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A Mobile Group I Intron from *Physarum polycephalum* Can Insert Itself and Induce Point Mutations in the Nuclear Ribosomal DNA of *Saccharomyces cerevisiae*

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Pp LSU3 is a mobile group I intron in the extrachromosomal nuclear ribosomal DNA (rDNA) of *Physarum polycephalum*. As found for other mobile introns, Pp LSU3 encodes a site-specific endonuclease, I-*Ppo*, which mediates "homing" to unoccupied target sites in *Physarum* rDNA. The recognition sequence for this enzyme is conserved in all eucaryotic nuclear rDNAs. We have introduced this intron into a heterologous species, *Saccharomyces cerevisiae*, in which nuclear group I introns have not been detected. The expression of Pp LSU3, under control of the inducible *GAL10* promoter, was found to be lethal as a consequence of double-strand breaks in the rDNA. However, surviving colonies that are resistant to the lethal effects of I-*Ppo* because of alterations in the rDNA at the cleavage site were recovered readily. These survivors are of two classes. The first comprises cells that acquired one of three types of point mutations. The second comprises cells in which Pp LSU3 became inserted into the rDNA. In both cases, each resistant survivor appears to carry the same alterations in all ~150 rDNA repeats. When it is embedded in yeast rDNA, Pp LSU3 leads to the synthesis of I-*Ppo* and appears to be mobile in appropriate genetic crosses. The existence of yeast cells carrying a mobile intron should allow dissection of the steps that allow expression of the highly unusual I-*Ppo* gene.

Group I introns are characterized by a series of conserved sequence elements that allow the intron RNA to assume a complex secondary structure, which then enables the intron to undergo autocatalysis in vitro, i.e., to "self-splice" from the primary transcript. The prototype self-splicing group I intron is Tt LSU1, from the extrachromosomal ribosomal DNA (rDNA) of *Tetrahymena* species (10). About 100 group I introns have been described to date (7, 9), most of which are found in the organelle genomes of lower eucaryotes. A few are also found in T-even bacteriophages, in procaryotes, and in nuclei. All of the rare nuclear group I introns are located in the genes coding for ribosomal RNA, i.e., rDNA. These include Tt LSU1 plus introns in *Physarum polycephalum* (34, 35), *Pneumocystis carinii* (19, 28), *Didymium iridis* (24), and several species of algae (13, 16). Many group I introns are "optional," i.e., are present in some but not all strains or species of a particular organism. A few group I introns are also mobile. In vivo, they rapidly and efficiently spread from a locus that contains the intron (I^+) to one that lacks the intron (I^-). This process, termed intron homing (17), is initiated by a double-strand break introduced in the I^- locus by a site-specific endonuclease encoded by the intron itself (reviewed in reference 4). Intron homing is believed to be mediated by a double-strand break repair mechanism similar to that proposed to explain unidirectional gene conversion and mating type switching in *Saccharomyces cerevisiae* (53). In this process, the I^+ allele serves as a template for the repair of the cleaved I^- allele. Once a DNA molecule has acquired the intron, it is no longer a substrate for cleavage by the endonuclease and can serve as an I^+ template for further homing events.

The only example so far reported of a mobile intron in the nucleus is Pp LSU3, an optional intron found in one strain of the acellular slime mold *P. polycephalum*. Pp LSU3 is inserted in rDNA in the gene coding for the large-subunit ribosomal RNA and thus is expressed in the nucleolus. rDNA in *P. polycephalum* exists as a collection of linear, 60-kb extrachromosomal DNA molecules. Mating of an I^+ strain carrying Pp LSU3 with an I^- strain leads to rapid conversion of all the ca. 150 I^- extrachromosomal rDNA molecules to an I^+ form (34). We showed previously, by in vitro translation and expression in *Escherichia coli*, that Pp LSU3 encodes an endonuclease, termed I-*Ppo*, in the 5′ half of the intron (33). The open reading frame of this enzyme extends into the upstream exon, i.e., the coding region for the large-subunit RNA. Translation of both the full open reading frame initiated in the upstream exon (160 amino acids) and a shorter form initiated at an AUG in the intron (138 amino acids) leads to proteins with similar endonuclease activities (33). As for other intron-encoded endonucleases, the recognition sequence of I-*Ppo* is large, comprising 13 to 15 bp (20). This sequence is located in a region of ca. 60 bp in rDNA that is 100% conserved in all eucaryotes.

The structure of Pp LSU3 indicates that it is closely related to the prototypic *Tetrahymena* group I intron. The 3′ half of the 941-bp Pp LSU3, which contains the sequence elements required for autocatalysis, has over 70% homology with Tt LSU1, making this the closest relative of Tt LSU1 in species other than those of the genus *Tetrahymena*. Both introns are also inserted into the same site in rDNA. However, only the *Physarum* intron encodes an endonuclease and is mobile under experimental conditions. There are a number of other examples of introns, like Pp LSU3 and Tt LSU1, which are optional and closely related in structure

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and whose parent species diverged long ago in evolution. Such introns are presumed to have been acquired somehow by horizontal transfer late in evolution, after divergence of the organisms in which they reside. The mobility of group I introns via double-strand break-repair provides a mechanism for the horizontal spread of these introns into new hosts.

We have developed an experimental system for the introduction of Pp LSU3 into *S. cerevisiae*, an organism that does not normally contain any nuclear group I introns. Transformation with plasmids carrying Pp LSU3 was found to be lethal in yeast cells because of the I-*Ppo* endonuclease, whose expression presumably results in cleavage of some of the ca. 150 tandem rDNA repeats on chromosome XII. However, transformants that are resistant to the lethal effects of I-*Ppo* appeared at a surprisingly high frequency. These transformants were shown to be of two classes. The first class acquired point mutations at the I-*Ppo* cleavage site, rendering all rDNA repeats resistant to cleavage. The second class acquired Pp LSU3 itself, again in all rDNA repeats. Cells of the second class, whose growth is only slightly impaired, were shown to produce readily detectable levels of I-*Ppo*. Mating experiments suggested that Pp LSU3 is mobile in yeast cells.

MATERIALS AND METHODS

Yeast strains and culture. Strains DBY1034 (*MAT***a** *his4-539 lys2-801 ura3-52*) and DBY947 (*MAT*α *ade2101 ura3-52*) were provided by T. Huffaker (Cornell University). Strain F808 (*MAT***a** *ura3-52 leu2-3,112 his4-519 ade1-100 GAL1*) was obtained from G. Fink (Massachusetts Institute of Technology). Strain 2262 (*MAT*α *ade1 ura1 gal1 his5 lys11 leu2* form II) (41) was obtained from the American Type Culture Collection (ATCC 42679). All strains were grown in YPD medium. In selections for the *URA* marker, complete minimal medium minus uracil (CM-ura) was used. For curing of strains with *URA*-containing plasmids, colonies were selected after plating on 5-fluoroorotic acid. All medium formulations are described elsewhere (3).

Yeast cells were transformed with LiCl (3). For selection of cleavage-resistant mutants, transformants were first plated on CM-ura medium plus glucose. *Ura*$^+$ colonies were then transferred onto plates containing the same medium but with galactose as the carbon source. Colonies that were able to grow on galactose were selected for DNA isolation, blotting, and sequence analysis.

