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Naegleria nucleolar introns contain two group I ribozymes with different functions in RNA splicing and processing

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ABSTRACT

We have characterized the structural organization and catalytic properties of the large nucleolar group I introns (NaSSU1) of the different *Naegleria* species *N. jamiesoni*, *N. andersoni*, *N. italica*, and *N. gruberi*. NaSSU1 consists of three distinct RNA domains: an open reading frame encoding a homing-type endonuclease, and a small group I ribozyme (NaGIR1) inserted into the P6 loop of a second group I ribozyme (NaGIR2). The two ribozymes have different functions in RNA splicing and processing. NaGIR1 is an unusual self-cleaving group I ribozyme responsible for intron processing at two internal sites (IPS1 and IPS2), both close to the 5′ end of the open reading frame. This processing is hypothesized to lead to formation of a messenger RNA for the endonuclease. Structurally, NaGIR2 is a typical group IC1 ribozyme, catalyzing intron excision and exon ligation reactions. NaGIR2 is responsible for circularization of the excised intron, a reaction that generates full-length RNA circles of wild-type intron. Although it is only distantly related in primary sequence, NaSSU1 RNA has a predicted organization and function very similar to that of the mobile group I intron DiSSU1 of *Didymium*, the only other group I intron known to encode two ribozymes. We propose that these twin-ribozyme introns define a distinct category of group I introns with a conserved structural organization and function.

Keywords: group I intron; ribosomal DNA; ribozyme; RNA circles; rRNA processing; self-splicing

INTRODUCTION

Nucleolar group I introns are relatively widespread among eukaryotic microorganisms, interrupting a number of conserved sites in either the small subunit (SSU) or the large subunit (LSU) rRNA genes (Johansen et al., 1996). Although the majority of the these introns are small and contain only the characteristic self-splicing group I ribozyme structure (Jaeger et al., 1996), others are larger due to insertions such as open reading frames (ORFs). Naturally occurring nucleolar group I ribozymes catalyze a number of reactions as naked RNA in vitro. These reactions are involved in two major processes, pre-rRNA intron splicing and processing of the excised intron RNA. Splicing has been studied intensively, with the best-studied example being the *Tetrahymena* LSU rDNA intron (TtLSU1) (Cech & Herschlag, 1996). Group I intron splicing is initiated by nucleophilic attack of a noncoded guanosine (G) cofactor at the 5′ splice site, leading to the covalent attachment of the G residue to the released 5′ end of the intron. In a subsequent reaction, the free 3′ end of the 5′ exon attacks the 3′ splice site, resulting in ligation of the exons and release of the linear intron RNA. Splicing thus is essential for generation of pre-ribosomal RNA.

By contrast, excised intron processing appears to be a selfish feature of the nucleolar introns, which probably evolved to allow expression of the intron-coded endonucleases (Johansen et al., 1996). RNA processing of the *Physarum* LSU rDNA intron (PpLSU3), which is closely related in sequence to TtLSU1, has been well characterized (Ruoff et al., 1992; Rocheleau & Woodson, 1994, 1995). Here, the excised intron RNA is cleaved into two halves at an internal processing site (IPS), resulting in a group I ribozyme RNA and an RNA species that contains the ORF. The latter RNA may represent the messenger for the I-*Ppo* I endonuclease,

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a protein known to be essential for intron homing in vivo (Muscarella & Vogt, 1989, 1993; Muscarella et al., 1990). A more complex RNA processing pattern has been described in a different nucleolar group I intron (DiSSU1) found in the SSU rDNA of *Didymium* (Johansen & Vogt, 1994; Decatur et al., 1995). Here, the excised linear intron RNA generates a full-length circle, which is subsequently re-opened and cleaved at an IPS. The latter reaction is due to a second small group I ribozyme present within the intron RNA. The internal processing is hypothesized to generate the 5′ end of the messenger RNA for the intron-encoded homing endonuclease I-*Dir* I (Decatur et al., 1995) and, similar to I-*Ppo* I, this enzyme appears to be important for intron homing in vivo (Johansen et al., 1997). At the RNA level, DiSSU1 consists of two distinct group I ribozymes, DiGIR1 and DiGIR2, in a unique twin-ribozyme organization (Decatur et al., 1995).

Ten species in the genus *Naegleria* have so far been described (see De Jonckheere, 1994a). These are all free-living amoebo-flagellates isolated from diverse sources such as human cerebrospinal fluid, polluted water, or hot springs. Some species harbor an optional group I intron in their extrachromosomal nucleolar DNA. Although most species and strains lack this SSU rDNA intron (De Jonckheere, 1993), others have either a large (Embley et al., 1992a; De Jonckheere, 1994b) or a small (De Jonckheere & Brown, 1994) version of the intron. The large versions encode a homing-type endonuclease (M. Elde, P. Haugen, N.P. Willassen, & S. Johansen, in prep.), but intron mobility has not been demonstrated in an in vivo experimental setting. Here we report that the large *Naegleria* intron (NaSSU1) shares several of the unusual structural and functional features of DiSSU1 (Decatur et al., 1995), despite extensive differences in primary structure and organization.

RESULTS

NaSSU1 is composed of an ORF and two distinct group I ribozymes, NaGIR1 and NaGIR2

A secondary structure model of a large *Naegleria* intron is proposed in Figure 1. The structure is based on sequence comparisons of the eight different *Naegleria* introns (Table 1) as well as known general and special features among group I intron structures (Cech et al., 1994; Decatur et al., 1995; Jaeger et al., 1996). All large *Naegleria* introns are homologous in structure. The overall level of sequence identity between introns ranges from 88 to 96%, with the 738-bp ORF being the most variable. The introns of *N. jamiesoni* and *N. andersoni* are almost identical. Phylogenetic analyses suggest that all *Naegleria* introns developed from a common ancestor within the genus (De Jonckheere, 1994b). Thus, we

