Requirements for self-splicing of a group I intron from Physarum polycephalum

Gail Auger Rocheleau and Sarah A.Woodson*
Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742-2021, USA

ABSTRACT

The third intron from *Physarum polycephalum* (Pp LSU 3) is one of the closest known relatives to the well-studied *Tetrahymena* group I intron. Both introns are located at the same position in the 26S rRNA gene, and with the exception of an open reading frame in Pp LSU 3, are highly homologous. While Pp LSU 3 has been shown to self splice, little is known about its activity *in vitro*. We have examined the requirements for self splicing in greater detail. Despite its similarity to the *Tetrahymena* intron, Pp LSU 3 is 1500-fold less reactive, demonstrates a preference for high salt, and exhibits a low K_m for GTP. Removal of the open reading frame results in a modest increase of activity. This system provides an opportunity to understand how sequence variations in two related introns alter the efficiency of autoexcision, and how this relates to adaptation of group I introns to their particular sequence context.

INTRODUCTION

Group I introns are a class of autocatalytic intervening sequences that are found in both bacterial systems and in the organellar and nuclear genomes of lower eukaryotes (1). A number of these have been shown to self splice *in vitro*, as typified by the well-studied intron from *Tetrahymena thermophila* rDNA (Tt LSU) (2). The self splicing mechanism common to all group I introns involves two phosphodiester transesterifications and depends on an exogenous guanosine or GTP cofactor and magnesium ion (3). Many of the interactions responsible for assembly of the catalytic site within the intron RNA and for correct positioning of the splice sites have been identified (2, 4). Nonetheless, sequence variation between introns results in widely differing reaction characteristics. A comparison of a number of examples is important for understanding the basis of RNA catalysis.

Excision of group I introns found in pre-ribosomal and transfer RNAs presents a further challenge. In these transcripts, the high degree of secondary structure in the exons has the potential to interfere with splice site recognition (5). For example, in Tt LSU precursor RNAs, ribosomal RNA structure 5' and 3' of the intron

can attenuate 5' splice site activity (6). This arises from inhibition of the P1 pairing between the 5' exon and the internal guide sequence (IGS) of the intron (7).

A ribosomal group I intron that is of particular interest is the third intron in the 26S gene of the slime mold *Physarum polycephalum* (Pp LSU 3). This optional intron is found in the Carolina strain of *P.polycephalum* (8), and interrupts the sequence of the 26S rRNA in the same location as the *Tetrahymena* intron (8, 9). Despite the fact that these species are not closely related, the catalytic cores for the two introns have a very similar secondary structure (see Figure 1) and about 90% sequence homology (9, 10). In addition, the predicted rRNA secondary structures near the splice junction are identical (11). Thus the Tt LSU and Pp LSU 3 introns would appear to be related in terms of both internal structure and exon context. They might be expected to have similar strategies for optimizing splice site selection and intron excision.

Pp LSU3 has been shown to self splice *in vitro* via the general group I splicing mechanism (10). In addition to cleavage at the 5' and 3' splice sites shown in Figure 2, a second G addition occurs at an internal processing site (IPS), dividing the intron RNA into two fragments. The 3' half contains the catalytic core and is similar to the *Tetrahymena* intron. The 5' half, however, was shown to contain a large open reading frame (ORF) followed by a 3' untranslated region (3' UTR) (8). The ORF encodes a DNA site-specific endonuclease that is responsible for homing of Pp LSU 3 into strains that lack the intron (8, 9). Other minor products arise from cleavage at an alternative 5' splice site 63 nucleotides upstream (10).

Although the *in vitro* reaction products of Pp LSU 3 have been identified (10), little is known about the requirements for self splicing. In order to provide a basis for comparison of Pp LSU 3 and other ribosomal RNA introns, we have undertaken a more quantitative characterization of the splicing reaction. Conditions were optimized, the effect of the ORF on 5' splice site activity was examined, and the apparent K_m for GTP determined. Pp LSU 3 is much less active than Tt LSU *in vitro*. The major structural difference between these introns is the ORF in L1. This insertion could affect recognition of the 5' splice site in *Physarum*. Surprisingly, removal of the ORF results in only moderate improvement of splicing activity.

