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Expression of the *Naegleria* intron endonuclease is dependent on a functional group I self-cleaving ribozyme

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ABSTRACT

NaSSU1 is a complex nuclear group I intron found in several species of *Naegleria*, consisting of a large self-splicing group I ribozyme (NaGIR2), which itself is interrupted by a small, group I-like ribozyme (NaGIR1) and an open reading frame (ORF) coding for a homing endonuclease. The GIR1 ribozyme cleaves in vitro transcripts of NaSSU1 at two internal processing sites about 400 nt downstream of the 5′ end of the intron, proximal to the endonuclease ORF. Here we demonstrate that self-cleavage of the excised intron also occurs in vivo in *Naegleria gruberi*, generating an ORF-containing RNA that possesses a short leader with a sequence element likely to be involved in gene expression. To assess the functional significance of self-cleavage, we constructed a genetic system in *Saccharomyces cerevisiae*. First, a mutant yeast strain was selected with a mutation in all the rRNA genes, rendering the rDNA resistant to cleavage by the *Naegleria* endonuclease. Active endonuclease, which is otherwise lethal, could be expressed readily in these cells. Endonuclease activity also could be detected in extracts of yeast harboring plasmids in which the endonuclease ORF was embedded in its native context in the intron. Analysis of the RNA from these yeast cells showed that the excised intron RNA was processed as in *N. gruberi*. A mutant intron constructed to prevent self-cleavage of the RNA failed to express endonuclease activity. These results support the hypothesis that the NaGIR1-catalyzed self-cleavage of the intron RNA is a key event in expression of the endonuclease.

Keywords: group I intron; homing endonuclease; ribosomal DNA

INTRODUCTION

Group I introns are phylogenetically widespread autocatalytic RNA elements sharing a common secondary and tertiary structure (Cech et al., 1994). Many group I introns are capable of self-splicing in vitro through a series of transesterification reactions (Cech, 1990), although proteins may aid splicing in vivo (Lambowitz & Perlman, 1990; Shaw & Lewin, 1997). About one third of group I introns harbor open reading frames (ORFs) coding for either structural proteins, maturases, or DNA endonucleases (Johansen et al., 1997a). The DNA endonucleases, which are the most common intron-encoded proteins, are usually involved in intron mobility at the DNA level (reviewed in Belfort & Roberts, 1997). The endonuclease creates a double-strand break at the intron-lacking allele, which is subsequently repaired in a gene-conversion event, using the intron-containing allele as a template. The resulting unidirectional transfer of intron sequences is termed intron homing because of its high specificity at a homologous site.

Only 5% of the ~300 reported nuclear rDNA group I introns contain ORFs, and among these are the optional PpLSU3 and DiSSU1 of the myxomycetes *Physarum polycephalum* and *Didymium iridis*, respectively (Muscarella & Vogt, 1989; Johansen & Vogt, 1994), and NaSSU1 in a few species of the free-living amoeba-flagellate *Naegleria* (De Jonckheere, 1994). These introns interrupt rRNA genes on extrachromosomal rDNA molecules (see Einvik et al., 1998a), and encode the functional site-specific His-Cys box homing endonucleases named I-*Ppo*I, I-*Dir*I, and I-*Nja*I, respectively (Muscarella et al., 1990; Johansen et al., 1997b; Elde et al., 1999). I-*Ppo*I and I-*Dir*I are involved in homing in their natural hosts after mating (Muscarella & Vogt, 1989; Johansen et al., 1997b).

NaSSU1 and DiSSU1 constitute a unique class of group I introns referred to as twin-ribozyme group I introns (Decatur et al., 1995; Einvik et al., 1997, 1998a).

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In these introns, the endonuclease ORF is found downstream of a small group I self-cleaving ribozyme (GIR1) that catalyzes hydrolytic cleavage of the RNA just upstream of the endonuclease ORF (see Fig. 1). These two elements are both embedded in a loop of a more regular group I self-splicing ribozyme (GIR2). Strikingly, some species of *Naegleria* contain a shorter version of NaSSU1 that lacks both the ORF and GIR1 (De Jonckheere & Brown, 1994; Einvik et al., 1997), suggesting that these two elements form a functional genetic unit, acquired or lost at the same time in evolution. Despite their small size of ~180 nt, the GIR1 elements maintain the global structural features of the group I ribozymes (Jabri et al., 1997; Einvik et al., 1998b).

The exact mechanism by which proteins encoded by group I introns embedded in eukaryotic rRNA genes are expressed has not been elucidated. In the case of the twin-ribozyme intron DiSSU1 in its natural host *D. iridis*, maturation of the endonuclease mRNA was found to include internal processing by the GIR1 ribozyme, as well as removal of a spliceosomal intron and polyadenylation (Vader et al., 1999). Perhaps the best-studied intron of this type to date is PpLSU3, artificially inserted into all the rDNA repeats of yeast (Lin & Vogt, 1998; 2000). In this system, it was inferred that the intact excised intron RNA, and not an RNA species that is processed further, is the source of the message for the I-*Ppo*I endonuclease, whose coding region starts 14 nt downstream of the 5′ splice site (SS). By contrast, in the twin-ribozyme group I introns, the protein-encoding region is not positioned close to the 5′ SS, and numerous AUG codons occur between the 5′ end of the RNA and the start of the ORF. According to the scanning mechanism of eukaryotic translation initiation, upstream AUGs should greatly attenuate or eliminate expression of the ORF (Sachs et al., 1997). Certain viral and a few nuclear messengers have evolved complex elements called internal ribosome entry sites to recruit the ribosome (Belsham & Sonenberg, 1996; Sachs et al., 1997), and hence *a priori* it is conceivable that nuclear group I introns use such a mechanism for translation as well.

We noted earlier in the course of in vitro splicing studies that ribozyme-mediated hydrolysis cleaves the intron RNA provocatively just upstream of the ORF in both the *Naegleria* and *Didymium* introns. This observation led to the hypothesis that GIR1-catalyzed cleavage plays a role in expression of the endonuclease