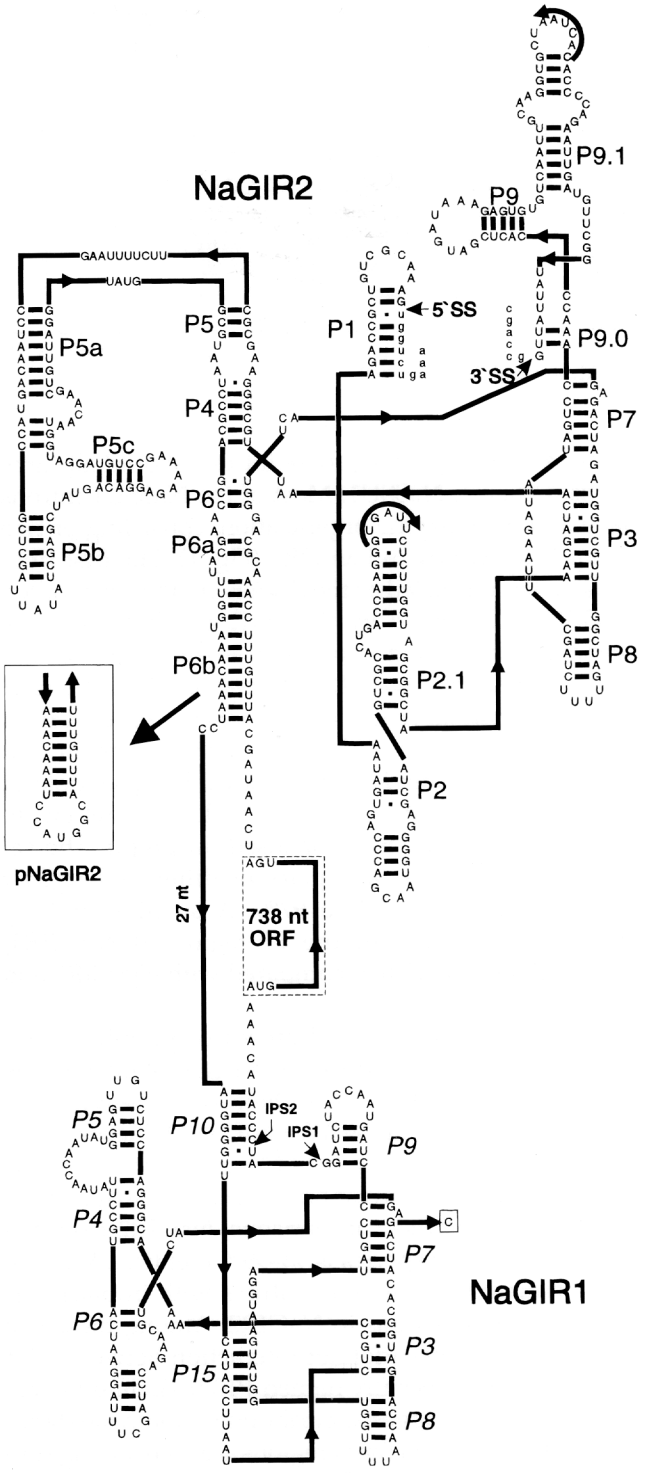


FIGURE 1. Secondary structure model of NaSSU1. Paired segments (P) are indicated in both the self-cleaving and self-splicing group I ribozymes NaGIR1 and NaGIR2, respectively. (GIR = Group I Ribozyme). The G → C mutation in the catalytic core segment *P7* in NaGIR1 is shown. The P6 deletion segment in pNaGIR2 constructs (pNjaGIR2 and pNanGIR2) is boxed. A putative pseudoknot interaction between loops of P2.1 and P9.1 (NaGIR2) is indicated by arrows. This interaction was recently named P13 (Lehnert et al., 1996). 5′ SS and 3′ SS, intron splice sites; IPS1 and IPS2, internal processing sites; ORF, open reading frame. The sequence of the Nja.SSU1 intron from *N. jamiesoni* rDNA is shown.

TABLE 1. *Naegleria* SSU rDNA introns.

Intron	Size (nt)	Species	Strain	Reference[a]
Nja.SSU1	1,308	*N. jamiesoni*	T56E	1
Nan.SSU1	1,309	*N. andersoni*	PPMFB-6	1
Nit.SSU1	1,316	*N. italica*	AB-T-F3	1
Ngr.SSU1	1,316	*N. gruberi*	CCAP1518/D	2
Ncl.SSU1	1,305	*N. clarki*	RU30	2
N434.SSU1	375	*Naegleria* sp.	NG434	3
N587.SSU1	375	*Naegleria* sp.	NG587	3
N650.SSU1	375	*Naegleria* sp.	NG650	3

[a]The references are: 1, this work; 2, De Jonckheere (1994b); 3, De Jonckheere and Brown (1994).

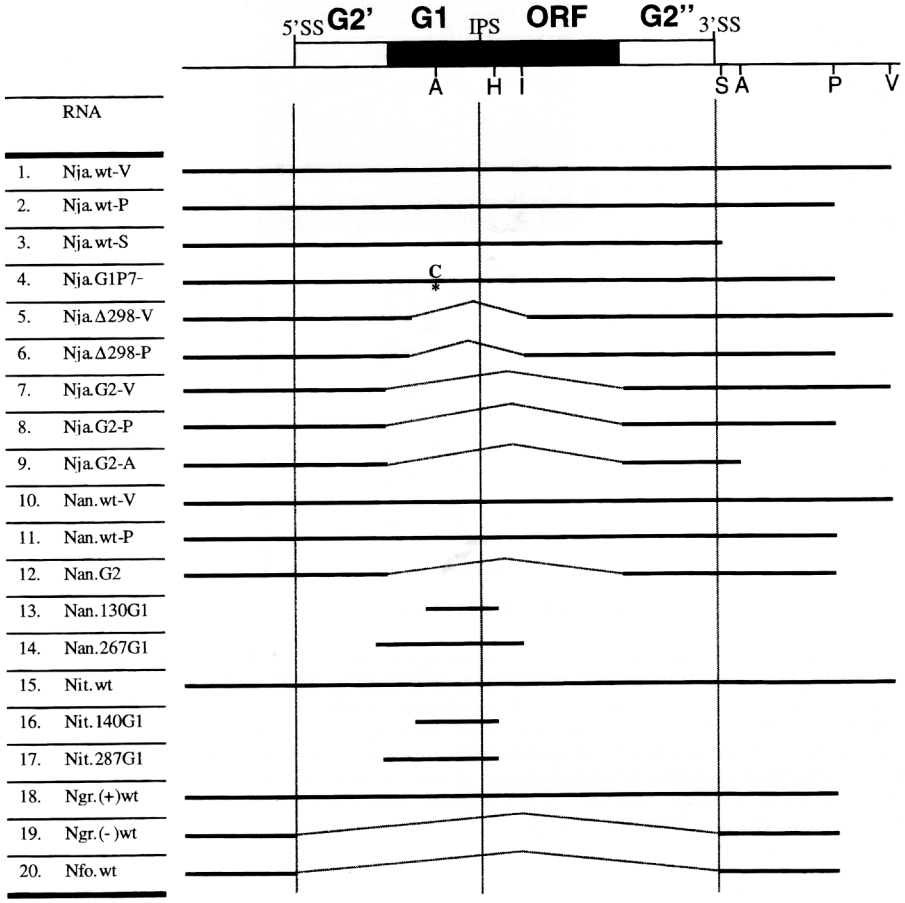
propose that, in general, these introns should all carry the same name, NaSSU1. Most of the NaSSU1 introns have a twin-ribozyme organization, consisting of two group I ribozymes and an ORF (Fig. 1). The first ribozyme to be completed as the RNA is synthesized, called NaGIR1, is followed by the ORF, and both of these elements are inserted into the P6 segment of a second group I ribozyme (NaGIR2). At about 180 nt, NaGIR1 is smaller than all of the known splicing group I ribozymes. It lacks a P1 and a P2 structure element, but contains an unusual P10 and a novel P15 pseudoknot within the catalytic core (C. Einvik, H. Nielsen, E. Westhof, F. Michel, & S. Johansen, in prep.). The sequence identity between pairs of different *Naegleria* GIR1s ranges from 93 to 97%. A small stem-loop insertion is present in the P5-region of Nit.GIR1, Ngr.GIR1, and Ncl.GIR1, compared with Nja.GIR1 and Nan.GIR1.

