coli*. (A) Expression of I-*Ppo* induced in *E. coli* as described for Fig. 4. The plasmids used were pI3-941 (lanes 1 to 3), pI3-tr742 (lanes 4 to 6), pI3-d9 (lanes 7 to 9) and pI3-d55 (lanes 10 to 12). Cells were infected with phage lambda and then incubated for 5 min, after which rifampin was added for various times as follows: 15 min (lanes 1, 4, 7, and 10), 30 min (lanes 2, 5, 8, and 11), and 60 min (lanes 3, 6, 9, and 12). The arrowhead indicates the position of the intron-encoded protein. kd, Kilodaltons. (B) Cleavage in vivo by deleted I-*Ppo*. Various plasmids were maintained in *E. coli* along with pPHR 17. Induction of I-*Ppo* and cleavage of the target plasmid was performed as described for Fig. 4B. The plasmids used were pI3-941 (lanes 1 to 4), two separate isolates of pI3-d9 (lanes 5 to 8 and lanes 9 to 12, respectively), and pI3-d239 (negative control; lanes 13 to 15). Cells were uninfected (lanes 1, 5, 9, and 13) or infected with the lambda phage CE6 for 15 min (lanes 2, 6, 10, and 14), 30 min (lanes 3, 7, 11, and 15), and 60 min (lanes 4, 8, and 12). By the 60-min time point, the samples lysed as a result of phage infection. The arrowhead indicates the 8-kb, linear form of pPHR 17. Positions of the open circular (oc) and supercoiled (sc) forms of the plasmid are indicated.

cleavage are shown in Fig. 6B. The assay, as described for Fig. 4B, was performed by using the full-length construct pI3-941 as a positive control (lanes 1 to 4), pI3-d239 as a negative control (lanes 13 to 15), and two independently isolated clones of pI3-d9 (lanes 5 to 8 and 9 to 12). A small amount of cleaved product, at most 1/10 that seen in pI3-941, was observed for both clones. We conclude that a low level of enzymatically active protein, below the limits of detection by ^{35}S labeling, was indeed produced from this deletion construct.

In cells expressing I-*Ppo*, an additional band much larger than the 8-kb linear form of pPHR 17 also was generated (Fig. 6B). We have not determined the identity of this band. However, since it frequently appears when *E. coli* C600, a *recA*$^+$ strain, is used but never appears when DH5α, a *recA* strain, is used (data not shown), we surmise that this species arises from a recombination event initiated by the double-strand break.

Cleavage site for I-*Ppo*. To determine the exact cleavage site for I-*Ppo*, we used a modification of the in vitro cleavage assay (Fig. 7A and B; a schematic representation is shown in Fig. 7C). For these experiments, a plasmid with a synthetic target site was constructed by cloning an oligonucleotide corresponding to 38 nucleotides of rDNA at the site of intron insertion. Sequencing ladders were generated for each strand, using either the reverse primer (top strand; Fig. 7A, lanes 5 to 8) or the −20 primer (bottom strand; Fig. 7B, lanes 5 to 8), as indicated. A portion of the oligonucleotide sequence is shown. A sample of each of these sequencing reaction mixtures was subjected to cleavage by I-*Ppo* translated in a reticulocyte lysate. The cleaved DNA was then electrophoresed on a high-resolution gel in parallel with the uncleaved reaction mixtures. A single radioactive band was produced in each set of reactions (Fig. 7A and B, lanes 1 to 4). The exact location of the cleavage site could be determined by alignment of this band, using a lighter exposure of the film (lanes 9 to 12), with the corresponding band in the sequencing ladder. The bands from both strands line up with an A in each sequencing ladder (indicated by arrowheads in Fig. 7A and B). Combining the data obtained for both strands, we conclude that I-*Ppo* introduces a staggered double-strand cut in the target DNA. The cuts were offset by three nucleotides and one nucleotide, respectively, from the site of intron insertion (Fig. 7C). The cleaved DNA could be ligated by T4 ligase (data not shown), indicating that it possesses a terminal 5′-phosphate.