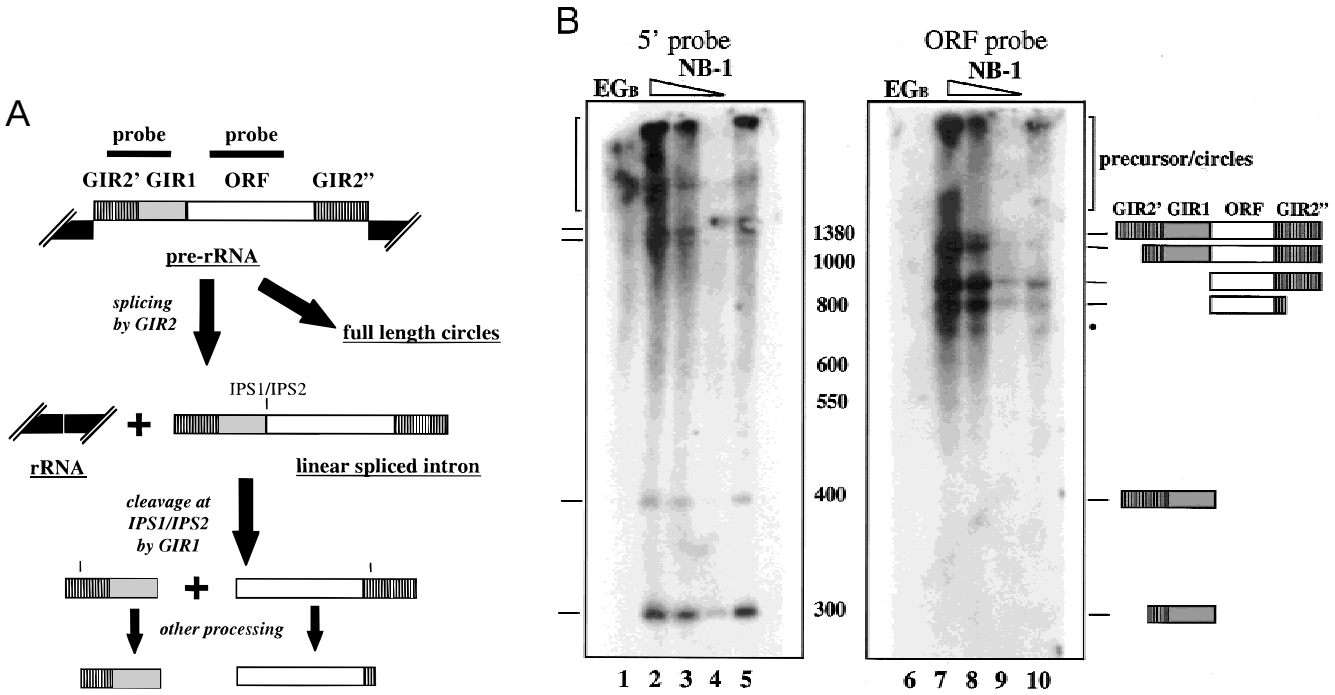

FIGURE 1. *N. gruberi* NaSSU1 organization and processing. **A**: Summary of the processing events of the NaSSU1 intron in *Naegleria*. The PCR products used to generate probes for the Northern blot are indicated as bars above the intron. The figure is drawn to emphasize processing in close proximity to the 5′ end of the ORF, and is not meant necessarily to signify temporal order. **B**: Northern blot analysis. Probes for the intron region upstream of IPS1/IPS2 (left panel) or for the ORF (right panel) were used to detect RNA species in *Naegleria*. Lanes 1 and 6: 9 μg of total RNA from control strain *N. gruberi* EG_B; lanes 2, 3, 4 and 7, 8, 9: 18 μg, 12 μg, and 3 μg, respectively, of total RNA from strain *N. gruberi* NB-1 RNA; lanes 5 and 10: 12 μg total RNA from a different *N. gruberi* NB-1 RNA preparation. Assignments for the spliced/processed intron RNAs are indicated on the right with schematic drawings, and the approximate migration of size markers is shown in the middle of the figure. A minor, uncharacterized RNA that contains the ORF sequence, yet migrates below the size of the entire ORF (738 nt) is indicated by a dot.

(Decatur et al., 1995; Einvik et al., 1997). In the present work we examined the pattern of rRNA processing of the NaSSU1 intron in its natural host *Naegleria.* Several processing events were detected, and among these was cleavage just upstream of the ORF, corresponding exactly to the self-cleavage event characterized in vitro. Additionally, we generated a mutant strain of *Saccharomyces cerevisiae* that enabled expression of the endonuclease ORF. Analysis of the *Naegleria* intron artificially introduced into these yeast cells showed that GIR1-catalyzed processing of the intron RNA was required for the synthesis of endonuclease. We conclude that the biological function of NaGIR1 is to create a 5′ end near the beginning of the ORF, thus promoting ribosome attachment and initiation.

RESULTS

Processing of the *Naegleria gruberi* intron in vivo

We have shown previously that the large intron inserted in the gene for the small subunit ribosomal RNA of certain *Naegleria* species (e.g., *N. gruberi* strain NB-1) consists of two group I ribozymes (Einvik et al., 1997). Specifically, the larger self-splicing ribozyme (NaGIR2) is formed by interactions between 5′ and 3′ segments of the intron. This arrangement places the small ribozyme NaGIR1 and the endonuclease ORF in the P6 loop of NaGIR2. The translation-initiation codon for the *N. gruberi* endonuclease (I-*Ngr*I) is over 400 nt downstream from the 5′ SS with six upstream AUG codons preceding the initiation codon. In vitro transcripts of the intron RNA undergo self-cleavage mediated by GIR1, thereby eliminating most of the RNA segment upstream of the start codon for I-*Ngr*I. Two closely spaced hydrolysis sites (internal processing sites IPS1 and IPS2) have been identified a few nucleotides upstream of the start codon (Einvik et al., 1997, 1998b). We reasoned that if this processing occurs in vivo, self-cleavage could influence expression of the homing endonuclease.

To examine whether NaGIR1 catalyzes internal processing of NaSSU1 RNA in its natural host organism, we performed Northern blot analysis on total RNA from NB-1 (intron +) and EG_B (intron −) amoebae. An exon probe as well as simple observation of ethidium-bromide-stained RNA confirmed that NaSSU1 is efficiently spliced from the preribosomal RNA to produce an abundance of NB-1 SSU rRNA equal in size to the 2,019-nt EG_B SSU rRNA (data not shown). Probes covering different portions of the 1.3-kb intron (shown schematically in Fig. 1A, top) yielded distinct patterns of bands on the blots (Fig. 1B). With an intron probe specific to the ORF, an abundant small RNA of ~900 nt was seen, as well as a slightly smaller and less abundant species of ~790 nt, in addition to large RNAs corresponding to the primary transcript and excised intron intermediates. With an intron probe specific to sequences upstream of the internal processing sites IPS1/IPS2, an RNA species of ~300 nt was seen, as well as a small amount of an RNA of ~400 nt, in addition to RNAs corresponding to the primary transcript and excised intron intermediates. The assignment of primary structure to these several bands is shown schematically in the margins of Figure 1. These same patterns of bands were also observed in Northern blots when RNA was extracted from *N. gruberi* NB-1 that had been induced to differentiate into flagellates by shaking washed amoebae in a nutrient-free suspension for 80 min (Fulton, 1970; data not shown).

The small RNAs visualized by the upstream probe imply that the intron RNA was further processed in the region proximal to IPS1/IPS2, and primer-extension analysis identified a 5′ end at nt 125 of the intron (data not shown). Accumulation of the 790-nt RNA seen with the probe against the ORF suggests that some of the liberated ORF-containing RNA fragments undergo an additional processing event. The Northern blot data combined with an S1 mapping assay (data not shown) are consistent with processing approximately 40 nt downstream of the ORF. Interestingly, the 790-nt *Naegleria* RNA appears analogous to the ~850-nt polysomal I-*Dir*I mRNA from the related twin-ribozyme intron DiSSU1 (Vader et al., 1999). The efficient formation of full-length intron circles has been observed in vitro with wild-type versions of the NaSSU1 intron, but not with deletion mutants lacking GIR1 and ORF (Einvik et al., 1997). To test if such RNA circles are also formed in vivo, we employed an RT-PCR approach like that designed previously (Einvik et al., 1997) to detect circle junctions. Direct sequencing of the products showed that NaSSU1 intron in *N. gruberi* circularized in vivo to form full-length intron circles (data not shown). Figure 1A summarizes our data on NaSSU1 splicing and subsequent processing of the excised intron.