DNA constructs. Plasmid pGAL656 was obtained from A. Bretscher (27). It contains the inducible *GAL10-GAL1* promoter region cloned into a 2μm plasmid. Plasmid pGAL-I3 was constructed by cloning an *Xba*I-*Sal*I fragment from pI3-941 (33) into the multiple cloning site of pGAL656. This fragment includes the entire 1.4-kb *Eco*RI-*Cla*I fragment from pI3-941, which contains all of intron 3 flanked by 376 bp of upstream 26S rRNA exon sequence and 24 bp of downstream sequence. Plasmid pGAL-Ippo was constructed by cloning the *Not*I-*Sph*I fragment from pI3-d9 (33) into the *Xba*I and *Sph*I sites of the multiple cloning region of pGAL656. The *Not*I and *Xba*I sites were blunted prior to ligation. Thus, pGAL-Ippo is deleted of all *Physarum* sequences upstream of nucleotide +9 in the intron and downstream of nucleotide +604. The open reading frame for the "short" form of I-*Ppo* is contained within this fragment, extending from nucleotides +14 to +427. Plasmid pGAL-tr604 was generated by digesting pGAL-I3 with *Sph*I and religating the resulting deleted fragment. Plasmid pGAL-del239 was constructed by subcloning an *Eco*RI-*Cla*I fragment from pI3-d239 (33) into pGAL656. A termination codon in the I-*Ppo* coding region was inserted into pGAL-I3 by blunt-end ligating an *Xba*I linker containing stop codons in all three reading frames (New England Biolabs) into a *Pfl*MI site located at nucleotide +100 in the intron to generate plasmid pGAL-term100.

DNA isolation and analysis. Genomic DNA was isolated from yeast spheroplasts (3). For blotting experiments, 2 to 5 μg of total DNA was digested with the appropriate restriction enzyme and electrophoresed in 0.8 to 1.0% agarose gels, depending on the experiment, in a Tris-borate buffer. The DNA was transferred to a Zeta-probe blotting membrane (Bio-Rad). The blots were prehybridized and then probed with nick-translated DNA fragments according to the manufacturer's recommendations. The probe for yeast rDNA was plasmid pSZ20, which was obtained from J. Szostak (54).

Polymerase chain reaction (PCR) amplification of a segment of yeast rDNA was carried out with primers corresponding to coordinates 2070 to 2086 (5′-GACCGTCGCT TGCTACA-3′) and 2299 to 2315 (5′-GTGACGCGCATGA ATGG-3′) of yeast 25S rRNA (21). The resulting 246-bp fragments for the cleavage site mutants and the ~1.2-kb fragments for the intron-containing strains were gel purified and either used directly for PCR sequencing or subcloned for plasmid sequencing.

For the cleavage site mutants, the 246-bp fragment from the PCR amplification was digested with *Hin*PI, which cuts twice within the fragment, on either side of the I-*Ppo* recognition sequence, generating a 72-bp fragment. This *Hin*PI fragment was cloned into the *Acc*I site of Bluescript KS (Stratagene) and then sequenced by standard methods. Alternatively, sequencing was performed directly on the gel-purified PCR fragments. For the cleavage site mutants, a primer corresponding to coordinates 2149 to 2170 of yeast 25S rRNA was used (5′-GCATTCGATGGTCAGAAAGTG-3′). For strains in which the intron was inserted into the rDNA, a sequencing primer corresponding to nucleotides 10 to 60 of intron 3 (33) (5′-CCCAGCTGTCAATCACAGCC-3′) was used.

Galactose induction and assay for I-*Ppo* activity. Cells transformed with pGAL-Ippo as described above were grown in CM-ura medium containing glucose to an optical density at 550 nm (OD_{550}) of ~1.0. Cultures were then diluted 1:10 into CM-ura medium containing 2% ethanol, and incubation was continued. When cultures reached an OD of ~1.0, galactose was added to a final concentration of 2%. Aliquots (10 ml) were taken at various time points. Cells were pelleted, and extracts were made and analyzed for I-*Ppo* activity. Galactose induction for detection of double-strand breaks in vivo was performed as above, but cultures were grown in glucose, washed three times in water, and placed directly in galactose-containing medium. Aliquots (10 ml) were collected at each time point, and DNA was prepared and subjected to blotting analysis.

Assays for I-*Ppo* endonuclease activity were done starting with cell pellets from 10 ml of culture. The pellets were resuspended in 0.2 ml of ice-cold breakage buffer (10% glycerol, 200 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 0.2 mM phenylmethylsulfonyl fluoride). Cells were transferred to a microcentrifuge tube containing 0.2 ml of glass beads (425 to 600 μm diameter) and vortexed for 5 min. The lysates were then spun in a microcentrifuge for 5 min at 4°C. Aliquots of the supernatant were assayed for the ability to cleave an end-labeled fragment that contained the I-*Ppo* recognition

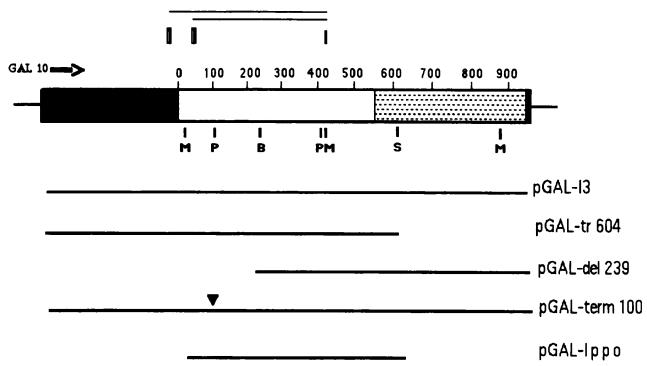

FIG. 1. Plasmids used in yeast transformation. Plasmid pGAL-I3 contains the entire 941 bp of intron Pp LSU3, flanked by 376 bp of *Physarum* 26S rRNA upstream sequence and 27 bp of downstream sequence, indicated by solid black regions. The 3′ portion of the intron, which contains the ribozyme and is homologous to the *Tetrahymena* intron, is indicated by a stippled rectangle. The 5′ portion of the intron, which contains sequences unique to the intron and includes the coding region for I-*Ppo*, is indicated by an open rectangle. Two possible methionines used for initiation of translation, one in the upstream exon and one within the intron, are indicated by open bars, and the site for termination of translation is indicated by the solid bar above the diagram. The direction of transcription from the *GAL10* promoter is indicated by the arrow. Plasmids derived from pGAL-I3 are shown, along with the nucleotide corresponding to either the 5′ deletion (del) or 3′ truncation (tr) of the intron. Restriction sites within the intron used to generate each construct: B, *Bam*HI; M, *Mse*I; P, *Pfl*MI; S, *Sph*I. The site of insertion of a stop codon is indicated by the solid triangle.

sequence. Cleavage reactions were performed and analyzed as described previously (33, 34).

