Self-Splicing of the Chlamydomonas Chloroplast psbA Introns

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We used α-32P-GTP labeling of total RNA preparations to identify self-splicing group I introns in Chlamydomonas. Several RNAs become labeled with α-32P-GTP, a subset of which is not seen with RNA from a mutant that lacks both copies of the psbA gene. Hybridization of the GTP-labeled RNAs to chloroplast DNA indicates that they originate from the psbA and rrn 23S genes, respectively, the only genes known to contain group I introns in this organism. Introns 1, 2, and 3 of psbA (with flanking exon sequences) were subcloned and transcribed in vitro. The synthetic RNAs were found to self-splice; splicing required Mg2+, GTP, and elevated temperature. In addition, the accuracy of self-splicing was confirmed for introns 1 and 2, and intermediates in the splicing reactions were detected. These results, together with our recent data on the 23S intron, indicate that the ability to self-splice is a general feature of Chlamydomonas group I introns. These findings have significant implications for the mechanism of group I intron splicing and evolution in Chlamydomonas and other chloroplast genomes.

INTRODUCTION

A number of chloroplast genes in plants and algae contain introns, many of which can be classified based on primary and secondary structure as belonging to group I or group II (reviewed by Plant and Gray, 1988), designations originally derived by comparing mitochondrial introns in fungi (Michel et al., 1982). A considerable amount of information is available about the splicing of group I introns in nonplant systems (reviewed by Cech, 1990). One of the most important findings is the discovery of RNA “self-splicing” by the rrn intron of Tetrahymena (Cech et al., 1981). Extensive study of the Tetrahymena nuclear rrn intron has revealed the general features of the self-splicing mechanism for this group I intron (Cech, 1990), and at least some of these features have been confirmed for group I introns from fungal mitochondria (Garriga and Lambowitz, 1984; Partono and Lewin, 1988) and T4 phage (reviewed by Belfort, 1990). Splicing is accomplished by means of two sequential transesterification reactions, the first being attack at the 5’ splice site by a free GTP (which results in cleavage), and the second, attack at the 3’ splice site by the 5’ exon, resulting in exon ligation. The liberated intron contains a noncoded G at its 5′ end. In addition, the excised intron may circularize by way of a conserved 3′-terminal guanosine reacting with one or more phosphodiester bonds near the 5′ end of the intron.

In chloroplasts, group I introns have been found in the trnL gene (cf. Steinmetz et al., 1982) and in rrn and psbA genes of several Chlamydomonas species (Erickson et al., 1984; Rochaix et al., 1985; Durocher et al., 1989; Turmel et al., 1989, 1991). Unlike the situation with fungal mitochondria or Tetrahymena, little is known about the mechanism of splicing of chloroplast group I introns or, for that matter, group II. Several authors have reported a lack of success in attempts to induce in vitro synthesized chloroplast preRNAs to self-splice, suggesting that splicing is protein dependent (Boyer and Mullet, 1988; Evrard et al., 1988; Plant and Gray, 1988). However, neither all group I nor many group II introns are capable of self-splicing (Cech, 1987). Thus, it is not safe to conclude that chloroplast introns do not self-splice (Plant and Gray, 1988; Xu et al., 1990). We have used a different approach that takes advantage of the fact that self-splicing group I introns can be detected using total RNA (Garriga and Lambowitz, 1984) and that identifies mutant strains of Chlamydomonas that overaccumulate unspliced transcripts. Using this approach, we recently found the rrn 23S intron of Chlamydomonas to be self-splicing (Herrin et al., 1990). An important question, however, is whether the 23S intron is unique in its capacity for self-splicing, or whether there are other self-splicing introns in Chlamydomonas. In the present study, we used GTP-labeling of total RNA from wild type and mutant strains to identify additional self-splicing introns, and we mapped them to the psbA gene. Using RNA produced by in vitro transcription of cloned DNA, we verified the self-splicing competence of these introns and have begun to characterize their biochemical abilities.