We used high-resolution mapping techniques to determine the exact sites of processing immediately upstream of the ORF. Primer extension showed a band corresponding to a 5′ end 10 nt upstream from the start codon of the ORF (Fig. 2A). This is the same site previously identified for RNA processed in vitro, and termed IPS2 (Einvik et al., 1997). The same band was seen in RNA preparations from both *N. gruberi* amoebae and flagellates (data not shown). To map the 3′ end of the complementary fragment, that is the upstream intron RNA, an S1 nuclease-protection assay was used. The probe consisted of a single-stranded DNA fragment complementary to RNA just proximal to the initiation codon of the ORF (Fig. 2B). The protection assay yielded two products, each present as a collection of fragments differing by 1 nt, as commonly seen in this type of

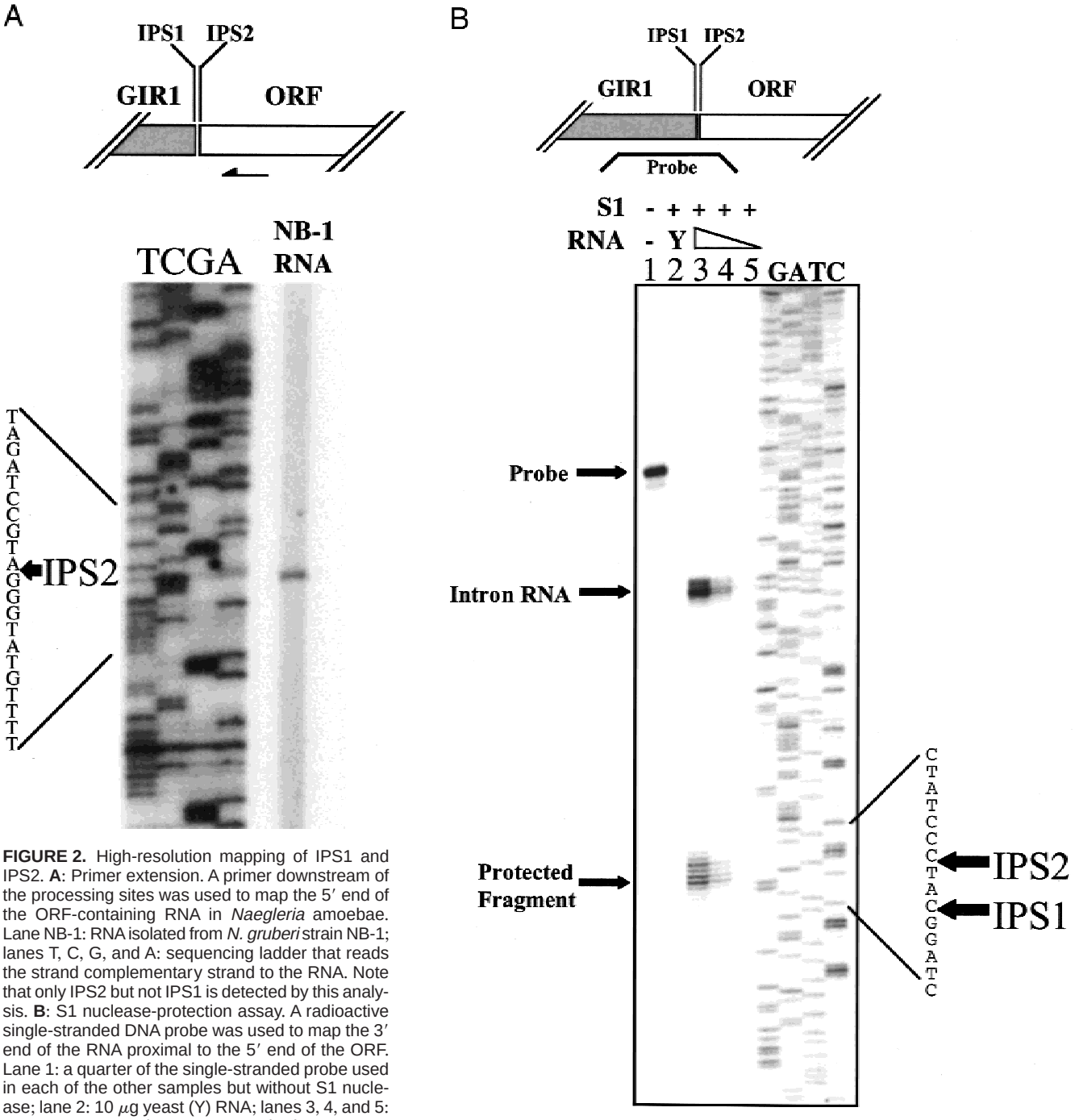

FIGURE 2. High-resolution mapping of IPS1 and IPS2. **A**: Primer extension. A primer downstream of the processing sites was used to map the 5′ end of the ORF-containing RNA in *Naegleria* amoebae. Lane NB-1: RNA isolated from *N. gruberi* strain NB-1; lanes T, C, G, and A: sequencing ladder that reads the strand complementary strand to the RNA. Note that only IPS2 but not IPS1 is detected by this analysis. **B**: S1 nuclease-protection assay. A radioactive single-stranded DNA probe was used to map the 3′ end of the RNA proximal to the 5′ end of the ORF. Lane 1: a quarter of the single-stranded probe used in each of the other samples but without S1 nuclease; lane 2: 10 μg yeast (Y) RNA; lanes 3, 4, and 5: 39 μg, 13 μg, and 1.3 μg *N. gruberi* NB-1 RNA, respectively. The full-length probe, probe fragment protected by unprocessed intron, and probe fragment protected by the 5′ product of intron processing are indicated.

assay. The larger product corresponds to unprocessed intron RNA. The smaller fragment corresponds to an RNA species with a 3′ end in the IPS region, close to or at IPS1, as the nuclease digestion does not proceed beyond this site. According to PhosphorImager quantitation of several S1 assays of RNA isolated from *N. gruberi*, at steady state in these cells, about 40% of the intron RNA is processed at IPS1 in vivo. To estimate what percentage of total cellular RNA this represents, we carried out parallel S1 protection assays with known amounts of NaSSU1 RNAs synthesized and processed in vitro. Comparisons led to the conclusion that total

intron RNA comprises about 0.2% of total *N. gruberi* RNA in the growing amoebae (data not shown).

Although processing at IPS1/IPS2 was readily observed in vivo, our attempts at detecting the product of the ORF, the *Naegleria* endonuclease, were unsuccessful. Using small, radiolabeled substrates, we were unable to find evidence of active endonuclease, either in extracts of amoebae or flagellates (data not shown). Mixing assays with partially purified, *Escherichia coli*-derived endonuclease fusion protein (see below) ruled out the possibility that crude extracts contained inhibitors of activity. Thus it appears that *N. gruberi* cells do not translate the processed intron RNA, at least not detectably under the growth conditions used in the laboratory, or that an unknown mechanism is used to suppress its activity.

Intron RNA processing in yeast

We examined intron splicing and processing in yeast to compare the fate of intron RNA in yeast and *Naegleria*, and ultimately to ascertain if processing is correlated with endonuclease expression as expected. In these experiments, RNA was examined from yeast cells carrying either of two plasmids, one with the wild-type intron and another with a mutant intron in which the GIR1 ribozyme had been crippled. pYGal-NjaSSU is a yeast 2 μm plasmid that has the wild-type NaSSU1, flanked by 365 bp of 5′ exon and 317 bp of 3′ exon, and driven by the *GAL*1 promoter (Fig. 3A). pYGal-G1$^-$Nja is identical, except for a single base pair change inactivating the critical G-binding pocket of the P7 segment of NaGIR1. This mutation was shown previously to abrogate self-cleaving activity (Einvik et al., 1997). For these experiments the yeast strain WD101 was used, which has a single base mutation in all the rDNA copies (see below). RNA was also analyzed from cells harboring pYGal-GIR2, an engineered version of NjaSSU1 that contains only the self-splicing ribozyme NaGIR2 with the same flanking exon sequences behind the *GAL1* promoter, but in the absence of the ORF and GIR1 (Einvik et al., 1997). Initial experiments employed RT-PCR to identify specific RNA species in yeast. RT-PCR with two exon primers produced a 275-bp product from RNAs transcribed from all three plasmids, with restriction digestion of the product showing that splicing had occurred accurately (Fig. 3B, lanes 2–4 and 12–14). In pYGal-GIR2 an additional 652-nt product was generated that corresponds to unspliced transcript (Fig. 3B, lane 2); equivalent bands for the other two versions of the intron presumably were not observed because of their much larger size. RT-PCR detected circles in all three cases (Fig. 3B, lanes 6–8). However, sequence analysis of the intron circles showed that in all cases the first 2 nt of intron had been lost. This result is similar to that reported for in vitro transcripts of the version of the intron containing only GIR2 (Einvik et al., 1997).