RESULTS

Plasmids containing the I-*Ppo* gene are lethal to yeast cells. In order to introduce Pp LSU3 into the rDNA of yeast cells, we first cloned the intron into pGAL656 (27), a 2μm plasmid containing the inducible *GAL1,10* promoter and a *URA3* selectable marker. The resulting construct, pGAL-I3, contains all 941 bp of the intron plus 376 bp of upstream exon and 24 bp of downstream exon. This construct and all constructs derived from it are illustrated in Fig. 1. Initially, pGAL-I3 and the control plasmid pGAL656 were transformed into the two yeast strains, DBY1034 and F808. The latter is inducible by galactose. The results of this experiment for DBY1034 are shown in Table 1 (trial 1). Transformation of haploids with pGAL-I3 followed by selection on CM-ura medium containing glucose yielded approximately 100-fold fewer *URA*$^+$ colonies than did the control, suggesting that the intron is lethal. Similar results were obtained for strain F808 haploids as well as for diploids of both strains. A lethal effect was also observed when the fragment carried by pGAL-I3 was cloned into the integrative vector Yip5 (data not shown). The lethality did not appear to be due to leakiness of the *GAL* promoter, since a derivative plasmid completely lacking this promoter, p656-I3, had the same effect (Table 1, trial 2). In all of these transformations, the rare *URA*$^+$ colonies containing Pp LSU3 that did grow took approximately twice as long to appear as did colonies containing the control plasmid (4 to 5 days versus 2 to 3 days, respectively). The lethality in the absence of the *GAL* promoter suggests that a cryptic promoter in the 376 bp of exon sequence preceding the I-*Ppo* open reading frame

TABLE 1. Transformation frequencies obtained with intron 3 constructs[a]

Plasmid	Transformants (no./μg of plasmid DNA)		
	Trial 1	Trial 2	Trial 3
pGAL656	>1,000	>1,000	>1,000
pGAL-I3	14/12	5	ND[b]
p656-I3	ND	5	ND
pGAL-tr604	ND	ND	0
pGAL-del239	ND	ND	>1,000
pGAL-term100	ND	ND	>1,000
pGAL-Ippo	>1,000/~500		

[a] All transformation frequencies shown were obtained with strain DBY1034 except for pGAL-Ippo, for which transformation frequencies are shown for both strains (DBY1034/F808).
[b] ND, not determined.

results in the production of enough endonuclease to cleave chromosome XII at one or more of the rDNA repeats, a presumably lethal event.

In order to verify that the I-*Ppo* endonuclease was responsible for the effects observed, additional plasmids were constructed and tested. These plasmids contain either a truncation of Pp LSU3 downstream of the coding region for I-*Ppo* (pGAL-tr604), a deletion of all upstream sequences, including the 5′ portion of the I-*Ppo* coding region (pGAL-del239), or an insertion of a stop codon within the I-*Ppo* coding region (pGAL-term100). The results implicate I-*Ppo* as the source of lethality, since only when the coding region for I-*Ppo* was perturbed (pGAL-del239 and pGAL-term 100) was the lethality abolished (Table 1, trial 3). By contrast, destruction of the ribozyme portion of the intron had no effect (pGAL-tr604).

In order to obtain tighter regulation of I-*Ppo*, a plasmid in which the coding region for I-*Ppo* is situated directly behind the *GAL10* promoter, without most of the intervening exon sequences present in previous constructs, was constructed. This plasmid, pGAL-Ippo, transformed both yeast strains on glucose-containing medium with high frequency (Table 1), suggesting that in this case, enzyme expression is appropriately suppressed on glucose.

Galactose induction leads to double-strand breaks. Since pGAL-Ippo appeared to lack deleterious effects on glucose-containing medium, the effect of I-*Ppo* on rDNA was studied in vivo by inducing cells with galactose. *URA*$^+$ colonies of strain F808 that had been transformed with pGAL-Ippo were selected on glucose. One such isolate was subjected to galactose induction as described in Materials and Methods. DNA isolated from aliquots of the culture at consecutive times was digested with the restriction enzyme *Eco*RI, and the resulting DNA was electrophoresed on an agarose gel and analyzed by Southern blotting with an rDNA-specific probe. The outcome for one such experiment is shown in Fig. 2. The two expected *Eco*RI fragments, of 2.4 and 2.9 kb, are visible in the uninduced control (Fig. 2A and 2B [t = 0]). The 2.9-kb fragment contains the I-*Ppo* cleavage site, which coincides with an *Afl*II site (indicated by an asterisk in Fig. 2A). Cleavage of this fragment with I-*Ppo* would generate fragments of 2.5 and 0.4 kb, as indicated. Upon induction of the yeast culture with galactose (Fig. 2B [t = 1 to 6]), a product of 2.5 kb appeared, consistent with cleavage by the intron-encoded endonuclease. (The expected 0.4-kb product would have run off the gel.) The intensity of the cleavage product increased over time, reaching a plateau at 4 h, suggesting either that the double-strand break induced by

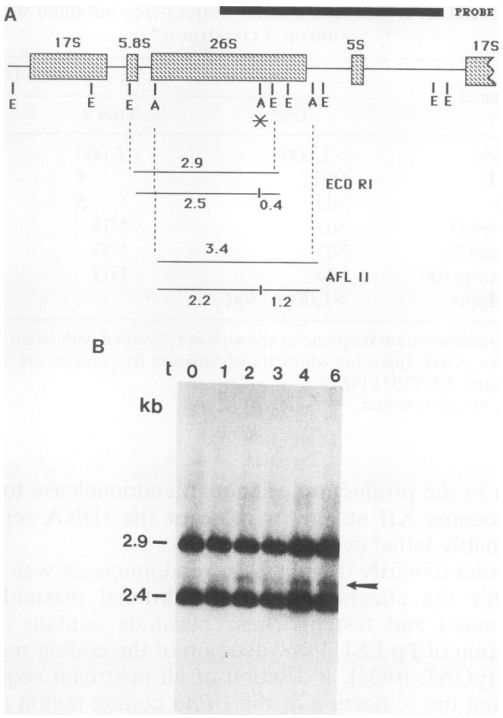

FIG. 2. Induction of double-strand breaks in rDNA in vivo. (A) Partial restriction map of yeast rDNA showing the *Eco*RI (E) sites and the *Afl*II (A) sites pertinent to these experiments. The *Afl*II site that is identical to the I-*Ppo* cleavage site in the 27S rRNA is indicated by an asterisk. Fragment sizes before and after cleavage by I-*Ppo* are indicated below the map. The horizontal black bar corresponds to the probe used. (B) Southern blot of cultures of yeast cells transformed with the construct pGAL-Ippo. Cultures were induced with galactose for 0 to 6 h, as indicated above each lane. Aliquots were taken for each time point, and the DNA was purified, digested with *Eco*RI, and electrophoresed in a 0.8% agarose gel. The DNA was transferred to a blotting membrane and hybridized with the probe indicated in panel A. The 2.9-kb *Eco*RI fragment is cleaved to a fragment of approximately 2.5 kb, indicated by the arrow. The other cleavage product of 0.4 kb is not present in this blot. The 2.4-kb fragment is an additional *Eco*RI fragment that hybridizes to the probe.

I-*Ppo* was repaired at the same rate as it was formed or that only a fraction of the rRNA genes were accessible to cleavage. We note that a similar level of rDNA breakage was observed previously in *P. polycephalum* after mating of I^+ and I^- strains (34).