*To whom correspondence should be addressed
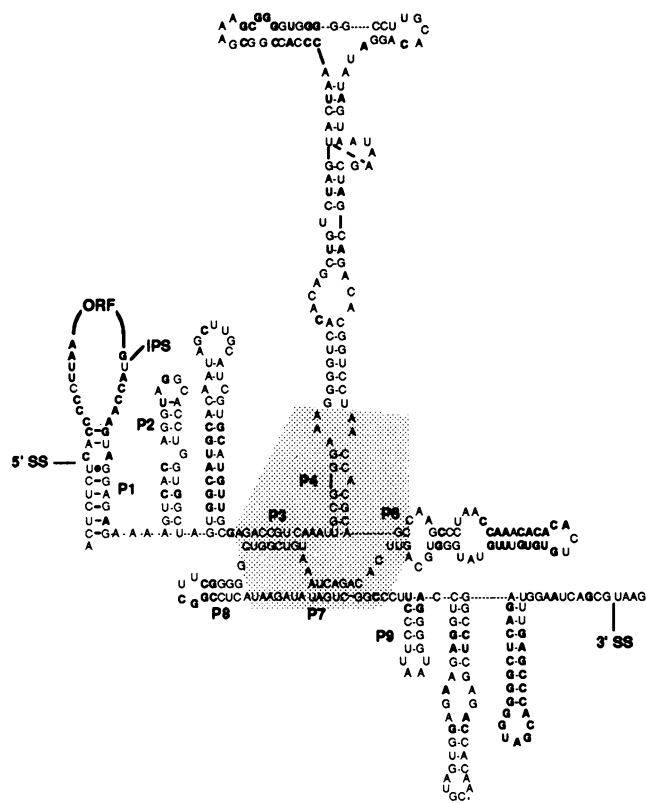
Figure 1. Predicted secondary structure of *Physarum* intron 3. Nucleotides that differ between Pp LSU 3 and Tt LSU are in bold print. The P8 helix is 2 bp shorter, P9.1 is 1 bp shorter and P9.2 is 9 bp shorter than the Tt LSU counterparts. The conserved core region is shaded, and the positions of the 5' and 3' splice sites, the internal processing site (IPS) and the open reading frame (ORF) are indicated. Structure adapted from refs 10 and 35.

MATERIALS AND METHODS

Construction of plasmids

Plasmids I3-BS and pI3Ex153 were the generous gift of V.Vogt. pI3-BS contains a *Eco*RI−*Cla*I fragment that encodes the 941 bp of Pp LSU 3 along with rRNA sequences beginning 378 bp upstream and ending 24 bp downstream of the splice junction (10). pI3Ex153 is the same, but with 153 bp of 5' exon sequence. The *Eco*RI−*Sal*I fragment of pI3-BS was inserted into the multiple cloning site of pTZ18U (BioRad) to create pI3TZ. This vector was chosen because it adds only 4 nucleotides to the transcribed sequences between the T7 promoter and the *Eco*RI site, as compared to pI3-BS, in which the *Eco*RI site is 64 bp away from the promoter.

The open reading frame of Pp LSU 3 was deleted in pI3ΔORFTZ by oligonucleotide-directed mutagenesis (12) and an *Nde*I site was created at the site of the deletion. Plasmid DNA containing the desired mutation was identified by restriction maps and dideoxy sequencing (Sequenase 2.0; US Biochemicals).

In vitro transcription

Uniformly radiolabeled precursor RNA was prepared by T7 RNA polymerase transcription of plasmid DNA linearized with *Hin*dIII or *Cla*I in the presence of [α-^{32}P]ATP (New England Nuclear). Transcription reactions were incubated at 30°C for 30 min (10), then immediately passed over a G-100 gel filtration spin column
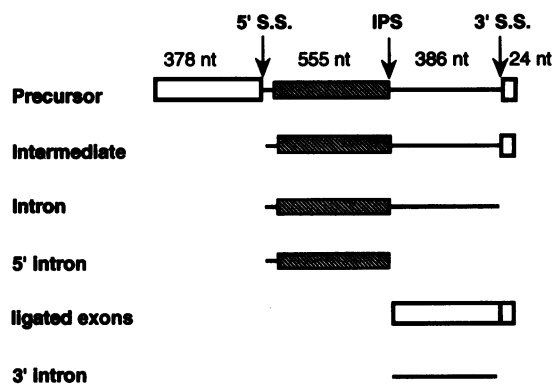
Figure 2. *P.polycephalum* precursor RNA and spliced products. The RNA precursor encoded by pI3TZ is shown with primary sites of reaction and the length of segments in nucleotides. The major products are illustrated below the initial transcript in decreasing length. Open rectangle, exons; striped rectangle, open reading frame; line, intron sequences. 5' S.S. and 3' S.S. indicates the 5' and 3' splice sites respectively; IPS, internal processing site.

(Clontech) equilibrated in 6 mM MgCl$_2$, 10 mM Tris−HCl, pH 7.5, 1 mM EDTA.

Self splicing reactions

Splicing reactions were carried out in 0.5 M KCl, 40 mM Tris−HCl, pH 7.5, 6 mM MgCl$_2$, 5 mM DTT, 2 mM spermidine unless stated otherwise. Reactions were initiated by the addition of GTP to a final concentration of 100 μM. Aliquots were removed at specified time intervals, added to equal volumes of 10 M urea plus tracking dyes, and electrophoresed on 8 M urea−4% polyacrylamide gels. The radioactivity in each lane was quantitated on a Molecular Dynamics PhosphorImager and the rate constants (k_{obs}) determined from linear fits to ln(1 −f_{sp}) vs. time as described previously (13). The fraction of spliced products (f_{sp}) is based on the appearance of ligated exons.