According to structural features of peripheral domains, NaGIR2 is grouped as an IC1 ribozyme, the same subgroup as the well-studied *Tetrahymena* group I intron TtLSU1 (Michel & Westhof, 1990; Jaeger et al., 1996). The small introns found in one of the *Naegleria* lineages (Table 1) correspond exactly in structure to NaGIR2. In pairwise comparisons, NaGIR2 from the different *Naegleria* species is 93–99% identical. We note some interesting structural features with implications for tertiary interactions in NaGIR2. Among these is a 5–6-bp putative pseudoknot interaction between L2.1 and L9.1 (Fig. 1; recently named P13 by Lehnert et al., 1996), which we suggest contributes to ribozyme stability. Similar complementary regions are seen among subsets of nucleolar group IC introns, including introns in *Tetrahymena* and *Didymium* (Nielsen & Engberg, 1985; Cech et al., 1994; Decatur et al., 1995; Lehnert et al., 1996). An L5b-P6b interaction between a GNRA (N for any nucleotide, and R for purine nucleotide) tetraloop and a receptor motif, respectively, is well documented as an invariant stabilizing feature of the P4-P6 domain among *Tetrahymena* wild-type introns (Nielsen & Engberg, 1985; Murphy & Cech, 1994; Cate et al., 1996). Remarkably, the corresponding L5b and P6b regions in NaGIR2 are the most variable parts of the structure. In fact, the L5b GNRA tetraloop is optional among the different *Naegleria* species. Both Nja.GIR2 and Nan.GIR2 lack an L5b tetraloop (see Fig. 1), as well as the tetraloop receptor in P6b (Costa & Michel, 1995). No obvious covariation is found between L5b and P6a/b with or without a GNRA-loop in L5b.

NaGIR2 is a regular self-splicing group I ribozyme

To analyze the catalytic activities of *Naegleria* intron RNA, we transcribed several different linearized rDNA plasmid constructs with T7 RNA polymerase. The results from splicing experiments of intron RNAs from *N. jamiesoni*, *N. andersoni*, *N. italica*, and *N. gruberi* are summarized in Figure 2. As an initial screening for in vitro self-splicing activity, an RT-PCR approach was used to identify ligated exons (Fig. 3A). The result of a representative experiment in shown in Figure 3B. Here, incubation of Nan.wt-V generated correctly ligated exons. Identical in vitro self-splicing results were obtained by the same approach with RNAs transcribed from Nja.wt-V (Fig. 2, line 1), from Nit.wt (line 15), and from Nja.Δ298-V (line 5). The latter RNA harbors a deletion of most of the Nja.GIR1 structure and parts of the ORF, and the result supports the conclusion that exon ligation is dependent only on the NaGIR2 ribozyme.

A different line of evidence in support of in vitro self-splicing activities was obtained from gel analysis. Processed RNA species, generated after incubating intron RNA under self-splicing condition, were identified by size and by comparison of patterns of RNAs transcribed from templates linearized at different sites in the 3′ exon (Fig. 2, e.g., lines 1–3 and 7–9). To confirm that NaSSU1 splicing is dependent only on NaGIR2, a mutation was introduced into the P7 guanosine binding site of Nja.GIR1 (Fig. 2, line 4), thus inactivating this ribozyme (Fig. 4A). The mutant RNA gave rise to ligated exon, but none of the RNA species defined by internal processing were observed (Fig. 4A, lane 4). To further characterize the NaGIR2-dependent splicing, we made a mutant construct of Nja.SSU1 lacking the entire GIR1 and ORF insertion. This construct (pNjaGIR2) corresponds in structure to the small *Naegleria* introns (Table 1). pNjaGIR2 templates linearized at different sites within the 3′ exon were transcribed (Fig. 2, lines 7–9), subjected to self-splicing conditions, and analyzed by electrophoresis (Fig. 4B, lanes 3–8). Seven major RNA species, including both ligated exon and excised intron, were identified based on size, 3′ exon size variability, and end labeling with radioactive GTP. In the latter experiment, RNAs carrying the 5′ end of the intron were identified by incubating unlabeled intron RNA in the presence of ^{32}P-GTP during the splicing reaction. G-addition is a unique



Self splicing	Internal processing	Circle formation	Template plasmid
+	+	n.d.	pNjaSSU
+	+	+ (FL)	pNjaSSU
+	+	n.d.	pNjaSSU
+	−	n.d.	pNjaG1P7-
+	Δ	n.d.	pNjaΔ298
+	Δ	+ (FL)	pNjaΔ298
+(G)	Δ	n.d.	pNjaGIR2
+(G)	Δ	+ (C-2)	pNjaGIR2
+	Δ	n.d.	pNjaGIR2
+	+	n.d.	pNanSSU
+	+	+ (FL)	pNanSSU
+	Δ	+ (C-2)	pNanGIR2
Δ	−	Δ	pNan130G1
Δ	+ (PE)	Δ	pNan267G1
+	+	n.d.	pNitSSU
Δ	−	Δ	pNit140G1
Δ	+ (PE)	Δ	pNit287G1
+	+	n.d.	pNgrSSU(+)
Δ	Δ	Δ	pNgrSSU(-)
Δ	Δ	Δ	pNfoSSU

FIGURE 2. Schematic presentation of RNA transcripts and summary of processing results. Schematic organization of intron and flanking rDNA sequences is indicated on top of the diagram. H, I, S, A, P, and V are the different restriction enzymes *Hind* III, *Hinc* II, *Sac* II, *Acc* I, *Pst* I (or *Sph* I), and *Pvu* II, respectively, used for template linearization. G1, NaGIR1; G2′, NaGIR2 5′ segment; G2″, NaGIR2 3′ segment; ORF, open reading frame; 5′ SS and 3′ SS, intron splice sites; IPS, internal processing site. Different RNA transcripts analyzed are presented below. All transcripts were obtained by in vitro transcription from the T7 promoter. Right, summary of RNA splicing and processing results. +, activity observed; −, no activity observed; n.d., not determined; Δ, processing site or sequence region is not present; G, guanosine-labeling experiment performed; PE, subjected to primer extension experiments; FL, full-length intron RNA circles; C-2, circles lacking the first two coded nucleotides of intron. A representative selection of the data is presented in Figures 3–7.

feature of group I introns, in which a noncoded G residue becomes covalently bound at the intron 5′ end during the first step in splicing. The result of a G-labeling experiment is presented in Figure 5 (lane 2). As expected, two of the RNA species became labeled during splicing, corresponding to an intron-3′ exon fragment and to the excised linear intron RNA (compare lanes 1 and 2). We conclude that NaGIR2 is a regular group I splicing ribozyme that carries out all the reactions associated with self-splicing.

NaGIR2 catalyzes full-length circularization of NaSSU1 intron RNA

Gel analysis of processed *Naegleria* intron RNA strongly suggests that intron circles were generated during splicing because the relative migration pattern of one of the major RNA species changed between 8% and 5% polyacrylamide gels (Fig. 4B, compare lanes 2 and 4). To analyze intron circle junctions, DNA copies of the self-spliced RNAs Nja.wt-P, Nja.Δ298-P, and Nja.G2-P were amplified by RT-PCR and then cloned and sequenced (see Materials and Methods). Representative results of Nja.wt and Nja.G2 circle junctions are shown in Figure 6. Four independent clones each from Nja.wt-P and Nja.Δ298-P were all identical in sequence, and implied the formation of full-length intron circles. By contrast, 9 of 10 analyzed cloned products from Nja.G2-P contained circle junctions lacking the first two coded nucleotides of the intron, a result similar to that reported for *Azoarcus* group I intron (Tanner & Cech, 1996). The tenth clone was highly truncated, and indicated a circle junction between a G-residue located 110 nt downstream from the 3′ splice site and an