Some transformants carry rDNA mutations. What allows the rare *URA*$^+$ colonies to grow when pGAL-I3 is transformed into *S. cerevisiae*? We considered two possible explanations for this survival. According to the first, the 2 μm plasmid acquired mutations in the I-*Ppo* gene, which led to an enzymatically inactive protein. To test this idea, plasmids were recovered from several independent yeast transformants, and the intron-containing fragment was transferred into an *E. coli* plasmid carrying the phage T7 promoter. The ability of the resulting DNA to give rise to catalytically active I-*Ppo* was then tested by in vitro translation (33). RNA synthesized in vitro by T7 RNA polymerase was used to program a rabbit reticulocyte lysate, which was assayed for endonuclease activity by incubating an aliquot of the translation mix with a DNA fragment containing the I-*Ppo* recognition sequence. All 10 plasmids tested, which were generated from independent yeast transformants, yielded active enzyme (data not shown). This result suggests that, at least in most of the surviving yeast cells, the I-*Ppo* gene itself had not become mutated, nor did it appear that other sequences on the plasmids responsible for I-*Ppo* expression were mutated, since these plasmids were still lethal when retransformed into yeast cells (data not shown).

According to the second explanation, the surviving cells became resistant to the lethal effects of the endonuclease because of alterations in the yeast DNA. This explanation is supported by experiments in which the transformants were cured of the *URA3* plasmid. Colonies were picked and grown on a medium containing the uracil analog 5-fluoroorotic acid, which allows only cells that have lost the I-*Ppo*-encoding plasmid to grow. Among more than 30 colonies tested, approximately 20% gave rise to cured cells that were subsequently able to be transformed at high frequency by the same pGAL-I3 plasmid. The colonies that did not transform with high frequency have not been characterized. We refer to the cured cells that were able to be retransformed at a normal high efficiency as high-frequency (HF) strains. They presumably carry chromosomal mutations that make them resistant to the action of I-*Ppo*. Initially, it seemed unlikely that the target site for I-*Ppo* could be a candidate for mutation, since it is present in each of the ca. 150 copies of rDNA. Nevertheless, we addressed this possibility by Southern blot analysis of DNA from the HF isolates. DNA was isolated and digested with the restriction enzyme *Afl*II, which fortuitously recognizes a 6-bp sequence exactly at the I-*Ppo* cleavage site (see Fig. 4E). *Afl*II was used in these experiments because of the limited availability of purified I-*Ppo*. However, as discussed below, later experiments performed with I-*Ppo* itself yielded identical results.

*Afl*II cleaves yeast rDNA at several sites. Two of the resulting fragments, 2.2 and 1.2 kb, lie within the 27S rDNA (Fig. 2A). Failure of *Afl*II to cleave at the common *Afl*II/I-*Ppo* site would result in a novel fragment of approximately 3.4 kb. Indeed, a band of this size was generated from the two HF DNAs (Fig. 3A, lanes 2 and 3), but not in the control DNA (Fig. 3A, lane 1). Similar cleavage-resistant isolates were obtained for both the F808 and DBY1034 strains. None of the HF strains showed evidence of the 2.2-kb and 1.2-kb fragments. Thus, we conclude that, within the limits of detection, all of the rDNA copies in these cells carry alterations at the I-*Ppo* cleavage site. These alterations appeared to be genetically stable; diploids generated by mating the cleavage-resistant strains with the control haploid strain (Fig. 3A, lanes 5 and 6) retained a mixture of cleavable and resistant rDNA.

Cleavage-resistant isolates were also obtained with pGAL-Ippo. Although this plasmid yielded the same number of transformed yeast cells on medium containing glucose, when the transformed cells were plated on galactose, approximately 100-fold fewer colonies were observed than in controls. Several colonies that grew on galactose were analyzed by Southern blotting after digestion of DNA with *Afl*II as described above. (In this case, the cells were not first cured of the plasmid.) Of the 12 colonies selected, 6 each from the DBY1034 and F808 strains all contained rDNA that was resistant to cleavage. Even at an input of 10 μg of total DNA, no 2.2-kb or 1.2-kb cleavage products were visible, suggesting that all of the rDNA repeats were altered (Fig. 3B).

In principle, either DNA modification or mutation could

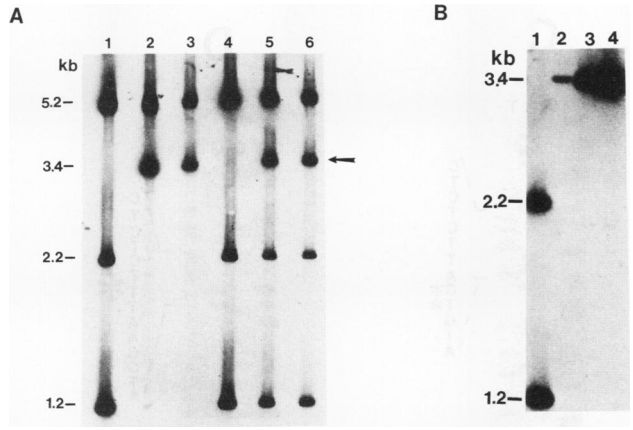

FIG. 3. Identification of cleavage-resistant yeast isolates. (A) Southern blot showing cleavage resistance in haploid and diploid yeast strains. DNA from control yeast strain DBY1034 (lane 1) and two isolates that had been transformed by pGAL-I3 (lanes 2 and 3) was digested with *Afl*II, electrophoresed in a 1% agarose gel, transferred to a blotting membrane, and hybridized with the probe indicated in Fig. 2A. The 2.2- and 1.2-kb *Afl*II fragments from the 27S rDNA of the control yeast strain (shown in Fig. 2A) are indicated. The 3.4-kb fragment, which is seen only in the cleavage-resistant isolates, is indicated by an arrow. The 5.2-kb fragment corresponds to an *Afl*II fragment elsewhere in the rDNA that hybridizes to the probe. DNAs from diploids of the parental control strain DBY1034 mated to isogenic control strain DBY947 (lane 4) and from diploids from the mating of strain DBY947 to each of the cleavage-resistant isolates (lanes 5 and 6) are also shown. (B) DNA from an isolate of DBY1034 that had been transformed with plasmid pGAL-Ippo and subsequently selected by growth in galactose was purified and subjected to the same analysis by blotting hybridization as described for panel A. Increasing amounts of DNA (2, 5, and 10 μg, lanes 2, 3, and 4, respectively) are shown. (The 5.2-kb fragment is not shown in this blot.) The 2.2- and 1.2-kb *Afl*II fragments in the control strain are indicated (lane 1), as is the 3.4-kb fragment from the cleavage-resistant isolate.

account for the cleavage resistance of these yeast rDNAs. Since DNA methylation has not been detected in *S. cerevisiae*, we assumed that the rDNA had acquired mutations. Such rapid acquisition of a mutation in all copies of the highly repeated rRNA genes has not been described previously. To test directly for rDNA mutations, we cloned and sequenced the region near the I-*Ppo* cleavage site in several isolates carrying cleavage-resistant rDNA. A short fragment was first amplified by PCR, cloned into an *E. coli* plasmid, and sequenced. The results of these analyses showed the rDNA to be mutated in one of three possible ways (Fig. 4). A representative sequencing ladder for each class of mutation is shown in Fig. 4A through D, and the mutations are summarized below (Fig. 4E). The first class (Fig. 4B) contains a single transition. This mutation was found in two independent isolates of DBY1034 and one of F808. The second class of mutation, which was found in one isolate of strain F808, contains both a transition and a transversion in adjoining base pairs (Fig. 4C). These first two classes were derived from cells transformed by the plasmid pGAL-I3. The third class of mutation (Fig. 4D) was found only when cultures were transformed with pGAL-Ippo and then selected on galactose. This mutation, an insertion, was recovered in all 12 independent isolates obtained with this plasmid (6 each for the two yeast strains).