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RESULTS

GTP Labeling of Total RNA

Total RNA from wild type and the 8-36c, ac-u-c 2-43, and ac20cr1 mutant strains of Chlamydomonas were incubated with α-32P-GTP under self-splicing conditions (see Methods). Strain 8-36c is a photosystem II mutant in which both copies of the psbA gene have been deleted (Bennoun et al., 1986); ac-u-c 2-43 is an ATP synthetase mutant that has lost one entire inverted repeat and most of the atpB gene (Myers et al., 1982); and the ac20cr1 mutant is a nuclear double mutant that is deficient in chloroplast ribosomes (Harris et al., 1974). Figure 1 shows that several RNAs became radioactively labeled with 32P-GTP, with qualitative and quantitative differences seen in the labeling profiles with the different strains; the GTP-labeled RNAs are ~900 nucleotides, ~1000 nucleotides, and several from ~1300 to 2000 nucleotides. Interestingly, the major GTP-labeled RNAs of ~1300 to 2000 nucleotides were absent from the 8-36c (psbA deletion mutant) profile (lane 2) even with a very long exposure of the gel (data not shown), but were obtained with RNA from wild type and the other mutants. This result suggests that the major GTP-labeled RNAs of ~1300 to 2000 nucleotides are derived from the psbA gene. In addition, the sizes of three of the GTP-labeled RNAs (1300, 1400, and 1870 nucleotides, respectively) were similar to the sizes of psbA introns 1, 2, and 4, which are ~1.3, 1.4, and 1.8 kb, respectively; psbA intron 3 is ~1.1 kb (Erickson et al., 1984). Figure 1 also shows that the labeling of these RNAs was substantially increased with the ac20cr1 mutant (lane 4), consistent with our previous report that this mutant overaccumulates unspliced psbA transcripts (Herrin and Schmidt, 1987).

In contrast to the larger sized RNAs, the ~900- and 1000-nucleotide GTP-labeled RNAs were not readily seen with wild-type RNA (lane 1) but were obtained with all three mutants, particularly ac20cr1 (lane 4). We have determined that both these RNAs are derived from the 23S intron by hybridization and partial sequencing (Herrin et al., 1990; Y.-F. Chen and D.L. Herrin, unpublished results).

The GTP-labeled RNAs obtained with ac20cr1 RNA were hybridized to chloroplast DNA (cpDNA) from Chlamydomonas that had been digested with EcoRI or BamHI. Figure 2, lanes 1 and 3, show the restriction patterns obtained with these enzymes, which were similar to those reported previously (Rochaix, 1978) except for the largest (>25 kb) BamHI fragments. B14 and B15; B15 (not labeled) was barely detectable and B14 was slightly reduced in intensity. We presume that some degradation of these large fragments occurred during restriction and electrophoresis, although we cannot rule out the possibility of degradation during cpDNA isolation. The degradation of B15, however, does not affect the interpretation of the results because it is completely overlapped by EcoRI fragments of ~3 to 12 kb in lane 1 (Rochaix, 1978). Lanes 2 and 4 of Figure 2 show that hybridization of GTP-labeled RNA occurred with EcoRI fragments R24 and R14/R16.
analysis (Figure 1) and further indicates that the GTP-labeled RNAs originate only from the \textit{psbA} and 23S gene regions. It should also be noted that nearly all of fragments R24, R14, and R16 have been mapped and/or sequenced (Erickson et al., 1984, and references therein) and are not known to encode other intron-containing genes.

To further map the GTP-labeled RNAs, hybridizations with plasmid pEC23, which contains EcoRI fragment R14 (Herrin and Michaels, 1985), were performed; R14 contains the first four exons and three introns of the psbA gene (Erickson et al., 1984). Figure 3 shows the results of hybridizing GTP-labeled RNA from the ac20cr1 and 8-36c strains to EcoRI-Xbal digested pEC23; hybridization to a plasmid (pBC1) that contains the B1 fragment, and the 23S intron (Figure 3, map), was included for comparison. Using GTP-labeled ac20cr1 RNA as the probe, hybridization occurred with all three restriction fragments of R14, although greatest with the ~1.8-kb Xbal fragment (lane 2). The faint hybridization signal in lane 2 that migrates slower than the ~3.2-kb EcoRI-Xbal fragment is unknown; it does not comigrate with a major stained band. In contrast, no hybridization to these fragments was detected with GTP-labeled 8-36c RNA (lane 3). Hybridization to the 23S intron-containing fragment was obtained with GTP-labeled RNA from both strains (lanes 5 and 6), indicating that the absence of hybridization to R14 with 8-36c RNA was not due to the experimental conditions. In view of the fact that intron 1 is completely and uniquely contained in the ~3.2-kb EcoRI-Xbal fragment (Figure 3, map) and the fact that hybridization occurred to that as well as the other fragments of R14, the data suggest that at least two of the introns on R14 are capable of self-splicing. The relatively strong hybridization signal with the ~1.8-kb Xbal fragment, which contains intron 2 and part of intron 3 (Figure 3, map), may indicate that intron 2 self-splices and to a greater extent than intron 1, at least in total RNA. Consistent with this suggestion is the fact that there is an intensely GTP-labeled RNA of ~1400 nucleotides (Figure 1), which is the size of intron 2 (Erickson et al., 1984). However, we do not have direct data on precisely which GTP-labeled RNA(s) hybridize to each restriction fragment of R14.