By Northern blotting with a probe to the intron ORF, the pattern of processed RNAs from pYGal-NjaSSU in yeast was similar to that for *N. gruberi* NB-1 amoebae (Fig. 3C, lanes 1 and 2 compared with lanes 3 and 4). However, a major difference was that the smallest ORF-containing RNA in yeast appeared shorter than the equivalent RNA in *Naegleria* (see below). Another difference was that the abundance of the excised, GIR1-defective intron RNA was much lower than that of the wild-type excised intron RNA (Fig. 3C, lane 4). Because mutations in the GIR1 ribozyme do not affect GIR2-catalyzed splicing in vitro (Einvik et al., 1997), the low levels of excised intron RNA seem surprising. In addition and as expected, no ORF-containing RNAs resembling the IPS1/IPS2-processed RNA were evident for the GIR1-defective intron.

To resolve the apparent discrepancy between the sizes of the processed, ORF-containing RNAs in *Naegleria* and yeast, primer-extension analysis was carried out with a primer from within the ORF. The results placed the 5′ end of the yeast-derived RNA species at positions 53 and 56 nt downstream of IPS2, that is, within the endonuclease ORF. These RNAs are unable to encode an active endonuclease. We speculate that upon GIR1-mediated cleavage at IPS1/IPS2, the ORF-containing RNA molecules become substrates for Rat1p/Xrn1p 5′ $\rightarrow$ 3′ exonucleases in yeast, which are specific for uncapped 5′ termini (Johnson, 1997).

Naegleria intron endonuclease expression in yeast with mutant rDNA

Previous work had shown that the nuclear group I intron PpLUS3 can home from a plasmid into the rDNA of *S. cerevisiae* (Muscarella & Vogt, 1993). We decided to use this tractable yeast system to study the *Naegleria* intron, and began by attempting to force homing of NaSSU1 into rDNA by "trans-integration" as described for PpLSU3 (Lin & Vogt, 1998). In our experiments with *S. cerevisiae*, we chose to use the homologous intron from *Naegleria jamiesoni* instead of that from *N. gruberi,* because the endonuclease encoded by the former (I-*Nja*I) has been better characterized (Elde et al., 1999).

NaSSU1 is inserted at position 516 (*E. coli* rRNA numbering) in a highly conserved segment of the SSU rRNA gene that is cleaved by the intron-encoded endonuclease (Elde et al., 1999). The *Naegleria* rDNA sequence surrounding this site differs from that in yeast and almost all other eukaryotes by a single C-to-G transversion 8 nt downstream from the intron insertion site, and a search of the yeast genome showed the existence of two closely related sequences at other locations (Table 1). To test if I-*Nja*I is able to recognize and cleave yeast DNA, we used yeast-genomic DNA and PCR to amplify DNA fragments containing the rDNA sequence and the two related sequences. The PCR

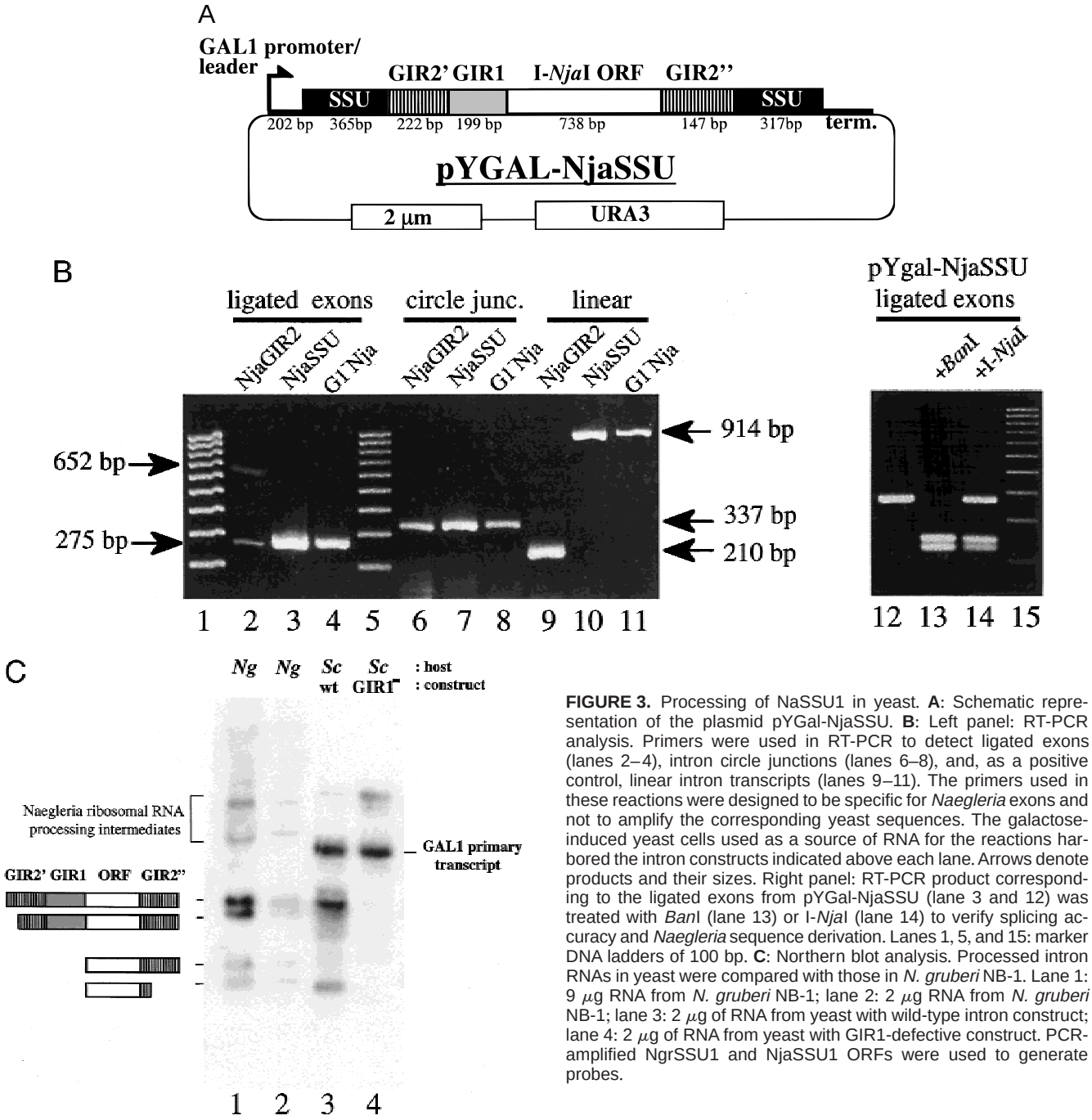

FIGURE 3. Processing of NaSSU1 in yeast. **A**: Schematic representation of the plasmid pYGal-NjaSSU. **B**: Left panel: RT-PCR analysis. Primers were used in RT-PCR to detect ligated exons (lanes 2–4), intron circle junctions (lanes 6–8), and, as a positive control, linear intron transcripts (lanes 9–11). The primers used in these reactions were designed to be specific for *Naegleria* exons and not to amplify the corresponding yeast sequences. The galactose-induced yeast cells used as a source of RNA for the reactions harbored the intron constructs indicated above each lane. Arrows denote products and their sizes. Right panel: RT-PCR product corresponding to the ligated exons from pYGal-NjaSSU (lane 3 and 12) was treated with *Ban*I (lane 13) or I-*Nja*I (lane 14) to verify splicing accuracy and *Naegleria* sequence derivation. Lanes 1, 5, and 15: marker DNA ladders of 100 bp. **C**: Northern blot analysis. Processed intron RNAs in yeast were compared with those in *N. gruberi* NB-1. Lane 1: 9 μg RNA from *N. gruberi* NB-1; lane 2: 2 μg RNA from *N. gruberi* NB-1; lane 3: 2 μg of RNA from yeast with wild-type intron construct; lane 4: 2 μg of RNA from yeast with GIR1-defective construct. PCR-amplified NgrSSU1 and NjaSSU1 ORFs were used to generate probes.

products were incubated with purified I-*Nja*I, which was found to cleave the rDNA site, but not the two related sites (data not shown). The same protein has previously been reported to cleave an oligonucleotide containing the yeast rDNA sequence, although at slightly lower efficiency than an oligonucleotide with the homologous *Naegleria* sequence (Elde et al., 1999). Together, these observations led us to expect that the endonuclease would specifically target the yeast rDNA repeats in vivo.