In some cases, sequencing was also done directly on the PCR product. In no case was a mixture of mutations observed. Thus, each isolate appears to have a single class of mutation in all of its rDNA.

The recognition sequences for intron-encoded endonucleases are about 18 bp long (5, 11, 12, 33, 56), and indeed, the size of the minimal sequence recognized by I-*Ppo* is about this long (20). Consequently, one might expect that any of a large number of mutations could prevent cleavage. Thus, the redundancy of the rDNA mutations actually obtained may seem surprising. However, several factors may impose restrictions on the kinds of mutations recovered. First, because the cleavage site is situated in a stretch of DNA that is completely conserved in all eucaryotes, many of the possible changes are likely to be incompatible with ribosome function. Second, although intron-encoded endonucleases recognize large sequences, unlike restriction endonucleases, they tolerate some alterations in their cognate sequences, at least in those cases that have been studied carefully (12, 32, 57). It may be that the cleavage-resistant rDNA mutations recovered, which cluster at or adjacent to the scission sites on the two DNA strands, are the mutations that are most resistant to cleavage by I-*Ppo* in vivo. Finally, the location of the observed mutations very near the ends of the broken DNA may reflect the mechanism by which they were induced. The DNA ends could be most prone to mutation because of repeated cycles of exonucleolytic degradation followed by repair, or perhaps because repair of the last few nucleotides at a double-strand break is intrinsically more error prone than repair of a sequence distant from an end.

It is unexpected that the different I-*Ppo*-encoding plasmids should lead consistently to different rDNA mutations. We speculate that this result reflects the levels of endonuclease generated under the different conditions. Cells transformed by pGAL-I3 were selected in the presence of glucose, leading to repression of the *GAL* promoter. In this case, I-*Ppo* was presumably synthesized from an mRNA initiated from a cryptic promoter between the *GAL* promoter and the open reading frame. Such an mRNA would likely be rare. By contrast, transformants obtained with pGAL-Ippo were plated first on glucose and then selected on galactose. The resulting transcript from the induced *GAL* promoter would be abundant, presumably leading to high levels of I-*Ppo*. It is plausible that insertion of a base pair confers a higher level of protection against cleavage by the endonuclease than do the other base changes, which may not be sufficient to completely block cleavage at high enzyme levels.

Some transformants have acquired Pp LSU3 in all copies of the rDNA. The introduction of point mutations into rDNA is not the only mechanism by which cleavage resistance was obtained. In four cleavage-resistant isolates (out of 20 obtained from experiments described in the previous sections), blotting analysis of *Afl*II-cleaved DNA revealed the presence of a fragment that was not only resistant to cleavage but also approximately 1 kb larger than the previously observed 3.4-kb cleavage-resistant fragment (Fig. 5A, compare lane 1 with lanes 3 to 7). When the same filter was stripped and subsequently rehybridized with a probe derived from Pp LSU3, only the larger, cleavage-resistant fragment was visible on the autoradiograph (Fig. 5B, lanes 3 to 7, arrow). This result provides strong evidence that Pp LSU3 was inserted into the yeast rDNA, apparently in all of the repeats.

To provide direct evidence for insertion of the intron into yeast rDNA, an *Eco*RI fragment containing the insertion site for Pp LSU3 was cloned from each of the isolates, and the 5′

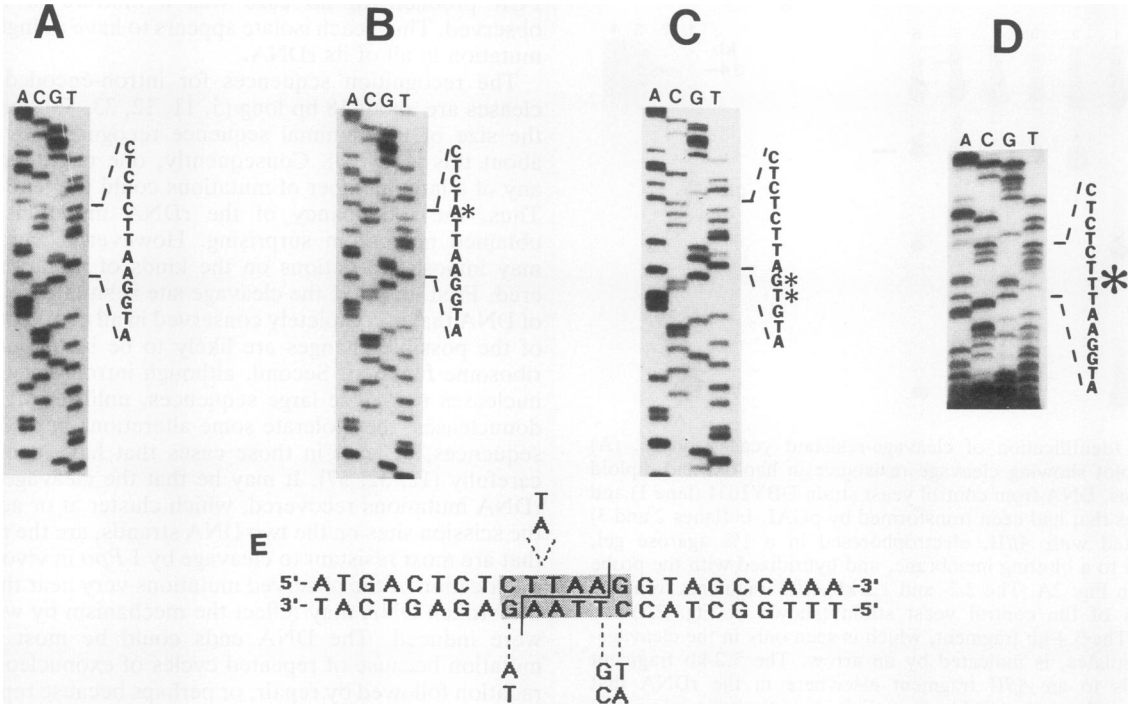

FIG. 4. Identification of point mutations in yeast rDNA. (A to D) Sequencing ladders of the region surrounding the I-*Ppo* cleavage site. Sequences for the wild-type control strain (A) and three representative cleavage-resistant mutants (B, C, and D) are shown. Asterisks indicate mutated bases for each isolate. Nucleotides corresponding to each sequencing track are indicated at the top of the autoradiographs. (E) Summary of mutations. The 4-bp 3′ overhang resulting from cleavage by I-*Ppo* is indicated by the solid line. The 6-bp recognition sequence for *Afl*II is shaded.