GTP labeling experiments were carried out under self splicing conditions as described above in the presence of [α-^{32}P]GTP (New England Nuclear) and non-radioactive precursor RNA. Samples from the reaction were added to 10 mM Tris−HCl, pH 7.5, 2 mM EDTA, 2 M NH$_4$ acetate, 0.05 μg/μl carrier RNA, and precipitated with 3 volumes of ethanol (14).

K_m determination for GTP

Uniformly labeled precursor RNA was prepared from pI3-BS, and self splicing reactions were carried out as described above. Reactions contained 0.15 μM RNA, and GTP concentrations were varied from 10 to 100 μM. Samples were removed at specified time intervals over 2 h and reactions were incubated overnight to reach an end point. Typically 30−40% of the precursor RNA was consumed in 2 h, depending on the initial concentration of GTP. Initial splicing rates, quantitated as above and normalized to the total extent at 24 h, were multiplied by the RNA concentration to give initial velocities (15). The K_m for three trials was obtained from a double reciprocal plot of initial velocity versus substrate concentration. GTP concentrations below 10 μM were not used, because the products became difficult to detect.

Trans-splicing reactions

Splicing reactions were carried out as described above, but in the absence of GTP and in the presence of 1−100 μM 5' exon

RNA, 5'-rGGCUCUCU. The 8 nucleotide RNA was prepared as described (16). Samples were electrophoresed on 8 M urea−6% polyacrylamide gels. Initial *trans*-splicing rates for 30 min were quantitated as described above, except f_{sp} is equal to the fraction of ligated oligonucleotide−3' exon in each lane, corrected for the specific activity of the product.

Sequencing of spliced products

RNA transcripts were prepared by T7 RNA polymerase transcription of 10 μg linearized plasmid DNA in a 1 ml reaction with 1 mM of each nucleotide. After 1 h at 30°C, KCl was added to a final concentration of 0.5 M. Following a further 2.5 h at 30°C, the RNA was precipitated by the addition of 3 volumes of ethanol. RNA products were separated on a 4% polyacrylamide−8 M urea gel, visualized by UV light and the bands excised from the gel. The RNA was soaked from the gel matrix overnight into 10 mM Tris−HCl, pH 7.5, 1 mM EDTA, 0.25 M NaCl, extracted with phenol and chloroform and precipitated with ethanol. Products were sequenced by primer extension using AMV reverse transcriptase (Life Sciences) in the presence of dideoxynucleotides (17).

For enzymatic sequencing, spliced products were prepared as described above except after splicing, the RNA mixture was passed over a Sephadex G-50 spin column (Pharmacia). The RNA was treated with calf intestinal phosphatase (Promega), extracted with phenol and chloroform and then 5' end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (New England Nuclear). The labeled products were isolated and purified as above. RNA was digested with riboendonucleases T1, U2, (Calbiochem) or Phy M (Pharmacia) or subjected to limited alkaline hydrolysis as described (18). Reactions were immediately loaded onto a 6 or 8% polyacrylamide sequencing gel.

RESULTS

Optimization of self splicing reactions

A more detailed investigation of the self splicing activity of Pp LSU 3 was carried out by using gel filtration to remove excess nucleotide triphosphates and salts without denaturation of the transcript. Isolation of precursor RNA from a denaturing polyacrylamide gel resulted in irreversible inactivation of Pp LSU 3. Attempts to renature the RNA by heating and cooling in the presence of Mg^{2+} failed to restore activity (data not shown), although this method is used successfully in the preparation of *Tetrahymena* pre-RNA (19).

Reaction conditions were optimized by varying the concentration of $MgCl_2$, monovalent salt, and temperature. Magnesium concentrations above 6 mM did not improve the extent of splicing at 2 h, and even appeared to slightly inhibit product formation (data not shown). The ability of monovalent ions to stimulate self splicing varies greatly among group I introns (20). Among the salts NaCl, NH_4Cl, $(NH_4)_2SO_4$ and KCl, NaCl and $(NH_4)_2SO_4$ appeared to inhibit splicing of Pp LSU 3, while 0.5 M KCl and 0.5 M NH_4Cl resulted in higher activity (Figure 3a). When the concentration of KCl was varied from 0.1 to 1 M, product formation was maximal between 0.5 and 1 M KCl.