To carry out the trans-integration experiment, we constructed two yeast plasmids. pYGal-INja has the I-*Nja*I ORF cloned behind the *GAL*1 promoter, and p423NjaSSU has the entire NjaSSU1 intron with 365 bp of 5′ flanking and 317 bp of 3′ flanking *Naegleria* exon sequence. Transformants carrying both plasmids were selected, and expression of the endonuclease was induced by plating cells onto galactose medium. The rDNA of several of the resulting colonies was examined for intron homing by PCR, with primers specific to yeast

TABLE 1. Sequences found in *S. cerevisiae* genome resembling the core sequence of the *Naegleria* endonuclease.

Yeast sequence resembling *Naegleria* endonuclease site (AAGTCTGGT•GCCAGCACCC[a])	Location (chromosome)	Gene
AAGTCTGGT GCCAGCAGCC[b]	XII	SSU rRNA; wt
AAGTCTAGT GCCAGCAGCC	XII	SSU rRNA; WD101
TCGTCTGGT GCCAGCAGCC	II	MET8[c]
AAATATGGT GCCAGCACCA	VII	ORF YGL010W[d]

[a]The optimal endonuclease recognition site (Elde et al., 1999); the bullet corresponds to the intron-insertion site in the *Naegleria* sequences.
[b]Underlined bases differ from the *Naegleria* sequence.
[c]Cherest et al., 1990.
[d]Johnston et al., 1994.

rDNA outside of the homologous region flanking the intron-insertion site. Despite multiple attempts, no integrations were detected by this method. However, by using an RT-PCR approach including one intron primer and one exon, we were able to detect what appeared to be rare exon–intron junctions, suggesting that at least the initial steps of intron homing were occurring in yeast (data not shown).

Expression of the *Physarum* intron homing endonuclease I-*Ppo*I is lethal in yeast. However, ~0.1–1% of the cells grow into colonies, and most of these have acquired mutations in all the rDNA repeats at the I-*Ppo*I cleavage site, rendering the rDNA resistant to cleavage (Muscarella & Vogt, 1993; Lin & Vogt, 1998). In a similar experiment, we analyzed surviving cells that grew on galactose plates after the induction of the *Naegleria* endonuclease from pYGal-INja, in the absence of any other plasmid. About 5–20% of the pYGal-INja-transformants grew on the galactose plates. PCR amplification and sequencing showed that 4 out of 34 survivors had a single G-to-A transition 3 nt upstream from the intron insertion site in most or all copies of the rDNA (Table 1). Cells that contained no mutation at the recognition site were not characterized further. The growth rate of the cells carrying pYGal-INja with mutated recognition sites did not appear to be impaired. One of these mutant colonies was picked and cured of the pYGal-INja plasmid, and this strain, called WD101, was used in the subsequent work described below. Analysis of this strain by PCR and restriction mapping showed that the mutation was stable even after growth for many generations in the absence of selection.

Strain WD101 was retransformed with either pYGal-INja or pYGal-INgr. The construction of the latter plasmid is similar to the former, except that it contains the endonuclease ORF from *N. gruberi* NB-1 instead of that from *N. jamiesoni*. Extracts of galactose-induced yeast carrying either plasmid showed specific endonuclease activity, when assayed either with the linearized substrate plasmid pCPR16 (Fig. 4, compare lanes 10–13 with lane 14) or with amplified intronless EG_B rDNA substrate (data not shown). The negative controls in this assay included extracts of the same strain without any plasmid, with the parental plasmid, and with the same pYGal-INja plasmid but grown in glucose (data not shown). Thus the *Naegleria* endonucleases can be expressed and analyzed in this derived yeast strain.

Dependence of endonuclease expression on GIR1

We used the WD101 yeast strain to address the function of the GIR1 ribozyme in endonuclease expression. In these experiments, endonuclease activity was as-

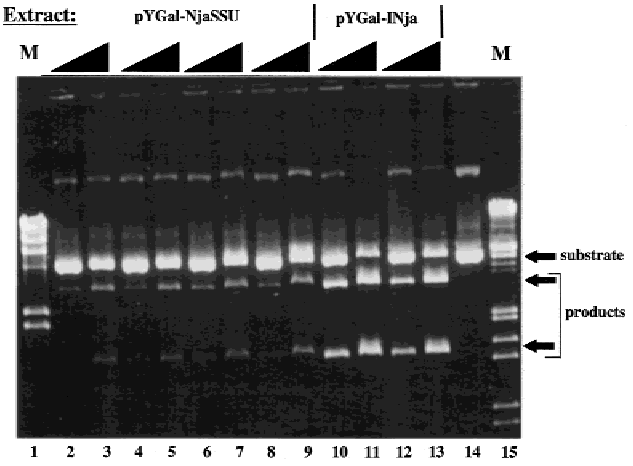

FIGURE 4. *Naegleria* endonuclease activity. Yeast cells carrying the rDNA mutation that confers resistance to the endonuclease were transformed with a galactose-inducible plasmid expressing the *N. jamiesoni* intron with flanking exon sequences (pYGal-NjaSSU), or with a galactose-inducible plasmid expressing just the endonuclease ORF (pYGal-INja). Independently isolated colonies were induced with galactose and assayed for activity. Lane 1: *Hin*dIII-digested λ DNA marker; lanes 2–13: linearized plasmid containing the cleavage site, incubated with approximately 3 μg (even numbered lanes) or 15 μg (odd numbered lanes) of total protein from colonies expressing pYGal-NjaSSU (lanes 2–9) or pYGal-INja colonies (lanes 10–13); lane 14: linearized plasmid incubated without extract; lane 15: *Eco*RI/*Hin*dIII-digested λ DNA marker.

sayed in extracts of yeast cells carrying either of two plasmids, again one with the wild-type intron and another with a GIR1-mutant. In these plasmids, the endonuclease ORF is more than 800 nt downstream of the site of transcription initiation (see Fig. 3A). Treatment of the linearized pCPR16 substrate DNA with extract from four galactose-induced yeast colonies harboring pYGal-NjaSSU1 led to site-specific cleavage (Fig. 4, compare lanes 2–9 with lane 14). Thus, intron RNA embedded within flanking *Naegleria* rRNA sequences is capable of expression of the endonuclease in yeast. As might be expected, however, enzymatic activity is much lower than in extracts of cells harboring plasmids with the ORF alone directly under galactose promoter control (Fig. 4, lanes 10–13).