exon-intron junction was sequenced by means of a primer within the 5′ region of the intron. A comparison of the sequence of the intron-exon junction in yeast cells and in *P. polycephalum* is shown in Fig. 5C. Pp LSU3 is inserted at exactly the same location in yeast cells as in *P. polycephalum*. The first 32 bp of rDNA immediately upstream of the intron are identical in *P. polycephalum* and *S. cerevisiae*, and hence the provenance of this DNA cannot be determined. Farther upstream, some divergence in nucleotide sequence occurs, including the absence in yeast cells of the exon-encoded AUG at the beginning of the long open reading frame for I-*Ppo* in *P. polycephalum*. In each of the Pp LSU3-containing yeast isolates analyzed, this upstream sequence was found to be identical to the published sequence of yeast rDNA (21). Homing of group I introns results in the frequent coconversion of sequences flanking the introns (18, 23, 58). In the case of Pp LSU3 in *P. polycephalum*, the coconversion tract extends to several hundred base pairs beyond the exon-intron junctions (34). By contrast, the gene conversion event that led to insertion of Pp LSU3 into yeast rDNA apparently led to conversion of at most 32 bp at the upstream exon-intron border. We have not analyzed the downstream intron-exon border of Pp LSU3 in *S. cerevisiae*. The construct used in all of our studies carries only 24 bp of exon sequence at this location. Thus, this short stretch of DNA, which is identical in *S. cerevisiae* and *P. polycephalum*, provides sufficient homology at the downstream end of the intron to allow gene conversion.

To address whether functional I-*Ppo* is expressed in the yeast cells carrying Pp LSU3 in their rDNA repeats, crude extracts were assayed for the specific endonuclease activity. First the intron-carrying (I^+) cells were cured of the plasmid by selection on 5-fluoroorotic acid. Plasmid loss was confirmed by the inability of the yeast cells to grow on medium lacking uracil and by the lack of plasmid sequences detectable by blotting analysis. Crude extracts from cultures of several cured strains were tested for their ability to cleave an end-labeled DNA fragment containing the I-*Ppo* recognition sequence. In parallel, to provide a positive control for synthesis of the endonuclease in yeast cells, galactose induction of cells carrying pGAL-Ippo was carried out. The results of these experiments are shown in Fig. 6A. Partially purified I-*Ppo* from yeast cells cleaved the 476-bp fragment into a 370-bp fragment (arrow) plus a smaller fragment (not shown). As expected, *Afl*II gave rise to the same product (Fig. 6A, lane 2). In the induction experiments, little or no cleavage activity was apparent in extracts of cells grown on glucose (Fig. 6A, lane 3). Activity became apparent in cells grown in a nonrepressing medium containing glycerol plus ethanol as the carbon source (Fig. 6A, lane 4) and increased after addition of galactose (Fig. 6A, lanes 5 to 7). No cleavage activity was detected in an untransformed, I^- control culture (Fig. 6A, lane 8). However, two independent I^+ isolates showed activity (Fig. 6A, lanes 9 and 10). Thus, I-*Ppo* is expressed from the copies of Pp LSU3 in yeast rDNA.

Does chromosome-to-chromosome homing of Pp LSU3 occur in yeast cells? Intron homing is defined as the copying of an intron from its site of residence into a homologous site that lacks the intron. Normally this process occurs after mating of I^+ with I^- cells and consequent fusion of the parental mitochrondria, chloroplasts, or nuclei. The experiments described above show that Pp LSU3 can home from a plasmid containing *Physarum* rDNA sequences to the rDNA genes on chromosome XII of *S. cerevisiae*. Can Pp LSU3

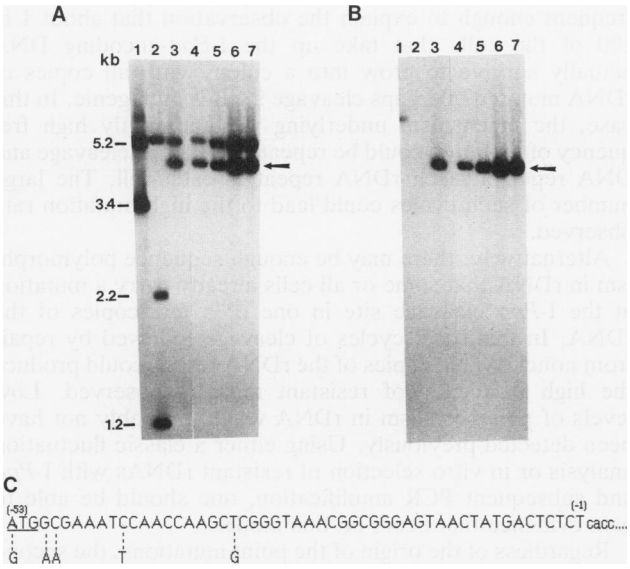

FIG. 5. Insertion of intron 3 into yeast rDNA. (A) Identification of larger, cleavage-resistant fragments in some isolates. DNA from a cleavage-resistant mutant (lane 1), from control strain DBY1034 (lane 2), and from five isolates of DBY1034 that were transformed with pGAL-I3 (lanes 3 to 7) are shown. DNA was purified, digested with *Afl*II, electrophoresed in a 1% agarose gel, transferred to a blotting membrane, and subjected to hybridization analysis. The probe used was the same yeast rDNA fragment used for the previous blots. The arrow indicates the cleavage-resistant fragment in the five new isolates (lanes 3 to 7). (B) Same filter as shown in panel A, stripped and reprobed with a fragment containing only intron 3 sequences. (C) Upstream rDNA exon sequence and the intron-exon junction from the intron-containing yeast strains shown above compared with that from *P. polycephalum* rDNA. The yeast sequence was obtained by using a primer from within intron 3. The sequence shown is that of *P. polycephalum*, which was determined previously (33, 35). Exon sequences are indicated by uppercase letters, and intron sequences are in lowercase letters. The exon-encoded methionine present only in the *Physarum* sequence is underlined. Nucleotides in *S. cerevisiae* that differ from those of *P. polycephalum* are shown below the *Physarum* sequence.