Reaction temperatures were also varied from 25 to 60°C (Figure 3b). Splicing activity, as judged by the amount of ligated exons and free intron RNA formed in 2 h, increased with temperature up to 50°C. Between 37 and 50°C, however, side reactions such as 3' splice site hydrolysis were also increased.
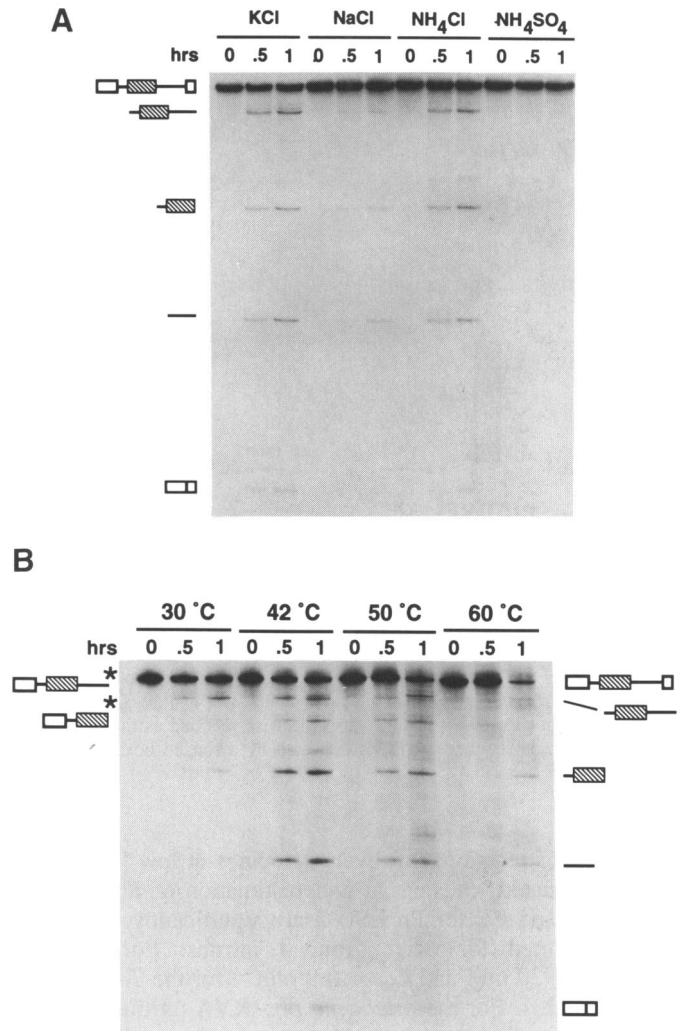

Figure 3. Effect of salt and temperature on self splicing of Pp LSU 3. (**A**) Uniformly radiolabeled pre-RNA (pI3153Ex) was incubated in 40 mM Tris−HCl, pH 7.5, 6 mM $MgCl_2$, 5 mM DTT, 2 mM spermidine and 100 μM GTP. The reaction mixture was supplemented with 0.5 M monovalent salt, as indicated above the lanes. Reactions were at 30°C for 0, 0.5, and 1 h. Samples were separated by 4% polyacrylamide gel electrophoresis. Products are keyed as in Fig. 1. (**B**) Self splicing reactions containing 0.5 M KCl were carried out as in (A). RNA was incubated at 30, 42, 50 and 60°C for 0, 0.5 and 1 h as indicated above the lanes. Reactions at other temperatures are not shown. Products of splicing are indicated on the right, while products of hydrolysis are labeled with an asterisk to the left of the figure.

For this reason, subsequent splicing reactions were carried out at 30°C. This temperature is also nearer to the optimal physiological temperature (22−26°C) for *P.polycephalum* (21).

K_m for GTP

Although the self splicing reaction does not proceed with multiple turnover, an apparent Michaelis constant can be determined for the guanosine co-factor (15) and reflects saturation of the G binding site. The apparent K_m for GTP was measured for Pp LSU 3 by a similar method used for the *Tetrahymena* intron (15). At 30°C, K_m(GTP) was 7 μM ± 1.3 and the average value for k_{cat} was 0.028 ± 0.001 h^{-1}. These values reflect the average of three determinations, and are reasonably precise. Due to the
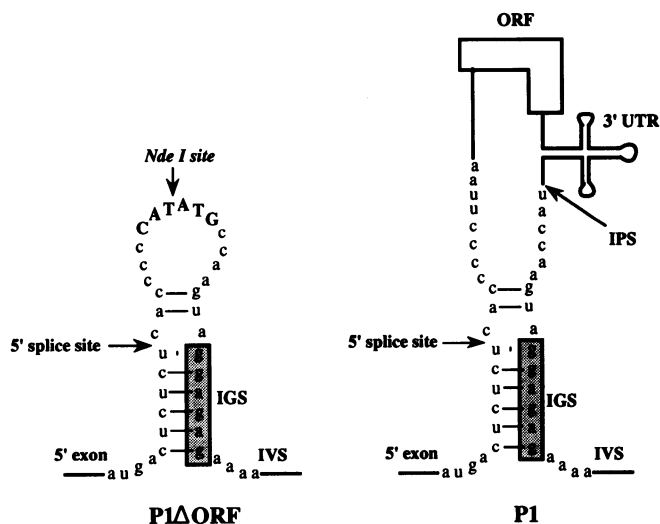
Figure 4. Structure of P1 in Pp LSU 3 and Pp LSU 3-ΔORF. The sequence of the wild-type P1 pairing is shown with the 550 nt ORF insertion and 3′ UTR (untranslated region) indicated schematically. The figure on the left shows the deletion of the ORF and insertion of an NdeI restriction site (bold type) in Pp LSU 3-ΔORF, creating a stem–loop similar in size to P1 in the Tt LSU intron. The natural sequence of Pp LSU 3 was otherwise retained as far as possible. The internal guide sequence (IGS) is indicated by a shaded box.

low extent of reaction, however, velocities at low [GTP] may be overestimated, causing an underestimation of K_m.