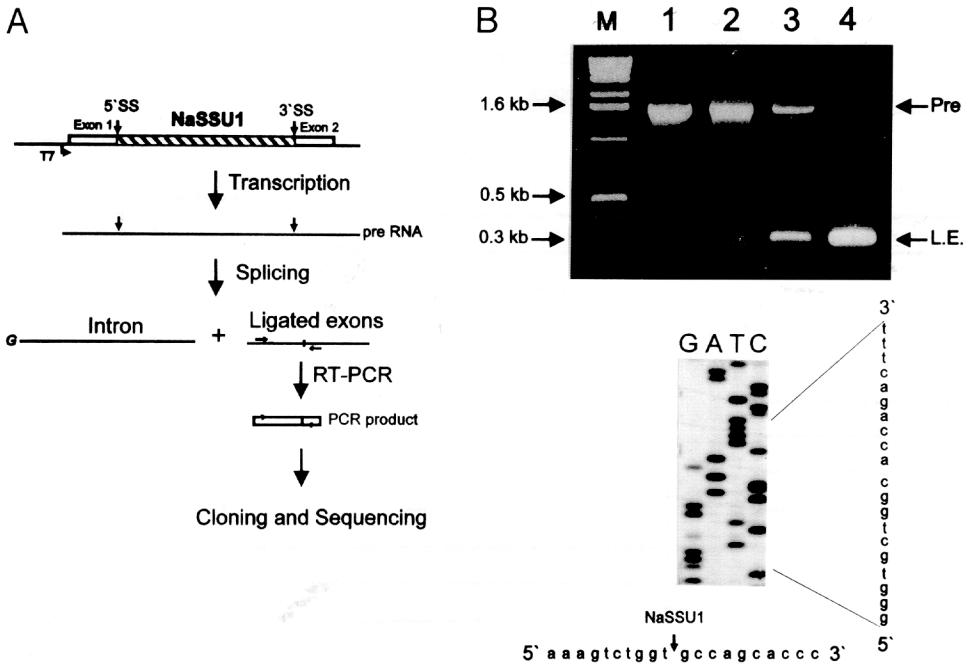


FIGURE 3. Self-splicing analysis by RT-PCR. **A:** Schematic presentation of the strategy. Transcribed RNA (preRNA) is subjected to self-splicing conditions, and ligated exons (LE) reverse transcribed, amplified by PCR, and subsequently cloned and sequenced. **B:** Top: Amplified products analyzed by agarose gel electrophoresis. Lanes 2 and 3 contain products generated from unincubated and incubated NaSSU1 intron RNA (Nan.wt-V) at self-splicing conditions, respectively, and lane 4 the intronless Nfo.wt RNA as a control. A small amount of ligated exon product is present in lane 2 due to self-splicing activity during transcription. Amplified pNanSSU DNA template is shown in lane 1. M, size marker. Bottom: Sequencing ladder of amplified ligated exon generated from Nan.wt-V. DNA sequence read from the reaction is complementary to the RNA sequence presented below. The intron insertion site is marked by an arrow.

A-residue at position 187 within the intron. We attribute this structure to a PCR artifact. A similar set of results was obtained for the intron constructs Nan.wt-P and Nan.G2, the former yielding full-length circles and the latter missing two nucleotides. All of these results are summarized in Figure 2, and lead to the conclusion that NaGIR2 is responsible for intron circle formation. Furthermore, full-length circles are observed only in intron constructs with large sequence insertions in P6.

NaGIR1 is responsible for internal processing of intron RNA

We presumed NaGIR1 to be responsible for internal processing because RNA from Nja.G1P7- was not processed at the IPS (Fig. 4A, lane 4). To confirm this function and to determine the approximate size of the functional NaGIR1, two small plasmid constructs were made to leave 130- and 267-bp upstream of the Nan.GIR1 IPS. The larger construct contains all the important sequences predicted from the secondary structure for NaGIR1 activity (Fig. 1), whereas the shorter was presumed to be inactive because it disrupts the P3, P10, and P15 structure segments. These assumptions were confirmed when the corresponding RNAs (Fig. 2, lines 13 and 14) were subjected to self-cleaving conditions. Here, only the larger RNA (Nan.267G1) cleaved at IPS (Fig. 7A, lanes 1 and 2). Identical results were obtained from Nit.GIR1 using the same approach (Fig. 2, lines 16 and 17; Fig. 7A, lanes 3 and 4). The functional size of Nan.GIR1, here estimated to 130–267 nt, has recently been delimited to about 180 nt (E. Jabri, pers. comm.). To precisely define the internal processing site, the self-cleaved Nan.267G1 and Nit.287G1 were subjected to primer extension analysis. Two internal processing sites (IPS1 and IPS2) located only 13-nt and 10-nt upstream of the ORF, respectively, were found (Fig. 7B). IPS1 and IPS2 correspond exactly to the internal processing sites observed in the related twin-ribozyme intron DiSSU1 in *Didymium* (Johansen & Vogt, 1994; C. Einvik, H. Nielsen, E. Westhof, F. Michel, & S. Johansen, in prep.).

DISCUSSION

We have shown that the *Naegleria* rDNA intron NaSSU1 consists of two distinct group I ribozyme elements, NaGIR1 and NaGIR2. NaGIR1 cleaves intron RNA at two internal sites close to the 5′ end of the ORF. NaGIR2 catalyzes intron excision and exon ligation, as well as full-length circle formation of excised NaSSU1 RNA. NaSSU1 has a twin-ribozyme organization, with NaGIR1 and the ORF being inserted into the L6b loop of NaGIR2 RNA.