DISCUSSION

We have characterized several features of I-*Ppo*, the endonuclease that mediates the mobility of intron 3 in the rDNA of *P. polycephalum*. I-*Ppo* is encoded in the 5′ half of intron 3. Two possible AUG initiation codons start its reading frame, one near the beginning of the intron and the other in the upstream exon. The polypeptide initiated in the exon is the major form translated in vitro in a reticulocyte extract, but by deletion analysis we showed that both forms possess endonuclease activity. The longer polypeptide also is preferentially synthesized in *E. coli*, where it cleaves plasmids carrying the target site. I-*Ppo* makes a four-base staggered cut in its rDNA target sequence, overlapping the site where insertion occurs.

In its enzymology, I-*Ppo* appears similar to the other intron-encoded endonucleases that have been characterized to date. The enzymes from group I introns found in yeast mitochondria (I-*Sce*I and I-*Sce*II, from the omega intron in mitochondrial rDNA and from the fourth intron of cytochrome oxidase subunit I, respectively), also make four-base staggered cuts near the sites where the introns can become inserted into an unoccupied homing site (8, 11, 17, 37). However, unlike I-*Ppo*, the T4 enzymes (I-*Tev*I and I-*Tev*II) cleave 13 to 26 nucleotides from the insertion site (32; M. Belfort, personal communication). In the large size of its recognition sequence, I-*Ppo* also resembles these other endonucleases. Our preliminary findings are that 16 to 18 nucleotides are required for efficient cleavage (unpublished observations), a size similar to that found for I-*Sce*I and