Both K_m and k_{cat} for Pp LSU 3 are significantly lower than values reported for other group I introns. For example, K_m(GTP) = 30 mM and k_{cat} = 0.9 min^{-1} for the *Tetrahymena* intron (22, 23). For the *Anabaena* pre-tRNA intron, K_m(GTP) = 240 mM and k_{cat} = 14 min^{-1} under similar conditions (24), and for sunY, K_m = 850 mM (25). Although we are unable to measure k_{cat}/K_m directly, from the Michaelis–Menton plot it is approximately 70 M^{-1} min^{-1}, two to three orders of magnitude below that of the other introns. Because k_{cat} is very low for Pp LSU 3, it is likely that the value of K_m is similar to or smaller than the dissociation constant for GTP.

Deletion of open reading frame

The poor reactivity of Pp LSU 3 in comparison with Tt LSU might be due to the presence of the large open reading frame (ORF) in L1. This insertion may destabilize P1 and impair 5′ splice site recognition. In other group I introns that contain open reading frames, such as the phage T4 introns and intron 5 of yeast mitochondrial cytochrome b pre-mRNA, it has been observed that the ORF is not necessary for self splicing activity (1, 26, 27). Accordingly, the ORF and the 3′ untranslated region (3′ UTR) were deleted from Pp LSU 3 as diagrammed in Figure 4. To minimize any effects from transcribed vector sequences on the splicing activity of the precursor RNAs, both the wild-type Pp LSU 3 and Pp LSU 3-ΔORF were expressed from the T7 promoter of pTZ18U.

Splicing reactions with the new precursor RNAs were carried out under the optimal conditions described above as shown in Figure 5a. Since the molecular weights of some of the expected products were similar to each other, experiments were also carried out in the presence of [α-^{32}P]GTP (Figure 5a) to identify
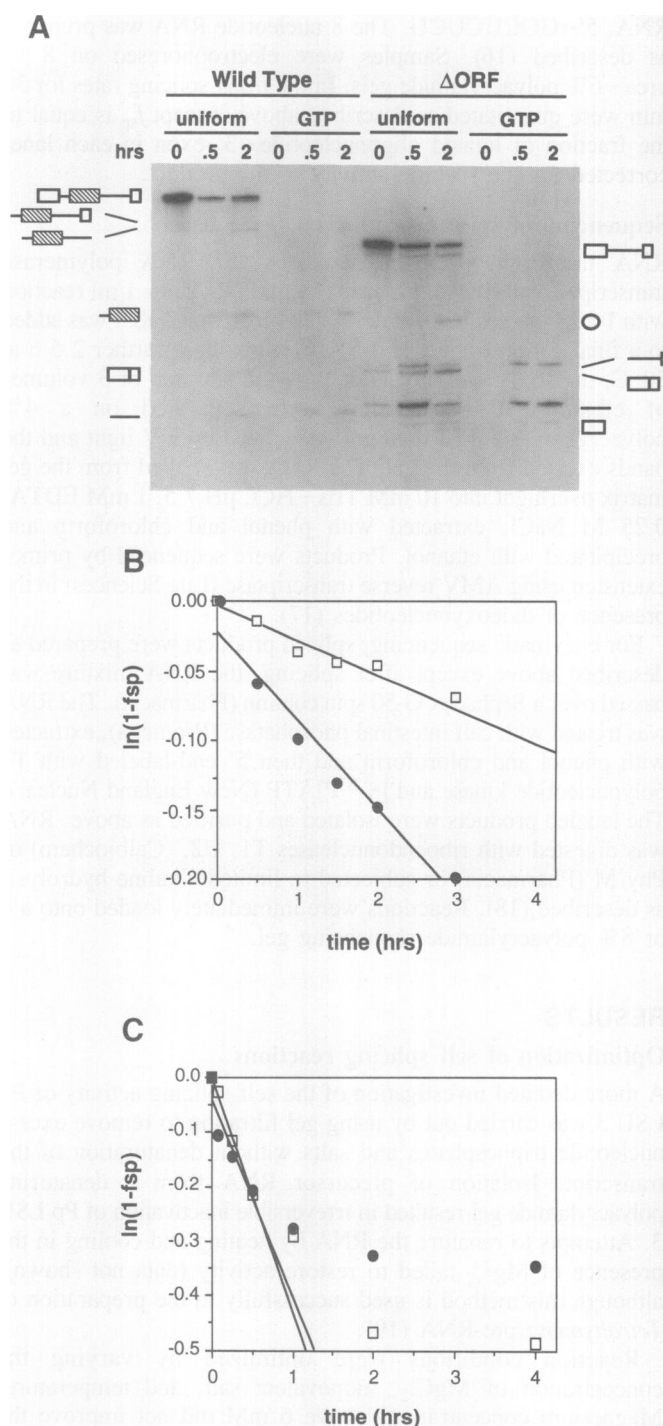
Figure 5. Splicing of wild-type and ΔORF pre-RNA. (**A**) Reactions were carried out at 30°C as described in Materials and Methods, and contained either uniformly radiolabeled precursor and unlabeled GTP (uniform) or unlabeled precursor RNA and radiolabeled GTP (GTP). Bands in the GTP-labeled lanes correspond to products that gain a guanosine during splicing. Reactions were incubated for the times shown (h) at the top of each lane prior to electrophoresis on a 4% polyacrylamide gel. Wild-type pre-RNA was transcribed from pI3TZ; ΔORF from pI3ΔORFTZ. RNA symbols keyed as in Fig. 1. (**B**) Splicing reactions were carried out as above and in Table I. The $\ln(1-f_{sp})$ is plotted versus time (h), where f_{sp} is the fraction of ligated exon product. □, wild-type (pI3TZ); ●, ΔORF (pI3ΔORFTZ). (**C**) *Trans*-splicing reactions were carried out as above, except in the absence of GTP and the presence of 100 μM rGGCUCUCU. Plot as in B, except that f_{sp} is the fraction of oligonucleotide–3′ exon product.