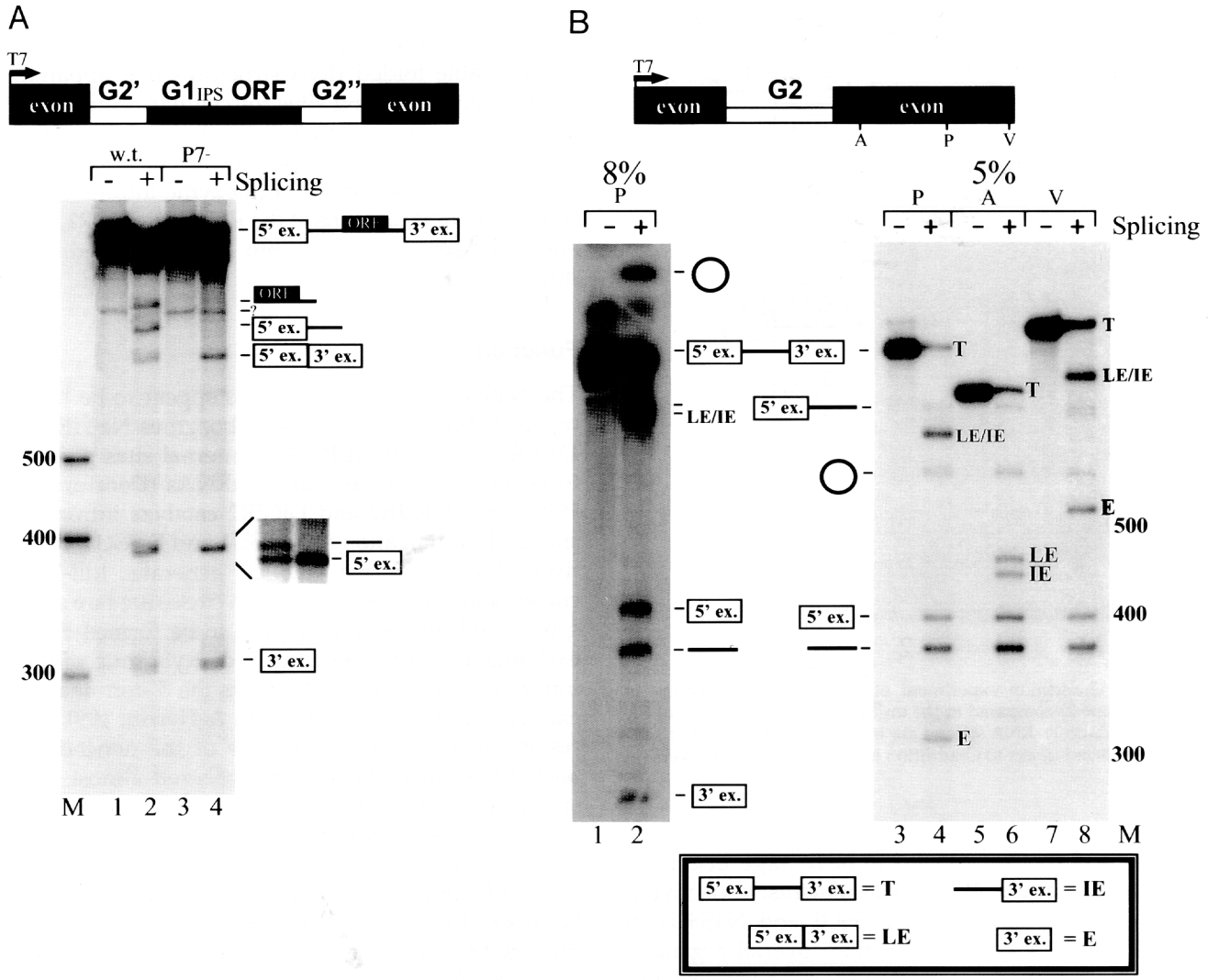


FIGURE 4. Gel analysis of self-splicing products. In vitro transcribed ^{32}P-labeled RNA was incubated at self-splicing conditions for 60 min at 50 °C. Schematic presentations of NaSSU1 and NaGIR2 are presented on top in A and B, respectively (see legend to Fig. 2). **A:** Analysis of NaSSU1 RNAs on a 5% polyacrylamide gel. Lanes 2 and 4 are wild-type (Nja.wt-P) and mutant (Nja.G1P7-) intron RNAs, respectively, incubated at splicing conditions (+). Lanes 1 and 3 show the unspliced samples (−) of the same RNAs. To the right, the 400-nt region is better resolved on an 8% polyacrylamide gel with the samples, left to right, corresponding to those in lanes 2 and 4, respectively. Certain species of RNA are indicated schematically. Intron circles appear to co-migrate with pre-RNA. An artifact that appears in each unspliced lane is also indicated (?). Lane M, RNA century size markers with size indicated to the left in nucleotides. **B:** Analysis of different NaGIR2 RNAs transcribed from pNjaGIR2; Nja.G2-P (lanes 3, 4), Nja.G2-A (lanes 5, 6), and Nja.G2-V (lanes 7, 8). Odd-number lanes are unspliced control reactions. All samples in lanes 3–8 were analyzed on a 5% polyacrylamide gel; pNja.G2-P in lanes 1 and 2 is analyzed on an 8% in order to distinguish topologically distinct species. P, A, and V are different restriction sites used in linearization of templates (see legend to Fig. 2). RNA species are represented schematically in between the two gels. Those species that involve the 3′ exon are diagrammed in the legend below and indicated by letters to the right on the 5% gel: primary transcript (T), ligated exons (LE), intron attached to 3′ exon (IE), and 3′ exon (E). To the right, where the RNA markers elsewhere on the 5% gel migrated is indicated in nucleotides. Minor band beneath the 5′-exon–intron band in lane 8 is uncharacterized.

Structure

In the collection of several hundred known group I introns, besides NaSSU1, there is only one other example of an intron with a twin-ribozyme organization. The mobile nuclear intron DiSSU1 in the slime mold *Didymium iridis* also has two ribozymes, one of which catalyzes splicing and the other hydrolytic cleavage upstream of the ORF for an endonuclease (Decatur et al., 1995). At the primary structure level, extensive differences are found between NaSSU1 and DiSSU1. They are located at different sites within SSU rDNA of *Naegleria* and *Didymium*, positions corresponding to 516 and 956, respectively (Johansen et al., 1996). The splicing ribozymes NaGIR2 and DiGIR2 are members of two more distantly related categories of group I ribozymes, the subgroups IC1 and IC4 (Michel & Westhof, 1990; F. Michel, pers. comm.). Finally, the

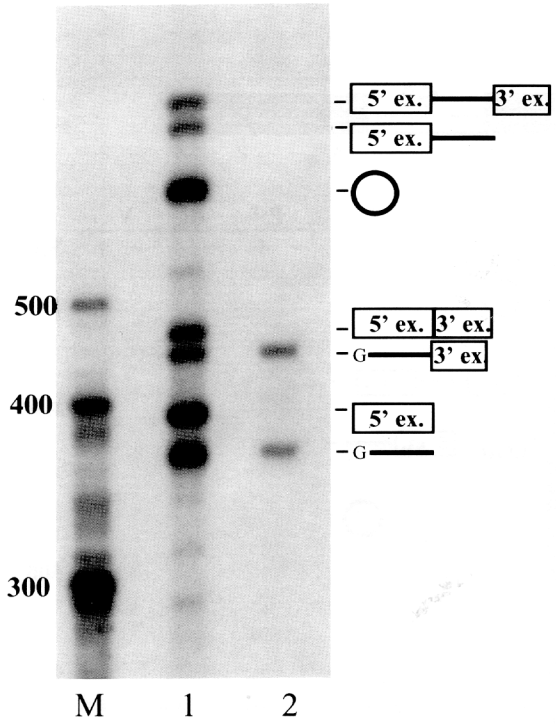


FIGURE 5. G-addition experiment of intron RNA. G-labeling of Nja.G2-A (lane 2) compared to the uniformed labeled pattern of the same RNA (lane 1). RNA species are indicated schematically to the right with those subject to G-addition indicated. M, size markers (in nucleotides).

NaGIR2 and DiGIR2 sequences have large insertions at different locations, P6 and P2 segments, respectively.

Despite these differences, DiSSU1 and NaSSU1 in fact are remarkably similar in their overall organization. In both introns, a small group I ribozyme followed by a homing endonuclease ORF is inserted into a splicing ribozyme. Both of the intron proteins contain the characteristic histidine- and cysteine-rich motif common among nuclear homing endonucleases (Johansen et al., 1993). The new secondary structure representation of group I introns, which includes information about tertiary interactions and orientation of helices (Cech et al., 1994), shows that both in NaSSU1 and DiSSU1 RNAs, the insertion of the ORF and small ribozyme is in one of the two parallel, or maybe more perpendicular (see Lehnert et al., 1996) helices that project in the same direction away from the catalytic core of GIR2 (Fig. 8). It has been observed previously that large ORFs always are located in the peripheral regions of group I intron RNAs, where they do not perturb RNA folding and ribozyme function. Although we have no direct evidence on the secondary and tertiary structure of the ORF in NaSSU1, sequence comparisons of the five NaSSU1 introns show what appear to be compensatory changes in predicted secondary structures in the ORFs (data not shown), consistent with a common folding pattern. It may be that the folding of the ORF RNA also is constrained by interactions with GIR1 or GIR2. For the mobile introns of archea, it has been reported that the ORF RNAs form highly stable folded RNA structures that apparently create specific binding sites for cellular proteins, consistent with the observed stability of excised intron RNA in vivo (Lykke-Andersen & Garrett, 1994). In summary, we propose that twin-ribozyme introns contain three structural domains at the RNA level (GIR1, GIR2, and ORF), all organized into a well-defined three-dimensional shape.