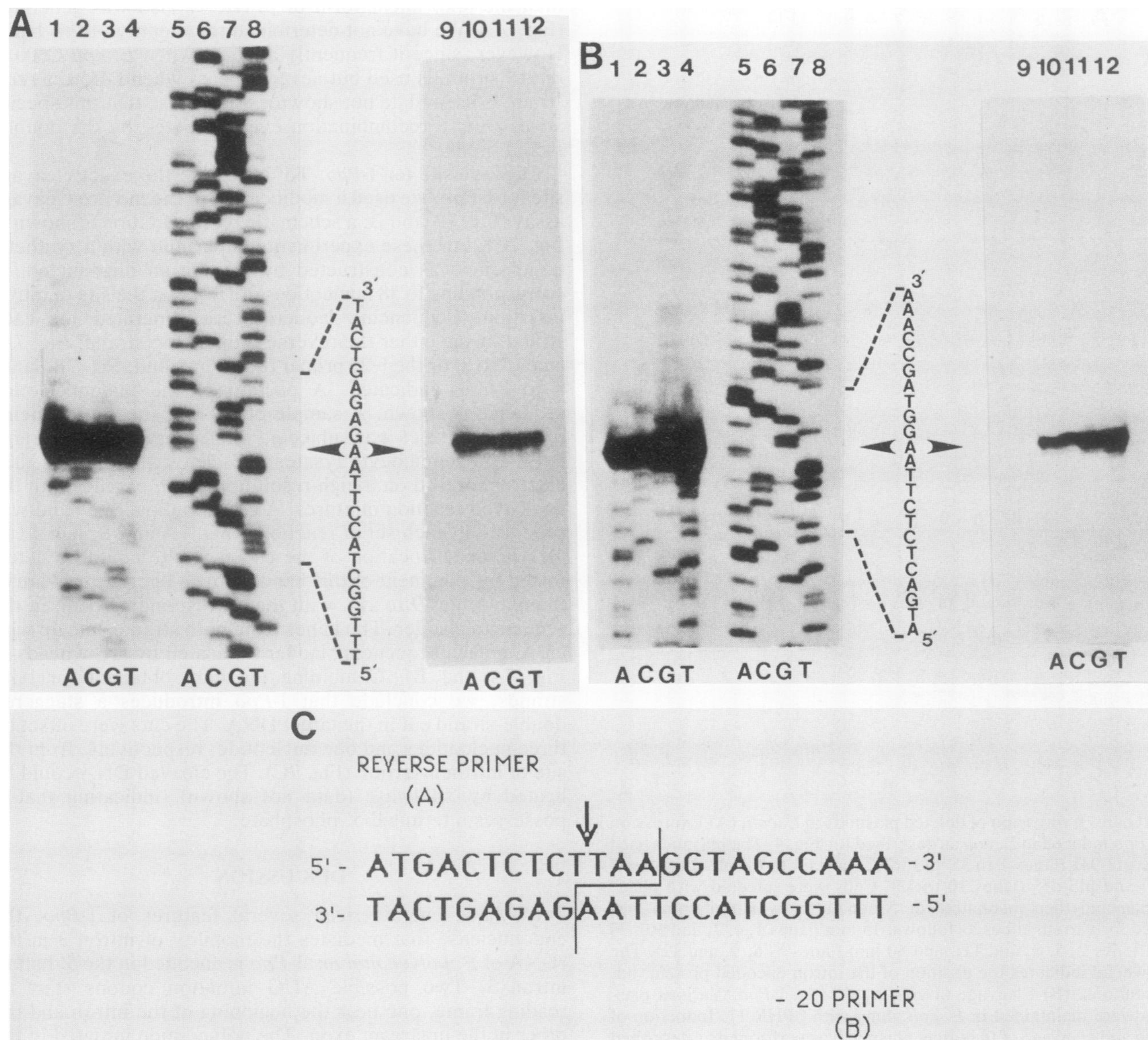

FIG. 7. Mapping of the cleavage site for I-*Ppo*. A synthetic oligonucleotide corresponding to 38 nucleotides surrounding the site of intron insertion was cloned into Bluescript as described in Materials and Methods. Sequencing ladders were generated by using either the reverse primer (A; top strand) or the −20 primer (B; bottom strand), as drawn in panel C. A partial sequence for each strand is shown (lanes 5 to 8). A portion of each reaction was subjected to in vitro cleavage by I-*Ppo* and electrophoresed along with the sequencing reactions. Exposures equivalent to those for the sequencing reactions (lanes 1 to 4) and a shorter exposure (lanes 9 to 12) are shown for each set of cleavages. The nucleotide that is adjacent to the cleavage site on that particular strand is indicated by an arrowhead. The cleavage sites on both strands, along with the site for intron insertion (arrow), are shown in panel C. The relative orientation for each of the sequencing primers of the Bluescript vector is also indicated.

I-*Sce*II (9, 27). Two other site-specific endonucleases from eucaryotic organisms, those encoded by the R2 retrotransposon-like element in the rDNA of *Bombyx mori* and by the *HO* gene that is involved in mating-type switching in *S. cerevisiae*, also recognize such a large sequence (29, 39). Despite the functional similarities it shares with these other endonucleases, I-*Ppo* is not structurally related to any of them. Our search in the data banks revealed no substantial amino acid sequence homology with any other proteins. In particular, the consensus motif LAGLI-DADG that is found in I-*Sce*I and I-*Sce*II is absent, as it is from the T4 enzymes. Thus, it appears that the *Physarum* enzyme does not share recent evolutionary ancestors with other intron-encoded endonucleases.

The reading frame for I-*Ppo*, which takes up most of the 5′ half of the intron, is separated from the conserved group I intron-specific sequences found in the 3′ half. Only the latter shows homology with the well-studied *Tetrahymena* intron. With the exception of the T4 enzymes, the genes for most other intron-encoded proteins also are separated from the sequences required for self-splicing (3, 12, 19). Another feature that I-*Ppo* shares with other intron-encoded proteins is the extension of the reading frame into the upstream exon. Intron-encoded maturases (i.e., enzymes required for splic-