Table I. Rates of self splicing

Precursor	Splicing $[k_{obs}$ $(h^{-1})]$	Intermolecular splicing $[k_{obs}$ $(h^{-1})]$			
		1 μM	10 μM	50 μM	100 μM
Wild-type (TZ)	0.024 ± 0.004	0.13 ± 0.03	0.20 ± 0.03	0.33 ± 0.09	0.47 ± 0.04
ΔORF (TZ)	0.064 ± 0.005	0.26 ± 0.05	0.28 ± 0.06	0.29 ± 0.05	0.35 ± 0.05
Wild-type (BS)	0.034 ± 0.013	ND	ND	ND	ND

Reactions were carried out in 40 mM Tris−HCl, pH 7.5, 6 mM MgCl$_2$, 5 mM DTT, 2 mM spermidine, 0.5 M KCl at 30°C, and contained either 100 μM GTP (Splicing) or no GTP and 1−100 mM 5'-rGGCUCUCU as indicated above each column (Intermolecular splicing). The concentration of precursor RNA was 0.15 μM. Rate constants were based on the appearance of ligated exon product for the first 2 h of reaction, normalized to the fraction of precursor RNA consumed in 24 h. Standard deviations are derived from the average of 3−10 independent trials. TZ designates precursors transcribed from pTZ18U as described in the text, and BS indicates pI3-BS (10). ND, not done.

products that gain an exogenous GTP during splicing. Spliced products were also purified from denaturing gels and sequenced as described in Materials and Methods (data not shown).

In addition to the full-length intron and ligated exon RNAs, a small amount of intron−3' exon intermediate was observed for both precursors. This suggests that the second step of splicing may be slow relative to the first. Addition of GTP to the IPS in Pp LSU 3 also gives rise to the 5' and 3' intron halves. This G addition reaction appears to occur only after intron excision. Products that would correspond to attack at the IPS before splicing, 5' exon−5' intron half (933 nt, compare to 941 nt intron band in Figure 5a) and 3' intron half−3' exon (410 nt, compare to 385 nt 3' intron half), were not seen at 30°C. A G-labeled 3' intron half−3' exon product was never detected.

No residual G addition at the IPS was detected in Pp LSU 3-ΔORF. However, a product that may correspond to a circular intron was detected for Pp LSU 3-ΔORF. This assignment is supported by the fact that the product migrates more slowly than the full-length intron, is not labeled with GTP, and lacks 3' exon sequences. Intron circularization is not observed with the full length *Physarum* intron (see also 10), but does occur in Tt LSU (28). Another surprising observation is that we are unable to detect products arising from G addition to the upstream alternative 5' splice site in either transcript. This splice site accounts for approximately 10% of spliced products in precursors expressed from pI3-BS (10). Usage of this minor 5' splice site may depend on transcribed vector sequences.

Observed rates of splicing were determined for precursors containing wild-type Pp LSU 3 and Pp LSU 3-ΔORF. Deletion of the ORF caused a three-fold increase in the rate of splicing, as shown in Table I and Figure 5b. Even in the absence of the ORF, the splicing rate remains 500-fold slower than that of Tt LSU at 30°C. Thus the presence of the ORF cannot account for the lower activity of the *Physarum* intron.