To increase the sensitivity of endonuclease detection, we designed a ligation-based PCR assay that takes advantage of the unusual five-base 3′ overhang produced by I-*Nja*I (Elde et al., 1999; Fig. 5A). The experimental logic is based on the ligation of a synthetic fragment with a five-base overhang complementary to one of the two expected cleavage products. The resulting ligated product then is amplified by PCR. In this assay, extracts of galactose-induced cells carrying pYGal-NjaSSU1 showed one product corresponding to the specific cleavage product, as well as a second product that is a PCR artifact (Fig. 5B, lanes 1–3). The artifactual band arises from uncut substrate reacting with oligonucleotide primers (data not shown); this band, which competes with the specific product, turned out to be convenient as a control. Using this assay, we examined expression of endonuclease from the GIR1-crippled plasmid pYGal-G1^{-}Nja. Up to 50 μg of extract from the galactose-induced cells failed to show detectable activity (Fig. 5B, lanes 5–10) under conditions where as little as 0.1 μg of wild-type extract yielded a detectable PCR product (data not shown). We interpret this result to mean that GIR1 activity is essential for ORF expression in yeast.

DISCUSSION

We previously identified a new class of group I ribozymes, represented by the GIR1 elements in the group I introns DiSSU1 and NaSSU1, that are the first examples of natural self-cleaving group I ribozymes with a function other than splicing (Decatur et al., 1995; Einvik et al., 1997). Several more recent studies have also examined the self-cleavage activity of NaGIR1 RNA produced in vitro (Jabri et al., 1997; Einvik et al., 1998b; Jabri & Cech, 1998). The hypothesized biological role of these novel ribozymes is to promote expression of

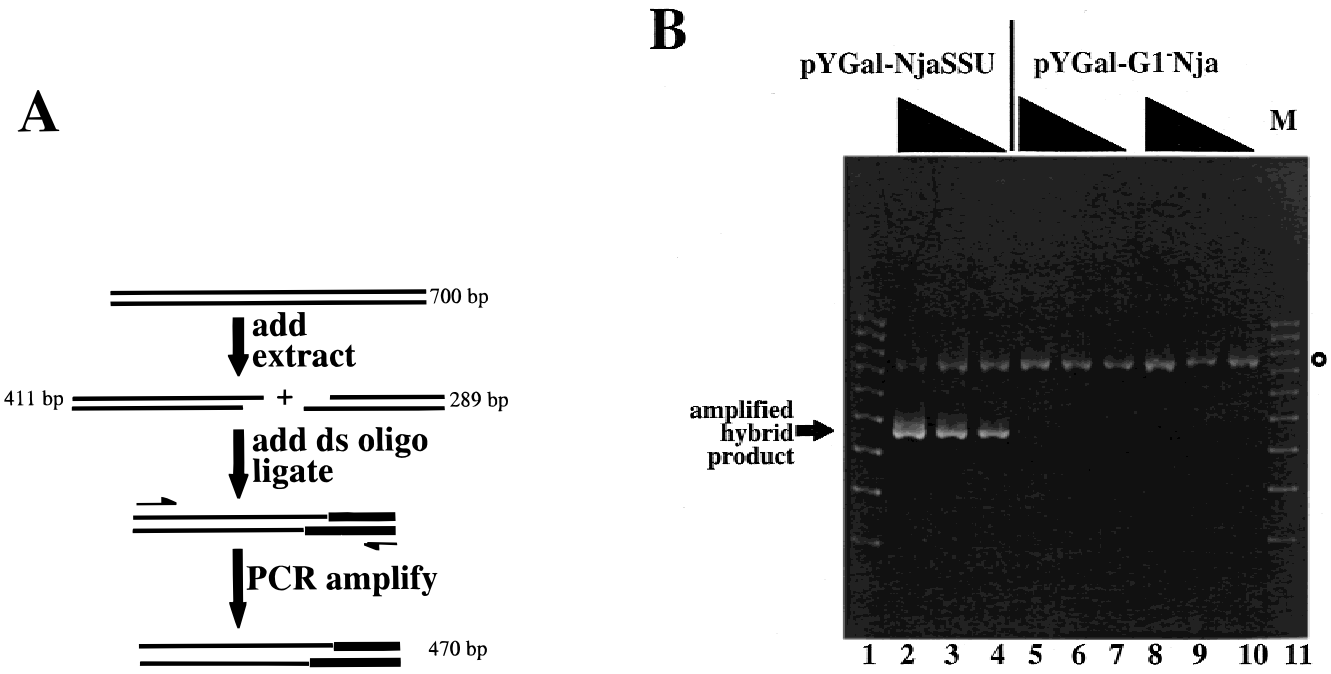

FIGURE 5. GIR1-dependence of endonuclease expression from the intact intron. **A**: Schematic drawing depicting the PCR-based endonuclease assay. A PCR product is incubated with extract. If endonuclease is present, it cleaves the substrate, producing the unique five-base 3′ overhangs specific to the *Naegleria* NaSSU1 endonuclease. A double-stranded oligonucleotide (indicated by dark bars) with overhangs complementary to one of the products is added and can be ligated to a cleavage product. The resulting DNA is amplified by PCR. Small arrows represent primers. **B**: Results of PCR based I-*Nja*I assay. Lanes 1 and 11: 100-bp ladder; lanes 2–4: 50, 25, or 5 μg, respectively, of total protein from galactose-induced cells carrying the wild-type plasmid pYGal-NjaSSU; lanes 5–7 and 8–10: 50, 25, or 5 μg, respectively, of total protein from independently transformed, galactose-induced cells carrying the GIR1-crippled plasmid pYGal-G1^{-}Nja. The specific product for the hybrid is indicated by the arrow. An ~750-bp band in each lane (indicated by a circle on the right) is a PCR artifact.

the intron-homing endonuclease. To address this hypothesis, we have examined intron-RNA processing in *N. gruberi*, a host organism for NaSSU1. The data presented here show that in vivo cleavage also occurs just upstream of the ORF, at both the IPS1 and the IPS2 sites identified in vitro. As primer extension showed only the 5′ end corresponding to IPS2, the sequential self-cleavages reported by Einvik and colleagues (1998b) in vitro must occur very efficiently in *Naegleria*. A result similar to ours was obtained in the analysis of DiSSU1 in its host organism *D. iridis* (Vader et al., 1999).

The absence of detectable endonuclease activity in *N. gruberi* NB-1 extracts suggests that the enzyme itself is produced in very small amounts, if at all. Because NaGIR1 clearly functions in vivo to generate the putative mRNA, the host cells may have additional mechanisms to regulate translation of the endonuclease or to restrict its activity. The fact that the steady-state level of total processed and unprocessed ORF-containing RNA is only 0.2% of total RNA, compared with ribosomal RNA at ~85% of total RNA (Walsh & Fulton, 1973), suggests that degradation of the intron RNA is rapid and thus might occur before transport of the RNA to the cytoplasm can take place. The biological significance of the apparent lack of endonuclease expression is uncertain. Flagellates have been suggested to be the mating form of *Naegleria* (Fulton, 1993), but in fact mating has never been observed in the laboratory. However, evidence does exist for genetic exchange in a natural population of *Naegleria* species (Pernin et al., 1992). Perhaps the failure to detect endonuclease stems from a narrow window in which flagellates in laboratory conditions are competent to mate (Fulton, 1970), and it is only in this hypothetical window that the endonuclease is active. In other organisms carrying mobile group I introns, homing endonuclease activity often is not detectable in crude extracts. For example, I-*Sce*I, I-*Sce*II, I-*Cre*I, I-*Dmo*I, and I-*Dir*I cannot be assayed in extracts from their wild-type host organisms (Dujon et al., 1985; Wenzlau et al., 1989; Dürrenberger & Rochaix, 1991; Dalgaard et al., 1993; A. Vader & S. Johansen, unpubl. results).