### In Vitro Synthesis and Self-Splicing of psbA Intron 2

To verify the GTP-labeling data and provide a system for further study, the psbA introns were subcloned for in vitro transcription. For intron 2, the ~1.8-kb Xbal fragment of R14 (Figure 3), which contains the 3’ end of exon 2 (80 nucleotides), intron 2, exon 3, and ~200 nucleotides of intron 3, was subcloned to generate pGEM.R14.2. Figure 4A shows a polyacrylamide gel analysis of the products of transcription of pGEM.R14.2 in the presence of \( \alpha \)-P-GTP at 28°C (lane 1) and 38°C (lane 3). The products expected
Figure 3. Hybridization of GTP-Labeled RNA to Cloned cpDNA Fragments.

~50 μg of RNA from the ac20cr1 (lanes 2 and 3) or 8-36c (which lacks both copies of the psbA gene) mutants (lanes 5 and 6) were GTP labeled as in Figure 1 and then hybridized to pEC23 restricted with EcoRI and XbaI (lanes 1 to 3) or pBC1 digested with BamHI (lanes 4 to 6). pEC23 contains the R14 fragment, whereas pBC1 contains B1. A map of this region is shown above the lanes; introns and exons are indicated by open and filled boxes, respectively. The Ncol (N) site is drawn on the lower line because it is a unique site only within R14. Lanes 1 and 4 are of the ethidium bromide-stained gel prior to DNA transfer, and lanes 2, 3, 5, and 6 are autoradiographs obtained after hybridization. The sizes in kb and positions of λ phage HindIII fragments used as size markers are indicated at the left. R, EcoRI; X, XbaI; B, BamHI; N, Ncol.

The identities of the putative 3′ cleavage products, E2-I2 and E3-I3, were investigated further. pGEM.R14.2 was digested with Ncol, which cuts in E3 (see Figure 3), transcribed in the presence of α-32P-GTP and the 3′ truncated precursor incubated for splicing. Analysis of the products showed reductions in the sizes of E3-I3 and E2-E3-I3 as predicted, and with no change in the mobility of the I2 or E2-I2 RNAs (data not shown). The putative E2-I2 molecule was examined by isolating it from the gel and, upon further incubation with GTP, self-cleaved to give a major product that comigrated with 12, also as predicted (data not shown).

The requirements for Mg2+ and GTP in intron 2 self-splicing were examined, and the results are shown in Figure 4B. For this experiment, gel-purified precursor was incubated for 30 min in the complete splicing mixture (lane 4), with GTP omitted (lane 2), or without Mg2+ (lane 3); lane 1 contained the untreated precursor. With the complete mixture, reasonably efficient splicing and some 3′ cleavage occurred, although there was relatively less self-cleavage compared to the RNA synthesized at 38°C (Figure 4A, lane 4). In the absence of GTP, there was for splicing and/or cleavage of the precursor at the 5′ or 3′ splice sites, respectively, are shown at the top above Figures 4A and 4B. The major product of both transcriptions is the size expected for the precursor, ~1840 nucleotides. However, four additional transcripts are seen at 38°C; these RNAs have the predicted sizes for, and were tentatively identified as: (1) the exon-ligation product (E2-E3-I3), (2) the excised linear intron (I2), and (3) the products resulting from cleavage of the precursor at the 3′ splice site (E2-I2 and E3-I3, respectively). Further characterization of these products is described below.