Trans-splicing of *Physarum* precursors

An intermolecular splicing reaction (*trans*-splicing) was used to study the second step of splicing directly and to gain more information about 3' splice site activation. This reaction uses an oligonucleotide complimentary to the IGS as the substrate for exon ligation in the absence of GTP (29). The products of this reaction are 5' exon−intron and oligonucleotide−3' exon RNAs. Precursor RNAs are able to *trans*-splice if the IGS is available for oligonucleotide binding and if the 3' splice site is positioned correctly for exon ligation. Precursors that do not form a stable intramolecular P1 helix will still *trans*-splice well if these two requirements are met (5).

Pre-RNAs were incubated in the presence of 1−100 μM free 5' exon substrate, 5'-rGGCUCUCU (Figure 5c). At high oligonucleotide concentrations, *trans*-splicing can be used to estimate the reactivity of the 3' splice site. Comparison of the rate constants listed in Table I shows that *trans*-splicing of Pp LSU 3 and Pp LSU 3-ΔORF is 20- and 5-fold faster than the corresponding intramolecular reaction. These results indicate that the 3' splice site is reactive towards exon ligation, despite the fact that 5' exon and intron−3' exon intermediates accumulate in typical self splicing reactions (100 μM GTP).

The observed rate of *trans*-splicing varies much less for ΔORF pre-RNA than for the wild-type, over the range of substrate concentrations tested (Table I). One possibility is that intermolecular splicing of Pp LSU 3-ΔORF is already near saturation at 1 μM oligonucleotide, suggesting that the oligonucleotide binds the ΔORF intron more tightly than the wild-type. One explanation is that the intramolecular P1 base pairing is less stable than the intermolecular complex in the ΔORF precursor, as compared to the wild-type. This is opposite to what we expect, however, based on the predicted stabilities of the stem-loops. A more probable explanation is that improved tertiary contacts between the shortened P1 stem-loop and the core of the intron in Pp LSU 3-ΔORF stabilize both the intra- and intermolecular P1 helices, leading to higher rates of *cis*- and *trans*-splicing. In this scenario, the equilibrium would favor the intramolecular P1 duplex, in agreement with the lower extent of *trans*-splicing for the ΔORF precursor as compared to the wild-type. Further experiments are required to address these points.

DISCUSSION

The optimal splicing conditions for *Physarum* intron 3, 40 mM Tris−HCl pH 7.5, 6 mM MgCl$_2$, 0.5 M KCl at 30°C, show a preference for high monovalent salt concentration. This is unlike the *Tetrahymena* intron, which is active in 50 mM NaCl (30). However other group I introns, such as *Ankistrodesmus* SSU, and nrdB and sunY from phage T4, also display a need for high salt (20, 31). This requirement may indicate that the folded structure needed for self splicing activity is not as stable as that of other introns. This is supported by the observation that Pp LSU 3 is inactive after denaturation. In the presence of high salt, however, the intron retains some activity at 60°C.

The large ORF located in L1 may contribute to the low stability of the intron. Deletion of the ORF resulted in a slight increase in splicing rate that may be due to enhanced stability of P1 due to improved tertiary interactions. This interpretation is consistent with the results from the intermolecular splicing assays. The

improvement in self splicing activity upon ORF deletion is quite modest, however, and cannot account for the large difference in reactivity of *Physarum* and *Tetrahymena* pre-RNAs.

The reactivity of the 3' splice site of Pp LSU 3 towards exon ligation is perhaps surprising given that the intron−3' exon intermediate accumulates in intramolecular splicing reactions. During self splicing, the intron must release the guanosine co-factor after cleavage of the 5' splice site to permit positioning of the 3' terminal G in the active site. This conformational change may be slow in Pp LSU 3, resulting in build-up of the intermediate. A slow conformational change would not affect *trans*-splicing, since the 5' splice junction is not cleaved.

The low K_m(GTP) would appear to suggest that Pp LSU 3 binds guanosine tightly. However, if the second step of splicing is signficantly slower than the first, K_m could be smaller than K_d by approximately the ratio of the rate constants (32). In fact, if initial velocities are based on the appearance of intermediate products rather than on ligated exons, a higher value of K_m is obtained (approximately $20-50$ μM compared to 7 μM). A slower rate of exon ligation would lead to a higher probability of premature 5' exon dissociation, and thus account for the build-up of intron−3' exon intermediate.

Physarum intron 3 self splices very slowly, at a rate of 0.024 h^{-1} or 4×10^{-4} min^{-1}, compared to the observed rate of 0.6 min^{-1} for Tt LSU intron (15, 33). This is somewhat surprising, given the high degree of similarity in the sequences of these introns. There are a few changes in P1, P10 and P9.0, and no differences in the central cores (see Figure 1). However, flanking rRNA sequences may also affect self splicing. For example, a *Tetrahymena* precursor containing a short 3' exon comparable to the length of these *Physarum* transcripts also splices slowly (0.06 min^{-1}; 13). Point mutations within the 3' exon can reduce the rate of splicing for Tt LSU even further, to 0.005 min^{-1} (13). Even this rate is 10-fold higher than the splicing rate for Pp LSU 3, suggesting that other factors are needed for efficient splicing in *Physarum*. For example, a maturase or additional rRNA sequences may be required to help the intron fold into a stable conformation. In *P.polycephalum* rDNA, a second group I intron is located only 24 nucleotides downstream from intron 3 (34), and may influence self splicing of intron 3. Experiments with longer precursor RNAs are in progress.