Under conditions used to force homing of PpLSU3 into yeast rDNA (Muscarella & Vogt, 1993; Lin & Vogt, 1998), NaSSU1 failed to home successfully in yeast. PCR analysis did not detect any intact introns in rDNA. However, this analysis did provide evidence of rare intron–exon junction fragments, suggesting that homing at least had been initiated. Either gene conversion then failed to proceed to completion, or after gene conversion was completed at least in some rDNA copies, the introns were deleted or partially deleted because of deleterious effects on cell growth. The latter possibility is more likely: we showed that when NaSSU1 was artificially built into a plasmid-born rDNA, preribosomal RNA expression from the plasmid was not able to rescue cell growth in the absence of RNA polymerase I transcription of the rDNA copies on chromosome XII (data not shown). It is possible that this failure is because of lack of splicing, or inefficient or incorrect splicing. Alternatively, the intron might affect some other aspect of ribosomal RNA maturation. The latter possibility is suggested by the observation that the *Tetrahymena* intron inserted into the ribosomal RNA genes of *Schizosaccharomyces pombe* prevented proper maturation of the 5.8S species, despite the absence of obvious splicing defects (Good et al., 1994). Presumably group I introns in nuclear rDNA have coevolved with rDNA to maximize splicing efficiency and to minimize disruption of other processes in their host organisms. It seems possible that minor sequence changes might allow them to adapt to new species, but this notion has not been tested experimentally.

To study NaSSU1 splicing, processing, and endonuclease expression in yeast, we developed a strategy that circumvented the lack of homing in this system. First, the endonuclease was expressed by itself from a plasmid, and among surviving colonies, a mutant yeast strain was identified that tolerates endonuclease activity. This strain had acquired a single mutation in the endonuclease target site in all the rDNA copies, analogous to the mutations described previously that confer resistance to the I-*Ppo*I endonuclease in yeast (Muscarella & Vogt, 1993; Lin & Vogt, 1998). Into this strain a plasmid was introduced that carries NaSSU1 with ~0.3 kb flanking rDNA 5′ exon and 3′ exon sequences, under control of the *GAL*1 promoter. Induction by galactose in this system led to the synthesis of an rRNA from which the intron spliced and further processed itself, in a manner similar to that seen in vitro and also in *Naegleria*. Furthermore, intron expression led to endonuclease activity, which could be detected in crude extracts either by a straightforward DNA-cleavage assay, or by a more sensitive PCR-based assay for the characteristic 5 nt sticky end produced by cleavage. An inactivating mutation in the NaGIR1 ribozyme abrogated endonuclease activity. This result thus supports the hypothesis that the function of NaGIR1 is to generate the mRNA for the *Naegleria* endonuclease.

NaSSU1 and DiSSU1 are the only known examples of twin-ribozyme group I introns. Despite differences in organization (Einvik et al., 1998a), comparison of processing reveals a number of shared features. First, in both the DiSSU1 and NaSSU1 systems, a GIR1-mediated cleavage is observed in vivo downstream of the ORF. Second, both DiSSU1 RNA (Vader et al., 1999) and NaSSU1 RNA form full-length circles in their host organisms. Circularization, like 3′ SS hydrolysis, is usually thought to be a side reaction of group I ribozymes, but this reaction may serve a biological function. Because full-length intron circles by definition retain all the genetic information of the autocatalytic intron, it has been speculated that they could play a key role in the evolutionary spread

of the intron by reverse splicing (Johansen & Vogt, 1994; Johansen et al., 1996).

The third feature shared by DiSSU1 and NaSSU1 is the presence of several sequence elements characteristic of RNA polymerase II-derived mRNAs or other RNAs destined to be translated. DiSSU1 is unprecedented in containing a 51-nt spliceosomal intron within the ORF (Vader et al., 1999). This intron within an intron may facilitate export of the mRNA to the cytoplasm. In addition, DiSSU1 contains an appropriately situated poly(A) signal, AAUAAA. Indeed, the polysome-associated mRNA is both polyadenylated and fully spliced by removal of the 51-nt sequence (Vader et al., 1999). Although the NaSSU1 ORF does not possess a spliceosomal intron, it does have a poly(A) signal 13 nt upstream of the stop codon, a feature that is conserved in all known large NaSSU1 introns.

Together, these data suggest a model for the production of a mRNA for a NaSSU1 endonuclease from the spliced intron in *Naegleria* cells. The model is similar to that proposed for DiSSU1 (Vader et al., 1999). Upon transcription of the intron, NaGIR2 catalyzes the self-splicing reaction. The excised intron is further processed efficiently by the self-cleaving NaGIR1 to liberate the 5′ end, which is the target of *trans*-acting factors that stabilize and/or localize the nascent transcript, as well as perhaps regulate its translation. Possibly, polyadenylation occurs closely following the stop codon of the ORF. Poly(A) tails can act as a translational promoter in the absence of a cap (Preiss & Henzte, 1998), and may facilitate productive translation of the unusual message derived from the twin-ribozyme group I introns.

MATERIALS AND METHODS

Growth of cells and plasmid constructions

Naegleria growth and maintenance were as described previously (Einvik et al., 1997). *S. cerevisiae* strain INVSc2 (*MATα, his3-Δ200, ura3-167*) was purchased from Invitrogen. Growth of yeast was performed as described in Sherman et al. (1986) and cells were transformed by the lithium acetate method (Ausubel et al., 1998). Normally, yeast cells were grown at 30 °C in synthetic minimal medium with the appropriate carbon source and supplemented with amino acids. Curing of *URA3*-based pYGal-NjaSSU was carried out by growth in YEPD culture and plating on 5′-fluoro-orotic acid plates. *E. coli* DH5α was used for basic DNA manipulations.

Plasmids were constructed by standard cloning methods (Sambrook et al., 1989). PCR reactions to construct pNaNOY were carried out with Pfu polymerase (Stratagene) and Vent polymerase (New England Biolabs) to enhance accuracy. p423NjaSSU was constructed by cloning the *Not*I/*Sal*I fragment from pRSNjaSSU into the *Not*I/*Sal*I-cut 2 μ, *URA3*-based plasmid pRS423 (Sikorski & Hieter, 1989). pRSNjaSSU consists of the *Nhe*I/*Pst*I fragment of pNjaSSU+Nhe (Einvik et al., 1997) in CEN-plasmid pRS315. Plasmid pYESM was built by replacing the *Eco*RI/*Xba*I fragment of pYES2 (Invitrogen) with a short synthetic fragment containing *Xba*I and *Sph*I sites. Plasmid pYGal-INjaΔ3′UTR (referred to simply as pYGal-INja elsewhere) was made by inserting an *Xba*I/*Sph*I-cut PCR product containing the I-*Nja*I ORF amplified from pYGal-INja#7, into *Xba*I/*Sph*I-cut pYESM. pYGal-INja#7 consists of the *Xba*I/*Pst*I fragment from pMAL-INja+Pst inserted into *Xba*I/*Sph*I-cut pYESM and thus has downstream intron sequences between the ORF and the vector polyadenylation site. pMAL-INja+Pst was created by inserting annealed oligonucleotides carrying a *Pst*I site into the *Sac*II site of pMAL-INjaI (Elde et al., 1999). pYGal-INgr is a construction similar to pYGal-INja, and was created by cutting a PCR product containing the endonuclease ORF, amplified from *N. gruberi* NB-1 DNA as template, with *Xba*I/SphI and inserting into pYESM. Plasmid pYGal-NjaSSU was built by replacing the *Xba*I/*Sph*I fragment of pYESM with the *Nhe*I/*Sph*I fragment from pNjaSSU (Einvik et al., 1997). pYGal-G1$^-$Nja was made by replacing the 347-bp *Xba*I/*Bgl*II fragment of pGal-NjaSSU with the 347-bp *Xba*I/*Bgl*II-cut fragment used to create pNjaG1P7$^-$ (Einvik et al., 1997). The *Nhe*I/*Sph*I fragment of pNanGIR2+Nhe (Einvik et al., 1997) is inserted into pYESM to make pYGal-GIR2. The NjaSSU1 intron was cloned into pNOY103 (Nogi et al., 1991b) via several steps to produce pNaNOY. The plasmids for the S1 assays were made by amplifying the respective region of the intron from *N. gruberi* NB-1 DNA and cloning into appropriately cut pGEM3Z (Promega).