ing) in yeast and other fungal mitochondria occur as in-frame fusions with the upstream exons (3, 12). Splicing destroys the maturase message, thus providing a negative-feedback control that guarantees the synthesis of these proteins only when they are needed. In the case of I-*Ppo*, the preferred initiation site in reticulocyte extracts as well as in bacteria is the exon AUG. But we have shown by deletion analysis that in vitro, and perhaps at a low level in *E. coli* as well, an enzymatically active protein also can be generated from the AUG 22 amino acids downstream near the beginning of the intron. At present it is not possible to predict which initiation site is used in *P. polycephalum*. The findings that both in vitro and in *E. coli*, similar amounts of I-*Ppo* are made from intact intron 3 constructs and from truncated constructs that prevent splicing argue that splicing is not required to generate an active mRNA.

The generation of the longer form of I-*Ppo* from the splicing-competent construct implies that in *E. coli* a substantial portion of the intron RNA must remain unspliced. The closely related *Tetrahymena* intron splices efficiently in *E. coli* (33). The major difference between these introns is the presence of the open reading frame in the *Physarum* construct. We hypothesize that translation by ribosomes initiating at the exon-encoded methionine inhibits the RNA folding required for splicing. Analysis of RNA species extracted directly from *E. coli* will be necessary to investigate this hypothesis. To explain the lack of translation from the intron AUG in *E. coli*, both in full-length and in the partially deleted constructs, we propose that ribosomes are unable to initiate because of the absence of a proper consensus binding site. However, it may also be that the shorter protein produced is less stable in vivo than is the full-length protein.

At present nothing is known about the form of the endonuclease made in *P. polycephalum* nor about the identity of its message. The nuclear location of intron 3 imposes special constraints on its expression. First, unlike all other intron-encoded proteins, which are made in vivo under conditions in which transcription and translation are coupled, I-*Ppo* presumably must be translated from a message that is first transported from the nucleolus through the nucleoplasm and nuclear membrane to the cytoplasm. Studies on *Tetrahymena* cells (1) showed that the half-life of the spliced intron RNA in vivo is only approximately 6 s. Given the transport times for typical messages, on the order of 20 min, it would seem impossible that such an unstable RNA could serve as a message. Perhaps some feature of intron 3 RNA serves to stabilize it, as appears to be true for the RNA of the phage T4 *td* intron, which is more stable than typical *E. coli* mRNAs (7).

Another constraint derives from the location of the intron in rDNA. We presume that the I-*Ppo* message is embedded in the pre-rRNA that is transcribed by RNA polymerase I. The only other example of a protein encoded in a polymerase I transcript is the endonuclease from the R2 element of *B. mori* rRNA (39). If splicing in *Physarum* is as rapid as it is in *Tetrahymena* cells, in which the intron-containing pre-rRNA has a half-life of only about 2 s (1), it would appear unlikely that the exon-encoded portion of the open reading frame could be included as part of the message for I-*Ppo*. Perhaps an alternative form of processing occurs in a fraction of pre-rRNA, leading to a population of more stable molecules. The omega intron of yeast mitochondrial rRNA provides a precedent for such alternative processing (40). Even though the coding region for I-*Sce*I is contained entirely within the omega intron, an unusual, omega-specific RNA species apparently is generated by a novel pre-rRNA processing event. This species is formed by two cleavage events, one within the 5′ rRNA exon and the other within the intron at a conserved mitochondrial RNA processing site immediately 3′ of the I-*Sce*I reading frame. The resulting fused exon-intron RNA has been hypothesized to the actual message used for the production of I-*Sce*I. Support for this hypothesis derives from a mutant that does not produce this novel RNA species (41). Even though large amounts of excised intron RNA accumulate in the mitochondria of these mutants, no functional omega endonuclease appears to be made. Perhaps a similar alternative splice product is made in I^+ *Physarum* strains. However, we cannot exclude the possibility that the message for I-*Ppo* is transcribed from an RNA polymerase II promoter that has yet to be identified. It will be important to investigate both of these possibilities in *Physarum*.

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