ACKNOWLEDGEMENTS

REFERENCES

1. Belfort, M. (1989) *Trends Genet.*, **5**, 209−213.
2. Cech, T. R. (1990) *Annu. Rev. Biochem.*, **59**, 543−568.
3. Cech T. R. and Bass, B. L. (1986) *Annu. Rev. Biochem.*, **55**, 599−629.
4. Cech, T. R., Herschlag, D., Piccirilli, J. A. and Pyle, A. M. (1992) *J. Biol. Chem.*, **267**, 17479−17482.
5. Woodson, S. A. and Cech, T. R. (1991) *Biochemistry*, **30**, 2042−2050.
6. Woodson, S. A. and Emerick, V. E. (1993) *Mol. Cell. Biol.*, **13**, 1137−1145.
7. Been, M. E. and Cech T. R. (1986) *Cell*, **47**, 207−216.
8. Muscarella, D. E. and Vogt, V. M. (1989) *Cell*, **56**, 443−454.
9. Muscarella, D. E., Ellison, E. L. and Vogt, V. M. (1990) *Mol. Cell. Biol.*, **10**, 3386−3396.
10. Ruoff, B., Johansen, S. and Vogt, V. M. (1992) *Nucleic Acids Res.*, **20**, 5899−5906.
11. Gutell, R. R., Schnare, M. N. and Gray, M. W. (1990) *Nucleic Acids Res.*, **18** (Suppl.), 2319−2330.
12. Kunkel, T. A., Roberts, J. D. and Zakour, R. A. (1987) *Methods Enzymol.*, **154**, 367−382.
13. Woodson, S. A. (1992) *Nucleic Acids Res.*, **20**, 4027−4032.
14. Garriga, G. and Lambowitz, A. M. (1984) *Cell*, **38**, 631−641.
15. Bass, B. L. and Cech T. R. (1984) *Nature*, **308**, 820−826.
16. Woodson, S. A. and Cech, T R. (1989) *Cell*, **57**, 335−345.
17. Zaug, A. J., Kent, J. R. and Cech, T. R. (1984) *Science*, **224**, 574−578.
18. Donis-Keller, H. (1979) *Nucleic Acids Res.*, **7**, 179−192.
19. Walstrum, S. A. and Uhlenbeck, O. C. (1990) *Biochemistry*, **29**, 10573−10576.
20. Davila-Aponte, J. A., Huss, V. A. R., Sogin, M. L. and Cech T. R. (1991) *Nucleic Acids Res.*, **19**, 4429−4436.
21. Mitermayer, C., Braun, R. and Rusch, H. P. (1965) *Exptl. Cell. Res.*, **38**, 33−41.
22. Williamson, C. L., Tierney, W. M., Kerker, B. J. and Burke, J. M. (1987) *J. Biol. Chem.*, **262**, 14672−14682.
23. Bass, B. L. and Cech T. R. (1986) *Biochemistry*, **25**, 4473−4477.
24. Zaug, A. J., McEvoy, M. M. and Cech, T. R. (1993) *Biochemistry*, **32**, 7946−7953.
25. Doudna, J. A. and Szostak, J. W. (1989) *Mol. Cell. Biol.*, **9**, 5480−5483.
26. Xu, M. Q. and Shub, D. A. (1989) *Gene*, **82**, 77−82.
27. Gampel, A. and Cech T. R. (1991) *Genes Dev.*, **5**, 1870−1880.
28. Zaug, A. J., Grabowski, P. J. and Cech, T. R. (1983) *Nature*, **301**, 578−583.
29. Inoue, T., Sullivan, F. X. and Cech T. R. (1985) *Cell*, **43**, 431−437.
30. Zaug, A. J., Grosshans, C. A. and Cech, T. R. (1988) *Biochemistry*, **27**, 8924−8931.
31. Hicke, B. J., Christian, E. L. and Yarus, M. (1989) *EMBO J.*, **8**, 3843−3851.
32. Fersht, A. (1985) Enzyme Structure and Mechanism. W. H. Freeman, New York, pp. 104−105.
33. Emerick, V. E. and Woodson, S. A. (1993) *Biochemistry*, **32**, 14062−14067.
34. Otsuka, T., Nomiyama, H., Yoshida, H., Kutika, T., Kuhara, S., Sakaki, Y. (1983) *Proc. Natl Acad. Sci. USA*, **80**, 3163−3167.
35. Murphy, F. L. and Cech, T. R. (1993) *Biochemistry*, **32**, 5291−5300.