Protein extraction and endonuclease assays

Yeast cells were grown to OD_{600} 1.0–1.5. Cells from a 5-mL culture were spun down and resuspended in 100 μL breakage buffer [10% glycerol, 200 mm Tris-HCl, (pH 8.0), 10 mm $MgCl_2$, 1 mm phenylmethylsulfonyl fluoride, 1 mm DTT]. One hundred microliters of glass beads (425–600 μm; Sigma) were added and the cells were vortexed for 5 min at 4 °C. After centrifugation at 4 °C for 15 min, the supernatant was spun again for 5 min, and the final supernatant was called the crude protein extract. *Naegleria* cells were grown on NM plates with bacteria as described (Fulton, 1970). For *Naegleria* protein extracts, about 100 μL of well-washed cells were processed as in the yeast protein extraction. Plasmid pCPR16 (Elde et al., 1999) was linearized with *Hin*dIII and used as substrate. Yeast crude protein extract was incubated with 300 ng linearized pCPR16 in WI buffer [50 mm Tris-HCl (pH 8.0), 10 mm $MgCl_2$, 50 mm NaCl, 1 mm DTT, 50 μg/mL BSA] at 37 °C for 1 h. Reactions were stopped, and the precipitated nucleic acid was run on a 1.0% agarose gel. For the I-*Ngr*I endonuclease activity assay with radiolabeled substrate and yeast extract, primers flanking the cleavage site were used to amplify a 520-bp fragment from EG_B DNA or a 1,826-bp fragment from NB-1 rDNA (Einvik et al., 1997) in the presence of 20 μCi α-^{32}P-dATP in a 100-μL PCR reaction. Aliquots of the PCR product were incubated with 28 μg crude yeast protein for 3 h at 37 °C in 50 μL. The reaction also included 4 μg poly [d(I-C)]. The products were analyzed by agarose gel electrophoresis and autoradiography. For the activity assays with *Naegleria* extract and radiolabeled substrate, the substrate was a 141-bp radioactive PCR product like that described above. The smaller substrate was used in this case to reduce the effects from the abundant nucleases in *Naegleria* extracts (Fulton, 1970), and these reactions included 50 ng lambda DNA in addition to poly [d(I-C)]. For

control reactions with purified *Naegleria* endonuclease, an MBP-I*Nja*I fusion protein expressed in *E. coli* was used (Elde et al., 1999).

For the ligation-based cleavage assay, the first-round substrate was a 700-bp PCR fragment containing the endonuclease recognition site. Fifty nanograms of this substrate were added to the extract to be examined, and incubated in WI buffer at 37 °C in a total of 50 μL. The reaction was phenol: chloroform extracted once, chloroform extracted once, and precipitated with glycogen as carrier. The pellet was resuspended in a total of 20 μL with 80 pmol each of two annealed oligomers of length 64 and 59 nt, creating the 3′ protruding end TGCCA. This reaction was incubated overnight at 15 °C with T4 ligase. The products were phenol:chloroform extracted once and precipitated, and then 1% was used in a 100-μL PCR reaction to amplify any annealed fragments.

RNA preparation and Northern blot analysis

Growth of yeast cells and yeast RNA preparation were carried out as described (Lin & Vogt, 1998). For RNA extraction from *N. gruberi* EG_B and NB-1 amoebae and flagellates, amoebae were grown on NM plates with bacteria, harvested, washed, and allowed to differentiate by starvation for 80 min shaking in 2 mm Tris at 30 °C if flagellates were desired (Fulton, 1970; Einvik et al., 1997). RNA was prepared from about 1×10^7 ($\sim$35 μL) well-washed cells using TRI REAGENT (Molecular Research Center, Inc.) according to the manufacturer's instructions. RNA was fractionated on a 5% polyacrylamide/8 M urea gel in 1$\times$ TBE and transferred by capillary transfer to GeneScreen Plus membrane (Du Pont NEN), and hybridized essentially as described (Lin & Vogt, 1998). DNA probes were made with the DECAprime II kit from Ambion, Inc. in the presence of α-^{32}P-dATP (Amersham) according to the manufacturer's instructions and passed over a Sephadex G-25 spin column. The templates for the probes consisted of 25 ng purified PCR products, obtained from amplifications of pNjaSSU (Einvik et al., 1997; probe for the NjaSSU1 ORF) or pNgrSSU(+) (Einvik et al., 1997; probes for the NgrSSU1 ORF and the NgrSSU1 intronic RNA upstream of IPS1).

RT-PCR, primer extension, and S1 assay

For RT-PCR, RNA from 2 OD_{600} of cells was annealed with 50 pmol primer and incubated in a 30-μL reaction using M-MuLV reverse transcriptase (Ambion) for 90 min. One third of the RT reaction was used in a 100-μL PCR reaction with 80 pmol of each primer. To analyze ligated exons, the primer used in the RT step was used again with an upstream primer to generate the amplified DNA fragment. All other RT-PCR reactions used a downstream primer in the amplification step that was different than the RT primer. Amplified circle junctions were sequenced by the Cornell Bioresource Center. Primer extension and associated sequence ladders were performed as described in Lin & Vogt (1998).

S1-assays were carried out using Ambion's S1-Assay kit according to the manufacturer's instructions, using α-^{32}P-dATP. The probes spanned the region upstream of the IPS1/IPS2 to about 70 nt downstream, a portion of the ORF, and the 3′ end of the intron. For each assay, 150 pg (1×10^6 cpm) probe was used with an average of 10 μg of RNA per sample. The probes were purified by elution from a 5% polyacrylamide/8 M urea gel. RNA to use as a standard for quantitating total ORF-containing RNA was generated by in vitro transcription (Einvik et al., 1997) with T7 RNA polymerase, with 1 μCi α-^{32}P-UTP, and 0.5 mm cold UTP to trace-label the RNA. To determine the exact positions of the 5′ ends, sequencing reactions (dsDNA Cycle Sequencing System; GIBCO-BRL) were carried out with a primer complementary to the 3′ end of the probe-hybridizing region of the intron.

Ectopic expression of the *Naegleria* intron

In the *trans*-integration experiment, the plasmids pYGal-INja and p423NjaSSU were transformed into the yeast-strain INVSc2 and plated on SD-Ura-His plates. Transformants were streaked on SGal-Ura-His plates to induce the expression of I-*Nja*I. Single colonies on SGal-Ura-His plates were grown in SGal-Ura-His liquid medium. DNA was extracted from the above culture and PCR was performed to screen for intron-integrated colonies, using primers that amplify the yeast rDNA segment corresponding to the NjaSSU1 insertion site, or primers that amplify yeast intron/exon junctions. For obtaining yeast expressing I-*Nja*I under control of the *GAL*1 promoter, pYGal-INja was transformed into INVSc2 yeast and selected on galactose. A colony that expressed I-*Nja*I as evidenced by the endonuclease assay was isolated, cured, and named strain WD101. This new strain was then the recipient for transformation by pYGal-INja, pYGal-NaGIR2, pYGal-NjaSSU, pYGal-G1$^-$Nja, or pYGal-Ngr. For *ts* complementation, the yeast strain NOY401 (Nogi et al., 1991b) was transformed with pNaNOY and selected on plates lacking uracil. Single colonies were streaked on SD and SGal plates and put at room temperature and 37 °C to assess complementation of the *ts* RNA pol I of NOY103 (Nogi et al., 1991a, 1991b) by intron-containing rDNA expressed from the *GAL*7 promoter.

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