Figure 4A also shows the effect of incubating the transcription products of pGEM.R14.2 for 30 min at 45°C in the presence of Mg2+ and nonradioactive GTP. A small fraction of the precursor synthesized at 28°C apparently splices under these conditions, producing the predicted intron and ligated-exon molecules (lane 2); longer exposure of the gel did not clearly reveal the putative 3′ splice site cleavage products. A substantial fraction of the precursor synthesized at 38°C also appears to splice during incubation at 45°C (lane 4). Comparison of lanes 3 and 4 shows that the 45°C incubation also increased the putative 3′ splice site cleavage reaction such that the cleavage products (E2-I2 and E3-I3) are even more prominent than the splicing products (E2-E3-I3 and I2). It appears that the intron 2 precursor synthesized at 38°C is more reactive toward self-cleavage, and possibly self-splicing, than the precursor synthesized at 28°C. Presumably, this reflects a different conformation for the molecule formed at 38°C versus 28°C. Lane 5 is a longer exposure of lane 4 and shows the presence of an RNA of extremely low mobility, which is characteristic of circular molecules (cf. Inoue et al., 1986); we have tentatively identified it as circularized intron (C).

The identities of the putative 3′ cleavage products, E2-I2 and E3-I3, were investigated further. pGEM.R14.2 was digested with Ncol, which cuts in E3 (see Figure 3), transcribed in the presence of α-32P-GTP and the 3′ truncated precursor incubated for splicing. Analysis of the products showed reductions in the sizes of E3-I3 and E2-E3-I3 as predicted, and with no change in the mobility of the I2 or E2-I2 RNAs (data not shown). The putative E2-I2 molecule was examined by isolating it from the gel and, upon further incubation with GTP, self-cleaved to give a major product that comigrated with I2, also as predicted (data not shown).
neither splicing nor cleavage of the precursor (lane 2). There was, however, a small amount of the putative circular RNA produced in the incubation without GTP (lane 2). Cyclization of precursor RNA has been reported to occur with a self-splicing preRNA from yeast mitochondria (Tabak et al., 1987). Alternatively, some of the precursor may have hydrolyzed at the splice sites (Inoue et al., 1986), producing linear intron molecules, which then cyclized. Incubation of intron 2 preRNA in the splicing mixture lacking Mg\textsuperscript{2+} resulted in no splicing and no side reactions (lane 3). This result indicates that Mg\textsuperscript{2+} is required to form a catalytically active molecule and is consistent with studies of the Tetrahymena rRNA intron (Cech and Bass, 1986). The unidentified bands on the autoradiograph are presumably due to degradation of some of the precursor molecules during isolation; there is no evidence that these are splicing products.

To confirm the identity of the excised intron (I2) and look for the predicted intermediate of the first step of splicing, the I2-E3-I3 molecule, nonradioactive precursor was produced from pGEM.R14.2. The unlabeled gel-purified precursor was incubated for self-splicing in the presence of \( ^{32} \text{P}-\text{GTP} \) and the products were coelectrophoresed with the uniformly labeled splicing products discussed above. Figure 4B, lane 5 shows that the major GTP-labeled band migrates with the size of the linear intron as expected and comigrates with a major uniformly labeled transcription/splicing product (I2 in lane 4), confirming the identity of this RNA. A minor GTP-labeled RNA is also seen in lane 5, which migrates slower than I2 and just ahead of the precursor. This transcript is the size expected for an intron-exon splicing intermediate that would be formed during the first step of splicing (i.e., I2-E3-I3). No GTP-labeled RNAs were evident other than the expected intron and intron-exon molecules.

One predicted intermediate, the 5' exon, was not seen on the autoradiographs in Figure 4 because of the small size (\( \pm 110 \) nucleotides; 80 nucleotides of exon 2 and 30 nucleotides of the plasmid polylinker) and small amount of label that would have accumulated in this product. However, electrophoresis of the splicing products on an 8% polyacrylamide gel (and for a shorter time) revealed an RNA of the expected size in the splicing mixtures with uniformly labeled transcripts (data not shown).

The products of splicing/cleavage of intron 2 were also characterized by primer extension and sequencing.
Figure 5. Primer Extension and cDNA Sequence Analysis of the Products of Intron 2 Self-Splicing.