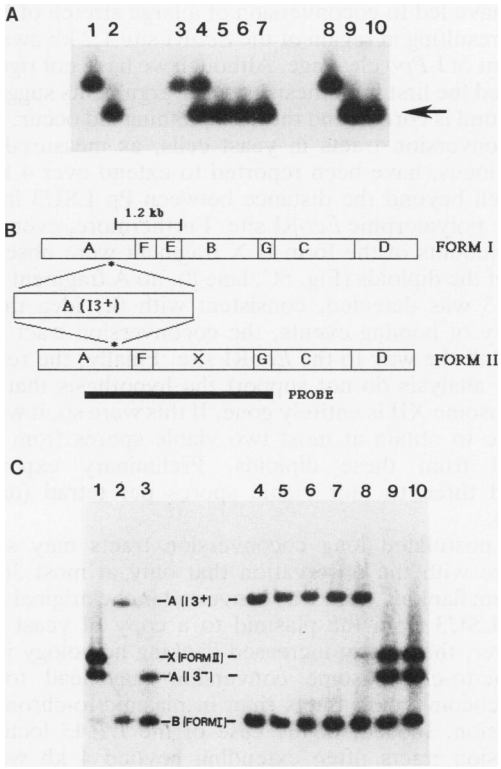

FIG. 6. Production of functional I-*Ppo* and possible homing of intron 3 in diploids. (A) Cleavage activity in yeast extracts. A 476-bp fragment from a clone of *Physarum* rDNA that contains the cleavage site for I-*Ppo* was end labeled and subjected to cleavage in vitro with extracts from *S. cerevisiae* as described in the text. After 30 min of incubation at 37°C, the reaction mixes were extracted with phenol, and the DNA was precipitated with ethanol and electrophoresed in a 1.5% agarose gel. The autoradiograph shows the uncleaved fragment (lane 1) and the same fragment cleaved with *Afl*II (lane 2). Activity in extracts derived from strain F808 that had been transformed with pGAL-Ippo and subjected to galactose induction is shown in lanes 3 to 7. The samples are as follows: glucose control (lane 3), glycerol plus ethanol (lane 4), and 1, 2, and 3 h following the addition of galactose (lanes 5, 6, and 7, respectively). Cleavage activity in extracts from strain DBY1034 before transformation (lane 8) and from two isolates that have acquired intron 3 in the rDNA (as determined by blotting and sequence analysis, described in the previous figure legend) and that were cured of the intron-containing plasmid as described in the text is shown in lanes 9 and 10. The arrow indicates the cleavage product of 370 bp. (B) *Eco*RI fragments from form I and form II rDNA. Intron 3, if present, is inserted into fragment A [A ($I3^+$)]. Fragments B and X are polymorphic in size between the two rDNA forms. Approximately 1.2 kb of sequence separates the site of I-*Ppo* cleavage from the polymorphic *Eco*RI site. (C) DNA from haploids or diploids derived from the matings described in the text was purified, digested with *Eco*RI, subjected to electrophoresis in a 1% agarose gel, transferred to a blotting membrane, and hybridized with the rDNA probe indicated in panel B. DNAs from haploids containing form II ($I3^-$) rDNA (strain 2262, lane 1), form I ($I3^+$) rDNA (lane 2), and form I ($I3^-$) rDNA (strain DBY1034, lane 3) are shown. (Note that form II rDNA contains approximately 10% form I B fragment). DNAs from diploids (lanes 9 and 10) generated from two control matings between strains 2262 (lane 1) and DBY1034 (lane 3) show all three expected *Eco*RI fragments. Five independent matings of strain 2262 with an $I3^+$ strain (lanes 4 to 8) show almost exclusively the B fragment and the A ($I3^+$) fragment from the form I strain.

homing also occur from one yeast chromosome XII to another? In an attempt to answer this question, we carried out matings between an I^- strain and I^+ strains that had been cured of the plasmid and then analyzed the DNA from the resulting diploids for the presence of the intron. In order to allow identification of both parental rDNA types in the diploid, strain 2262 was used as the I^- parent. This strain bears an *Eco*RI restriction site polymorphism in its rDNA, which leads to the fusion of fragments B and E to yield fragment X (Fig. 6B). The rDNA with the *Eco*RI site is referred to as form I, and the rDNA lacking that site is referred to as form II (41, 42). Control matings of form I (I^-) strains with the form II (I^-) strain resulted in diploids displaying all three expected fragments, A, B, and X (Fig. 6C, lanes 9 and 10; compare lanes 1 and 3). By contrast, diploids from several independent matings of the form I (I^+) strain (Fig. 6C, lane 2) with the form II (I^-) strain showed almost exclusively the A fragment containing Pp LSU3 plus the B fragment (Fig. 6C, lanes 4 to 8).

We considered two possible hypotheses to explain the absence of the form II X fragment. First, I-*Ppo*-mediated cleavages of rDNA on chromosome XII could have led to the destruction of the I^- version of this chromosome.

Second, gene conversion accompanying homing of Pp LSU3 could have led to coconversion of a large stretch of flanking DNA, resulting in regain of the *Eco*RI site 1.2 kb away from the point of I-*Ppo* cleavage. Although we have not rigorously excluded the first hypothesis, several arguments suggest that the second is correct and thus that homing did occur. Mitotic gene conversion tracts in yeast cells, as measured at the *URA3* locus, have been reported to extend over 4 kb (25), i.e., well beyond the distance between Pp LSU3 insertion and the polymorphic *Eco*RI site. Furthermore, even though trace amounts of the form II X fragment were observed in some of the diploids (Fig. 6C, lane 8), no A fragment lacking intron 3 was detected, consistent with the idea that in a minority of homing events, the coconversion tract did not extend all the way to the *Eco*RI site. Finally, the results of genetic analysis do not support the hypothesis that the I^- chromosome XII is entirely gone. If this were so, it would be possible to obtain at most two viable spores from tetrads derived from these diploids. Preliminary experiments showed three to four viable spores per tetrad (data not shown).

The postulated long coconversion tracts may seem at variance with the observation that only at most 36 bp of upstream flanking exon was converted in the original homing of Pp LSU3 from the plasmid to a copy of yeast rDNA. However, the greatly increased flanking homology in chromosome-to-chromosome conversion may lead to much larger coconversion tracts than in plasmid-to-chromosome conversion. Indeed, in the case of the *URA3* locus, long conversion tracts often extending beyond 4 kb were observed in diploid yeast cells (25). In sum, we interpret the results of these experiments to indicate that homing of this foreign intron proceeds rapidly and efficiently in the nuclei of yeast cells after mating, in a manner similar to that of other homing introns.

DISCUSSION

We have shown that the expression of I-*Ppo*, the site-specific endonuclease encoded by intron Pp LSU3 from *P. polycephalum*, is lethal in *S. cerevisiae*, an effect that can be attributed to cleavage of the rDNA on chromosome XII. Colonies that are resistant to this lethal effect were isolated at surprisingly high frequency. The resistance was shown to result from disruption of the cleavage site, either by point mutations or by homing (i.e., insertion) of the intron. In both cases, apparently all copies of the rDNA repeats were affected. The relative frequency of point mutations observed was approximately fivefold higher than that of intron insertions. This frequency is likely to vary depending on the extent of rDNA sequence homology in the donor plasmid. In our experiments, fewer than 60 bp surrounding the intron insertion site were identical in yeast rDNA on one hand and in the donor plasmid that carried Pp LSU3 and surrounding *Physarum* rDNA on the other. Since the frequency of homologous recombination in rDNA and in other genes is proportional to the extent of sequence homology (1, 50) and since 60 bp of homologous DNA is near the lower size limit for recombination in yeast cells (52), the frequency of intron homing could probably be increased by using a plasmid engineered to contain much larger regions of identical flanking sequences.

Point mutations in rDNA. The generation of point mutations in all copies of the yeast rDNA is likely to be a two-step process. The first step is the formation of a point mutation in a single rRNA gene. How could this mutation event be frequent enough to explain the observation that about 1 in 100 of the cells that take up the I-*Ppo*-encoding DNA actually survive to grow into a colony with all copies of rDNA mutated? Perhaps cleavage itself is mutagenic. In this case, the mechanism underlying the apparently high frequency of mutation could be repeated cycles of cleavage and DNA repair at each rDNA repeat in each cell. The large number of such cycles could lead to the high mutation rate observed.