Function

The NaSSU1 and DiSSU1 RNAs appear to be homologous in function. The small ribozymes NaGIR1 and DiGIR1 cleave intron RNA at internal sites proposed to generate 5′ ends of intron mRNAs (Decatur et al., 1995), and NaGIR2 and DiGIR2 catalyze intron excision and exon ligation of NaSSU1 and DiSSU1, respectively. We found that NaSSU1 generates full-length circles of intron RNA. Full-length circles are rare among group I introns, and suggested to be formed by a G exchange reaction where the hydroxyl group of the 3′ intron terminal G residue attacks the 5′-terminal G of excised intron RNA (Thompson & Herrin, 1991). This results in the circle and release of the noncoded G. Such RNA circles have been observed almost exclusively in protein-coding group I introns, including the DiSSU1 intron (Thompson & Herrin, 1991; Decatur et al., 1995; Beagley et al., 1996). Different from DiSSU1, the full-length circles in NaSSU1 appear somehow to be linked to wild-type organization of RNA because the NaGIR2 construct with deleted ORF and GIR1 generated RNA circles truncated by two nucleotides. Formation of full-length RNA circles is a well-known property of endonuclease-coding archea rDNA introns (Kjems & Garrett, 1988; Dalgaard & Garrett, 1992; Burggraf et al., 1993), and has been suggested to be involved in intron transfer among archeal cells (Lykke-Andersen & Garrett, 1994; Aagaard et al., 1995). The fact that the entire genetic information of the intron is preserved in such RNA circles, and that circular RNAs appear to be stable in vivo, make these RNA species interesting as candidates for horizontal transfer of introns between species. In this manner, they might be analogous to the infectious full-length RNA circles of viroids and virusoids (Diener, 1989; Johansen & Vogt, 1994).

Evolution

Naegleria and *Didymium* are genera that belong to different deep branching protist phyla, the Zoomastigina and the Myxomycota, respectively (Margulis & Schwartz, 1988), but their haploid amoebo-flagellate stages are remarkable similar. *Didymium* has an additional diploid stage initiated by sexual fusion between

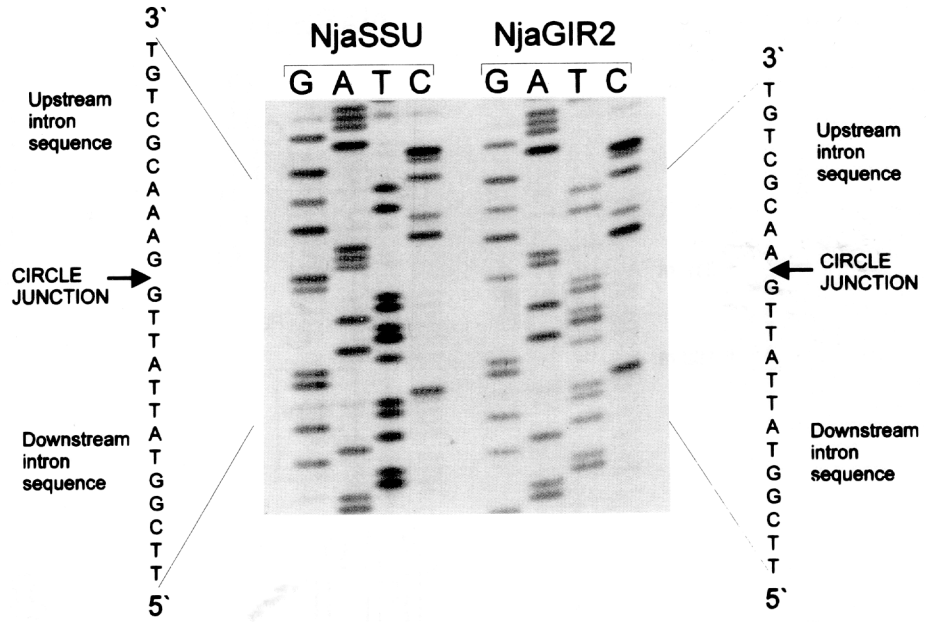


FIGURE 6. Analysis of intron RNA circle junctions. Regions corresponding to circle junctions were amplified by an inverse RT-PCR strategy from spliced intron RNA, and subsequently cloned and sequenced. Left: sequence of full-length intron circle junction from wild-type intron NjaSSU (Nja.wt-P RNA). Right: sequence of intron circle junction generated from NjaGIR2 (Nja.G2-P RNA). The corresponding DNA sequences are shown.

haploid amoebae of different mating types. DiSSU1 intron mobility was demonstrated recently by mating experiments. The intron was found to home into intronless alleles of SSU rDNA, a process initiated by a double-strand break made by the intron-encoded homing endonuclease (Johansen et al., 1997). By contrast, *Naegleria* species are not known to reproduce sexually, thus maybe preventing NaSSU1 homing. However, evidence for genetic exchange in natural populations of one intron-lacking *Naegleria* species (*N. lovaniensis*) has been reported, suggesting occasional sexual reproduction (Pernin et al., 1992). The distribution of introns in the *Naegleria* genus sheds light on the natural history of these genetic elements. De Jonckheere (1994) compared the phylogenetic pattern of five NaSSU1 introns with that of the corresponding SSU rRNA gene itself, and concluded that the origin of NaSSU1 was ancestral in *Naegleria*. A small version of NaSSU1, corresponding to NaGIR2 in our study, was observed in one lineage and explained by ORF deletion (De Jonckheere & Brown, 1995). We have extended these analyses to include all available NaSSU1 intron sequences, and have made separate comparisons of the three intron domains (NaGIR1, NaGIR2, and ORF), and SSU rDNA sequences with or without NaSSU1 (De Jonckheere, 1994a, 1994b; data not shown). The simplest interpretation is that, following an ancestral insertion of NaSSU1, the intron was subsequently lost in all but a few lineages during the evolution of the *Naegleria* genus. No supporting evidence was found for either shuffling of intron-domains between existing NaSSU1 introns, or intron-gain by horizontal transfer between lineages. Thus, it is remarkable that the present-day NaSSU1 introns found in this purported asexual eukaryotic microorganism still express conserved nonsplicing functions, namely internal processing of the intron RNA and homing endonuclease activity (M. Elde, P. Haugen, N.P. Willassen, & S. Johansen, in prep.). The long-term maintenance of these functions, which are known to be or expected to be essential only for intron homing, suggests either that sexual fusion occurs within these species, or that intron functions might have some beneficial function for the organism. These possibilities remain to be explored.