(A) Primer extension. pGEM.R14.2 was transcribed with T7 RNA polymerase and the transcripts incubated for 30 min in the complete splicing mixture (similar to Figure 4B, lane 4). An end-labeled 21-mer oligonucleotide, complementary to a region in exon 3, 27 nucleotides downstream from the 3’ splice site, was annealed to the transcription/splicing products and extended with reverse transcriptase; Figure 5A shows a polyacrylamide gel analysis of the extension products. Except for a 52-nucleotide product, the sizes and overall profile of the major cDNAs are as expected and consistent with their being derived from the unspliced precursor (~1558 nucleotides), the intron-3’-exon splicing intermediate (~1448 nucleotides), the ligated exons (~158 nucleotides), and the 3’ splice site cleavage product (48 nucleotides). To assess the accuracy of self-splicing, the 158-nucleotide cDNA was excised from the gel and sequenced using Maxam-Gilbert reactions. The sequence surrounding the exon 2-exon 3 splice junction is shown in Figure 5B and is identical to that determined previously (Erickson et al., 1984), indicating that splicing is accurate.

The major cDNA of 52 nucleotides (Figure 5A) has not been fully characterized and its origin is unclear. It could result from heterogeneity in 3’ splice site cleavage or, alternatively, it may be an artifact caused by a strong stop for reverse transcriptase in the ligated exons (E2-E3-I3). The 52-nucleotide cDNA does not appear to derive from the precursor because this product was only obtained with RNA incubated for self-splicing (data not shown).

In Vitro Synthesis and Self-Splicing of psbA Intron 1

To test whether psbA intron 1 is capable of self-splicing, the ~3.2-kb EcoRI-XbaI fragment of R14 (see Figure 3) was subcloned to create pGEM.R14.1. Transcription of pGEM.R14.1 in the presence of α-32P-GTP at 28°C produced a ~3.2-kb RNA and, after purification from a low-melting agarose gel, was assayed for self-splicing. Figure 6 shows a formaldehyde/agarose gel analysis of the intron 1-containing preRNA before (lane 1) and after (lane 2) incubation of the RNA under self-splicing conditions. After incubation, RNAs of ~1.9 and ~1.3-kb were produced concomitant with a decrease in the amount of the ~3.2-kb precursor; ~1.9 and ~1.3 kb were the sizes expected for the ligated exons, E1-E2 (E1 contains exon 1 and sequences upstream; see Figure 3 map), and linear intron, I1, respectively. To confirm the identity of the products, transcription, gel purification, and splicing of unlabeled precursor were performed in parallel with the

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and, moreover, indicating that splicing had occurred correctly.

The requirement for GTP and Mg\(^{2+}\) in self-splicing of intron 1 was examined, although in this case the products were analyzed by PAGE. Figure 7 shows the results obtained when \(^{32}\)P-labeled intron 1 precursor was spliced at 45°C using the complete mixture (lane 4), the complete

![Diagram](image)

**Figure 6.** Self-Splicing of Intron 1 Analyzed by Agarose Gel Electrophoresis and Hybridization.

The \(\sim 3.2\)-kb EcoRI-XbaI fragment of R14 that contains intron 1 and flanking sequences (see Figure 3) was subcloned to create pGEM.R14.1. The new plasmid was transcribed at 28°C with T7 RNA polymerase in the presence, or absence, of \(\alpha\)-\(^{32}\)P-GTP. After gel purification, the precursor transcripts were incubated for 30 min at 45°C in the standard splicing mixture and then electrophoresed on a \(\sim 1.5\%\) agarose/formaldehyde gel and blotted. Lane 2 contains \(^{32}\)P-RNA, whereas the nonradioactive RNA is loaded in lanes 3 and 4; untreated \(^{32}\)P-precursor is loaded in lane 1. Lane 3 was hybridized to a \(^{32}\)P-labeled intron 1 specific probe (see Methods), and lane 4 to an exon 1-exon 2 splice-junction probe. The diagram above explains the major predicted splicing products and their approximate sizes (in nucleotides). *E. coli* and Chlamydomonas rRNAs were used as molecular size markers. Pre, precursor (runoff transcript); E1-E2, exon-ligation product; I1, intron 1.