Alternatively, there may be enough sequence polymorphism in rDNA that some or all cells already carry a mutation at the I-*Ppo* cleavage site in one or a few copies of the rDNA. In this case, cycles of cleavage followed by repair from noncleavable copies of the rDNA repeat could produce the high frequency of resistant mutants observed. Low levels of polymorphism in rDNA would probably not have been detected previously. Using either a classic fluctuation analysis or in vitro selection of resistant rDNAs with I-*Ppo* and subsequent PCR amplification, one should be able to gain evidence that bears on this model.

Regardless of the origin of the point mutations, the second step in the generation of the observed mutant rDNA is likely to be the rapid and efficient spread of the mutation to the entire population of rDNA repeats. This process must be fast enough so that the rDNA breaks on chromosome XII are healed by the first mitotic division, to prevent the lethal loss of the distal arm of the chromosome. The mechanism responsible for spreading the original point mutation to all rDNA copies is presumably the same as that used by homing introns, i.e., gene conversion initiated at the double-strand break. The gene conversion events would be driven in the direction of fixation of the mutation, because wild-type rDNA keeps being cut by the endonuclease. Each time that a mutant copy of rDNA is used by chance as a donor to repair the break, the mutation would become fixed into a new copy. The kinetics of this process are predicted to be complicated, rising rapidly in the early stages. Careful kinetic analyses of intron homing under natural conditions have not been reported; published data are not easy to interpret because of the lack of synchrony of the mating process and thus always give an overestimate of the time required for homing to be complete. In the case of Pp LSU3 in its natural environment, where mating is not synchronous, we estimate that homing occurs in less than 24 h (34). Some of the yeast strains we have derived, which allow rapid induction of I-*Ppo* expression by galactose induction, should facilitate a dissection of the kinetics by which point mutations are spread.

Although gene conversion occurs in diverse genetic environments, special processes might be involved in the spread of the observed rDNA point mutations as well as in intron homing itself. Recombination in the cluster of rDNA repeats is known to differ from that in other parts of chromosomes in several ways, in part perhaps because of the evolutionary pressure for maintenance of sequence homogeneity of the rDNA genes. Although meiotic recombination in rDNA is greatly reduced compared with that in the rest of the genome (41, 43), unequal sister chromatid exchange occurs at a high frequency, as evidenced by the duplication or loss of a *LEU2* marker inserted into the rDNA (42, 54). A transcription-dependent recombination hot spot, *HOT1*, has been identified in the rDNA promoter (26, 55). Preferential amplification of sequences that have been inserted into or adjacent to rDNA has also been reported (40). Finally, it has been reported that under appropriate selective pressure, a plasmid inserted into rDNA can become amplified greater than

100-fold, whereas the same plasmid inserted elsewhere in the genome does not become amplified (29, 30). It is not known whether the mechanisms underlying any of these phenomena contribute to the generation or spread of the rDNA mutations that we have observed.

Other well-studied processes in *S. cerevisiae* could also shed light on the mechanisms underlying the spread of I-*Ppo* cleavage site mutations to all copies of the rDNA. Double-strand breaks have long been known to be highly recombinogenic, as evidenced by the stimulation of mitotic recombination in cells exposed to X rays (45–47) and by the enhanced frequency of transformation of cells by linear DNA fragments (36–38, 53). Mating type switching is initiated by a double-strand break introduced at the *MAT* locus by HO endonuclease (51). After induction of HO endonuclease, site-specific recombination is also observed in experiments in which double-strand breaks are introduced at other chromosomal locations, including rDNA (39), or into plasmids into which the HO cleavage site has been inserted artificially (48). More recently, similar results have been obtained with a yeast mitochondrial intron-encoded endonuclease, I-*SceI* (44). In all of these examples, recombinational repair of the double-strand break is dependent on the *RAD50* and *RAD52* gene products. However, a *RAD52*-independent repair pathway, which appears to be unique to rDNA and to other repeated genes, has been identified recently (39). This pathway is believed to involve strand recision and reannealing, resulting in the loss of one or more rDNA repeats. Elucidating the pathway by which rDNA cleavage site mutations are generated after I-*Ppo* expression will require a combination of genetic and biochemical analyses.

Insertion of intron 3 into rDNA. With the exception of the presumed transfer in evolution of a small remnant of a mitochondrial group I intron into a telomeric region (31), no group I introns have been identified in nuclear yeast genes, nor, to our knowledge, has artificial transfer of a group I intron from one species to another been reported. Several conclusions can be drawn from the existence and properties of these cells. First, splicing must proceed normally in order for rRNA to be formed properly. Although numerous group I introns self-splice in vitro, as does Pp LSU3 (49), accessory proteins are believed to facilitate splicing in vivo for many introns (2, 6, 8, 15, 22). With few exceptions (for example, the tRNA synthetase in *Neurospora crassa* mitochondria [2]), little is known about how such proteins function. Apparently, splicing of Pp LSU3 in vivo does not require such ancillary proteins, or else these putative proteins are conserved in function between *P. polycephalum* and *S. cerevisiae*. An alternative possibility that cannot be excluded is that I-*Ppo* itself promotes splicing, as does a mutated form of the I-*SceII* endonuclease encoded in the fourth intron in the yeast mitochondrial *coxI* gene (14). It should be possible to introduce genetically altered forms of Pp LSU3 into yeast rDNA to test some of these ideas.

We showed originally, by translation in vitro and in *E. coli*, that both the longer and shorter forms of I-*Ppo* can be made and are catalytically active (33). The longer form is initiated in vitro from an AUG codon 53 nucleotides upstream into the exon, relative to the 5′ end of the intron. In vitro self-splicing analyses demonstrated a minor 5′ splice site (G addition site) at nucleotide −63, i.e., just upstream of this AUG (49), consistent with the idea that a minor spliced RNA capable of encoding the long form of I-*Ppo* might be formed in vivo. *S. cerevisiae* does not have an AUG at nucleotide −53, and thus presumably the long form of I-*Ppo* could not be formed in yeast cells. Thus, a second conclusion from the properties of the yeast clones that we have derived is that the longer form of I-*Ppo* is not required for the biological activity of either the endonuclease or the intron.

The natural transcription by RNA polymerase I of a protein-encoding gene embedded in rDNA is unprecedented. Thus, it seemed possible that *P. polycephalum* might have evolved a special mechanism to allow the I-*Ppo* endonuclease to be expressed from pre-rRNA. The observation that yeast cells carrying Pp LSU3 in their rDNA also synthesize catalytically active I-*Ppo*, as measured by the presence of this activity in crude extracts, argues against this possibility. Hence, a third conclusion is that the machinery to generate an mRNA from the pre-rRNA is not restricted to the species in which the intron was found. However, the experiments presented here do not exclude the formal possibility that a copy of the I-*Ppo* gene inserted elsewhere in the genome is responsible for the enzymatic activity observed. From analysis of self-splicing products self-splicing in vitro, we have proposed that the I-*Ppo* messenger consists of the proximal half of the excised intron RNA (49). The 3′ end of this RNA has an unusual secondary structure that we hypothesize functions in stabilization or transport of this RNA. This hypothesis should be testable directly by creation of yeast strains carrying appropriate mutations in Pp LSU3.

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