MATERIALS AND METHODS

Naegleria species and intron sequences

DNA from the *Naegleria* species *N. jamiesoni*, *N. andersoni*, *N. italica* (species definitions according to De Jonckheere, 1994a; Table 1) and *N. fowleri* (strain MCM) were kindly provided by Dr. S. Kilvington (Royal United Hospital, Bath, UK). The SSU rDNA segments of interest were amplified by PCR using the forward primer 1Ef and reverse primer 892r (Table 2) described by Embley et al. (1992b). After PEG precipitation, products were sequenced directly on both strands using manual TAQ cycle sequencing according to Embley (1991). The Nja.SSU1, Nan.SSU1, and Nit.SSU1 intron sequences were identical to the corresponding introns in *N. jamiesoni* (strain T56E), *N. andersoni* (strain A-2), and *N. italica* (strain AB-T-F3), respectively, reported by De Jonckheere (1994b), except the following positions. (Nja.SSU1): C-insertion at A_4, $C_{421} \rightarrow T$, $G_{475} \rightarrow C$; (Nan.SSU1): $T_{885} \rightarrow C$;

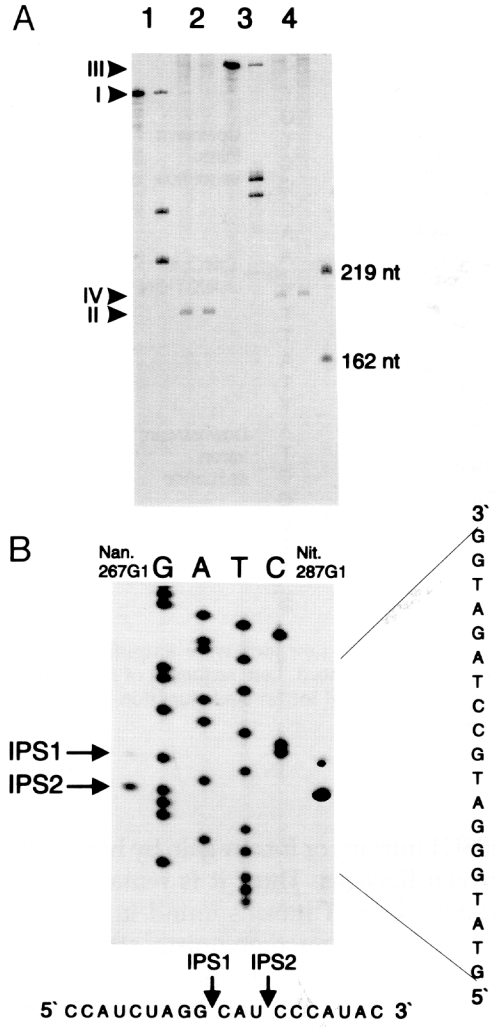


FIGURE 7. Gel analysis of NaGIR1 self-cleavage. **A:** In vitro-transcribed ^{35}S-labeled RNAs were uncleaved (−) or incubated at self-cleaving conditions (+) for 60 min at 50 °C. Lanes 1–4 contain the RNAs Nan.267G1, Nan.130G1, Nit.287G1, and Nit.140G1. The primary transcripts of Nan.267G1 (528 nt), Nan.130G1 (192 nt), Nit.287G1 (634 nt), and Nit.140G1 (202 nt), are indicated by I, II, III, and IV, respectively. Calculated sizes observed after self-cleaving of Nan.267G1 and Nit.287G1, respectively, are: 5′ fragment (290 nt and 308 nt); 3′ fragment (238 nt and 326 nt). Note that the 5′ fragments of Nan.267G1 and Nit.287G1 contain 23 nt and 21 nt, respectively, of vector sequences. M is a size marker. **B:** Primer extension mapping of the internal processing sites IPS1 and IPS2. Primer extension products generated from Nan.267G1 and Nit.287G1 were analyzed together with the DNA sequence of pNan267G1. All reactions were primed by OP-27 (Table 2). The DNA sequence (right) is complementary to the RNA sequence (below).

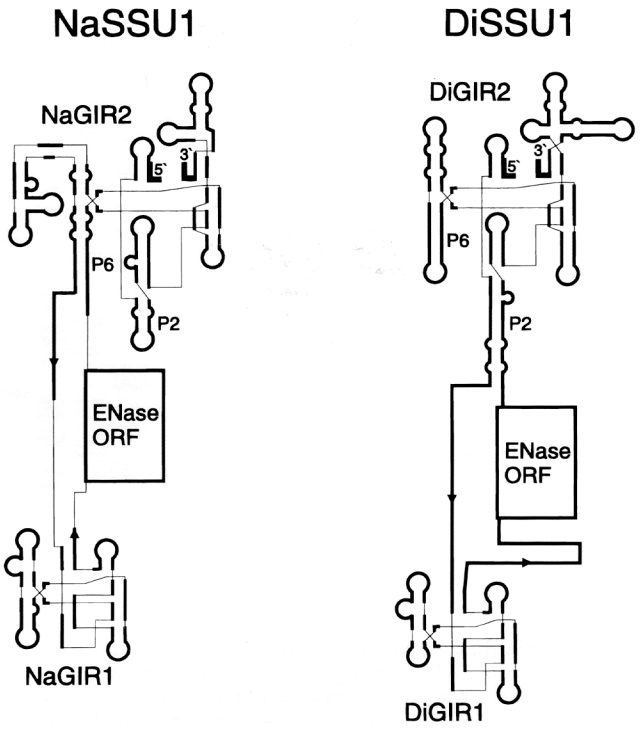


FIGURE 8. Conserved RNA structure features of the *Naegleria* (NaSSU1) and *Didymium* (DiSSU1) twin-ribozyme introns. Only the GIR2 paired segments P2 and P6 are labeled. ENase ORF, open reading frame encoding a functional homing-type endonuclease. The secondary structure presentation is according to Cech et al. (1994).

(Nit.SSU1): $A_{131} \rightarrow \Delta$, $C_{132} \rightarrow \Delta$, $G_{135} \rightarrow \Delta$, $A_{371} \rightarrow G$, $A_{402} \rightarrow T$, $T_{1048} \rightarrow A$, $A_{1095} \rightarrow C$. Numbering of intron positions is according to De Jonckheere (1994b). The rDNA sequences of *N. jamiesoni*, *N. andersoni*, *N. italica* (see Table 1), and *N. fowleri* (strain MCM) have been assigned the accession numbers U80250, Z16417, U80249, and U80059, respectively, in the EMBL/GenBank/DDBJ databases. After digesting amplified rDNA products with *Nhe* I and *Pst* I, intron-containing products from *N. jamiesoni*, *N. andersoni*, and *N. italica* rDNA (2 kb) and intronless product of *N. fowleri* (0.7 kb) were cloned into *Xba* I–*Pst* I-cut pGEM3Zf+ (Promega Biotech), yielding pNjaSSU, pNanSSU, pNitSSU, and pNfoSSU, respectively. In order to isolate DNA from the *N. gruberi* strains, the amoebae were grown according to established protocols (Fulton, 1970) and DNA prepared from amoebae scraped off plates essentially by a method described for yeast DNA isolation (Ausubel et al., 1989). *N. gruberi* strain EG_B (ATCC 30133) was obtained from the American Type Culture Collection and strain NB-1 was kindly provided by Dr. C. Walsh. The SSU rDNA segments of interest were amplified by PCR using primers WD-118 and WD-48 with a mixture of *Pfu* polymerase (Stratagene) and Vent Polymerase (New England Biolabs). The products (0.7 kb for intronless EG_B and 2 kb for NB-1) were cut with *Nhe* I and *Pst* I and cloned into *Xba* I–*Pst* I-cut pGEM3Z (Promega) to yield pNgrSSU(−) and pNgrSSU(+), respectively. The rDNA sequence of a clonal derivative of EG_B, NEG-M, was assigned GenBank accession number M18732 (Clark & Cross, 1988). The sequence of a segment of the SSU rDNA of another member of the same species cluster as NB-1 has been reported by De Jonckheere (1994a, 1994b).