![Diagram](image)

**Figure 7.** Self-Splicing of Intron 1 Analyzed by PAGE.

\(^{32}\)P-labeled and nonradioactive intron 1 precursor transcripts were prepared as described in the legend to Figure 6. The \(^{32}\)P-precursor was incubated in the complete splicing mixture (lane 4), and in the complete mixture minus GTP (lane 2) or minus Mg\(^{2+}\) (lane 3). Lane 5 contains the nonradioactive precursor incubated with \(\alpha\)-\(^{32}\)P-GTP, and lane 1 contains untreated \(^{32}\)P-precursor. The diagram above explains the predicted splicing/cleavage products and their approximate sizes (in nucleotides). Electrophoresis was performed on a 3% polyacrylamide/urea gel run in 0.4 × TBE buffer at 55°C. Molecular size markers are the same as in Figure 4. C, putative cyclized RNAs; PRE, precursor (runoff transcript).
mixture minus GTP (lane 2), or minus Mg\(^{2+}\) (lane 3). Splicing of the unlabeled precursor with \(\alpha\)-\(\text{32P}\)-labeled GTP was also performed, and the products were electrophoresed in lane 5. The separation of these RNA species on denaturing polyacrylamide gels was quite different from the agarose gel system. Not unexpectedly, the smaller ~1.3-kb RNA separated into two species that corresponded in size to the predicted linear intron (I1) and intron-exon intermediate (I1-E2), respectively. The identity of these molecules was confirmed by the fact that they specifically labeled with \(\alpha\)-\(\text{32P}\)-GTP during splicing of unlabeled precursor (lane 5). Although the I1 and I1-E2 molecules accounted for the vast majority of GTP labeling, there are some other faintly labeled RNAs visible in lane 5.

The reason for the anomalous behavior, migrating as a diffuse band behind the precursor (Figure 7), was confirmed by hybridization with the E1 -E2 splice-junction probe (data not shown). The reason for the anomalous migration of these RNAs is not known, but we suspect that it has to do with the relatively large stretch of sequence 5' to the beginning of the psbA gene that is contained in the construct (see Figure 3); this segment would occur in the precursor and ligation products only. Erickson et al. (1984) sequenced a portion of the region 5' to psbA and found that it contained a number of repeated sequences. It is possible that these sequences may not be fully denatured during electrophoresis despite the presence of 8 M urea and temperatures of 50 to 60°C. However, in spite of the anomalous migration of the exon 1-containing RNAs on polyacrylamide gels, the results of Figure 7 indicate that both GTP and Mg\(^{2+}\) are required for efficient self-splicing of intron 1.

Three RNA bands with very low mobility characteristic of circular molecules are easily seen with the complete psbA intron 1 splicing mixture (Figure 7, lane 4), suggesting that this intron cyclizes at one or more sites after excision; multiple cyclization products have been observed for other group I introns (Cech and Bass, 1986; Partono and Lewin, 1988; Michel et al., 1990). We have determined that all three bands hybridize with the intron-specific probe (Y. Bao and D. Herrin, unpublished results), but further work will be necessary to determine precisely the nature and origin of these molecules. It should also be mentioned that the putative circular molecules were not labeled during splicing of the unlabeled precursor in the presence of \(\alpha\)-\(\text{32P}\)-GTP (lane 5), which is the expected result for cyclization of a group I intron.

In Vitro Synthesis and Self-Splicing of psbA Intron 3

To assay intron 3 for self-splicing, the Ncol-EcoRI fragment of R14 was subcloned; the Ncol-EcoRI fragment contains the 3' half of exon 3, intron 3, exon 4, and ~200 nucleotides of intron 4 (see Figure 3). The new plasmid, pGEM.RI4.3, was transcribed in the presence of \(\alpha\)-\(\text{32P}\)-GTP at 28°C; pilot experiments indicated that the precursor would splice during transcription at 37°C (data not shown). The \(\alpha\)-\(\text{32P}\)-labeled precursor, ~1800 nucleotides, was incubated in the standard splicing mixture for 30 min and then analyzed by PAGE. Figure 8 shows an autoradiograph of the gel. A comparison of lane 4 (complete mixture) with lane 1 (untreated precursor) shows that the incubation resulted in the appearance of two new transcripts whose sizes were similar to those expected for the excised linear intron (I3) and ligated exon (E3-E4-I4) molecules, respectively, and with a concomitant reduction in the amount of precursor. Erickson et al. (1984) originally reported that intron 3 was ~1.1 kb based on the DNA, but in our hands the intron RNA migrated as ~1200-nucleotide species. The intron 3 precursor was also incubated in reaction mixtures that lacked either GTP (lane 2) or Mg\(^{2+}\) (lane 3); both conditions resulted in no discernible splicing products. These data indicate that intron 3 of psbA is capable of self-splicing and that Mg\(^{2+}\) and GTP are necessary cofactors. The many unidentified RNAs in Figure 8 are presumably due to some degradation of the RNA during preparation because they are present in the untreated preRNA (lane 1). A longer exposure (data not shown) of the gel (Figure 8) revealed the presence of a very low mobility, putative circular RNA band in lanes 2 and 4, similar to that seen with intron 2 (Figure 4B).