Plasmid constructions

The plasmids pNjaG1P7$^-$, pNjaΔ298, pNjaGIR2 were all generated from pNjaSSU. pNjaG1P7$^-$ is identical to pNjaSSU1 except a G → C mutation at the guanosine binding site of Nja.GIR1 (Fig. 1) and was made by inserting a 347-bp *Xba* I–*Bgl* II-cut PCR product into pNjaSSU. The PCR product had been generated using primers WD-96 and WD-87 (Table 2)

TABLE 2. Primer sequences.

Name	5′-3′ sequence	Position[a]
1Ef	AYCTGGTTGATYYTGCCAG	E-20
892r	AGAATTTCACCTCTG	E-1061C
OP-25	CTCGAATTCGCTCTTGGAGCTGGAATTA	E-663C
OP-26	ACGAAGCTTATTTCTAAGCCT	I-444C
OP-27	CTCGAATTCCAAAACGGGACCTCTG	I-292
OP-28	CAGAGGAGTTTCTTACCTATCA	E-388
OP-51	GCAAGGTGCTAATCACAC	I-1282
OP-188	GCTTGGCTGCGGATTACG	I-192C
WD-42	TGAATTCCGGAGAGGAGC	E-464
WD-48	GCCTGCAGTCCCATGCTAAATCGCT	E-940C
WD-51	AATTGACGCTAGCGTC	NA
WD-61	GCACCTTGCAATTGACACAC	I-1254C
WD-71	TCAACTAGTGGGTTTGGACT	I-1228C
WD-77	AATGCCATGGATTTGTTTACCAAATG	I-209C
WD-78	ATCCATGGCATTTGTTTCCAAGCGCAG	I-1180
WD-87	GATCGTCCTAGCATCAGGAT	I- 931C
WD-96	GAACGTCTAGACACTACACGGTAGACC	I-360
WD-118	ATCGACCGCTAGCAGGTGC	E-294

[a]Primers are numbered relative to the position of the 3′ nucleotide of the reported *N. gruberi* SSU rRNA gene exon sequence (*E*; Clark & Cross, 1988) or the Nja.SSU1 intron sequence (*I*; this work). *C*, indicates a complementary primer to those sequences. The base substitution in WD-96 used to introduce the mutation in the P7 helix of GIR1 is underlined. NA, not applicable.

with pNjaSSU as template. pNjaΔ298 contains an internal 298-bp *Xba* I–*Hinc* II deletion, disrupting most of NjaGIR1 and 5′ end of the ORF. pNjaGIR2 was made by replacing all the sequences corresponding to Nja.GIR1 and ORF with an *Nco* I site, resulting in a short P6b stem-loop structure at the RNA level (Fig. 1). This was accomplished by two rounds of PCR. First round consisted of two PCRs with pNjaSSU template and primer combinations of WD-42 with WD-77 and WD-61 with WD-78. Second round used those two products with primers WD-42 and WD-71. Finally, the *Bss*H II–*Afl* II-cut product was inserted into cut pNjaSSU+Nhe. pNjaSSU+Nhe was made as an intermediate to facilitate future cloning by replacing the *Eco*R I site just downstream of the T7 promoter in the multiple cloning site of pNjaSSU with self-hybridized oligo WD-51. pNjaGIR2 is identical to NjaGIR2, but in the original pNanSSU background. pNan267G1 was made from pNanSSU by deleting all the rDNA sequences upstream the internal *Xho* I site, leaving the complete Nan.GIR1 (267-bp upstream IPS1) and all downstream sequences in the construct. Plasmids pNit287G1 was made by the same approach, but from pNitSSU using an internal *Kpn* I site instead of *Xho* I. pNan130G1 and pNit140G1 were made from pNanSSU and pNitSSU, respectively, by PCR using primers OP-26 and OP-27. *Hind* III–*Eco*R I-cut fragments were cloned into the corresponding sites of pGEM3Zf(+). All plasmid manipulations were confirmed by DNA sequencing.

In vitro transcription, splicing and processing reactions

In vitro transcription, RNA splicing, and RNA processing were performed essentially as described previously (Johansen & Vogt, 1994; Decatur et al., 1995). Here, RNA was transcribed by T7 RNA polymerase off the linearized DNA either unlabeled or with [α^{35}S]ATP (10 μCi/μL; Amersham) or [α^{32}P]UTP (10 μCi/μL; Amersham) as the label and was subjected to either self-splicing or self-cleaving conditions. Self-splicing was performed at 50 °C for 30–60 min 40 mM Tris-HCl, pH 7.5, 7 mM $MgCl_2$, 125 mM KCl, 2 mM spermidine, 5 mM dithiothreitol, and 0.2 mM GTP. The G-labeling experiment was conducted as described in Johansen and Vogt (1994) in the presence of 50 μCi [α^{32}P]GTP (10 μCi/μL; Amersham) following use of a G-50 Sephadex spin column (Boehringer Mannheim) to separate the cold nucleotides from the transcripts. RNA subjected to self-cleaving was incubated for 60 min at 50 °C in high salt conditions similar to self-splicing, but at 500 mM KCl, 25 mM $MgCl_2$, and without added GTP. Processed RNA was analyzed on gels as described by Decatur et al. (1995), by primer extension, or RT-PCR (see below).

Primer extension analysis

pNan267G1 and pNit287G1 were linearized with *Hinc* II and *Hind* III, respectively, in vitro transcribed, and subjected to self-cleaving conditions. The RNAs were annealed to primer OP-26 (Table 2), and RT reactions were performed as described previously (Ruoff et al., 1992; Johansen & Vogt, 1994) using the Superscript Reverse-transcriptase kit (Gibco BRL) and [α^{35}S]dATP (12.5 μCi/μL; New England Nuclear) as the label. Sequencing reactions of the pNanG1-267 were prepared in parallel using OP-26 and run adjacent to the primer extension products as markers. Sequences of pNan267G1 and pNit287G1 are identical in the analyzed region.

RT-PCR and computer analysis

To analyze the ligated exon and circle-junction sequences, transcribed and self-spliced intron RNAs were subjected to reverse transcription using the First-Strand cDNA Synthesis kit (Pharmacia) and a downstream primer. The ligated exon region was reverse transcribed from primer OP-25, the purified cDNA amplified from primers OP-25 and OP-28 (Table 2), cloned into pUC18 using the SureClone Ligation Kit (Pharmacia Biotech), and sequenced in both directions using the PCR Product Sequencing kit (US Biochemicals). A similar approach was used to analyze circle-junctions, except that the primer OP-188 was used in the RT-reaction and OP-51 and OP-188 (Table 2) in PCR. The amplified products were cloned into pUC18 and sequenced using the AmpliCycle Sequencing kit (Perkin Elmer). Computer analysis of nucleic acid sequences was performed using the software package programs from the Genetics Computer Group (Madison, Wisconsin), DNASTAR, Inc. (Madison, Wisconsin), and GeneCompar, Applied Maths Inc. (Kortrijk, Belgium).

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