DISCUSSION

Self-Splicing Introns in cpDNA of Chlamydomonas

The psbA gene encodes the ~36-kD protein of the photosystem II core complex and what is probably the most abundant mRNA in Chlamydomonas (Herrin et al., 1986). The psbA gene of Chlamydomonas, unlike higher plants, contains introns and is located within the inverted repeat region of the chloroplast genome (Erickson et al., 1984). The introns, which are ~1.2 to 1.3 (intron 1), ~1.4 (intron 2), ~1.1 to 1.2 (intron 3), and ~1.8 kb (intron 4) in size, have structural features characteristic of group I introns (Erickson et al., 1984), although the complete sequences of the introns have not yet been published.

Previously, we showed that the ac20cr1 double mutant of Chlamydomonas, which is deficient in chloroplast ribosomes (Harris et al., 1974), contains significantly increased levels of unspliced psbA transcripts (Herrin and Schmidt, 1987); this mutant also has increased levels of unspliced
The ~1.8 kb Ncol-EcoRI fragment of R14 (see Figure 3) was subcloned to create pGEM.R14.3, which was transcribed with T7 RNA polymerase in the presence of $\alpha^{-32}$P-GTP at 28°C. The full-length runoff transcript (PRE, precursor) was incubated in the complete splicing mixture for 30 min at 45°C (lane 4), and in the complete mixture minus GTP (lane 2) or minus Mg$^{2+}$ (lane 3); lane 1 contains untreated 32P-precursor. A map of the major predicted splicing products and their approximate sizes are shown above the autoradiograph. Electrophoresis was on a 5% polyacrylamide/urea gel and the size markers are the same as in Figure 4.

**Figure 8.** Self-Splicing of Intron 3.

The ~1.8 kb Ncol-EcoRI fragment of R14 (see Figure 3) was subcloned to create pGEM.R14.3, which was transcribed with T7 RNA polymerase in the presence of $\alpha^{-32}$P-GTP at 28°C. The full-length runoff transcript (PRE, precursor) was incubated in the complete splicing mixture for 30 min at 45°C (lane 4), and in the complete mixture minus GTP (lane 2) or minus Mg$^{2+}$ (lane 3); lane 1 contains untreated 32P-precursor. A map of the major predicted splicing products and their approximate sizes are shown above the autoradiograph. Electrophoresis was on a 5% polyacrylamide/urea gel and the size markers are the same as in Figure 4.

23S preRNA (Herrin et al., 1990). In this report, we have used $\alpha^{-32}$P-GTP-labeling of total RNA preparations from ac20cr1 (and other mutants) to identify self-splicing psbA introns. Several RNAs of ~1.3 to 2.0 kb became labeled with $\alpha^{-32}$P-GTP using RNA from ac20cr1 and, to a lesser extent, the ac-u-c 2-43 mutant, which lacks one inverted repeat (Myers et al., 1982). In contrast, these RNAs were not labeled when preparations of RNA from a psbA deletion mutant, 8-36c (Bennoun et al., 1986), were used, suggesting that they originated from the psbA gene. Hybridization of GTP-labeled RNA to restriction digests of cpDNA and specific cloned fragments confirmed that these RNAs originated from psbA. In addition, the fact that the profile of GTP-labeled RNA obtained with the ac-u-c 2-43 mutant is similar to that of ac20cr1 precludes the possibility that heterogeneity among the introns in the two copies of the psbA gene accounts for any of the GTP-labeled RNAs.

The ability of psbA introns to self-splice was confirmed by subcloning introns 1, 2, and 3 into transcription vectors and synthesizing the RNA precursors in vitro. Each of the intron-containing transcripts showed evidence of self-splicing activity, and precise splicing was confirmed for introns 1 and 2; no attempt was made to confirm the precision of splicing for intron 3. With respect to psbA intron 4, although we have no direct data on intron 4 self-splicing, it seems likely that the ~1.8-kb RNA, which was prominent among the GTP-labeled products of total RNA (Figure 1), is derived from intron 4 (also ~1.8 kb). It should be stated, however, that we have not conclusively determined corresponding relationships between psbA introns and specific GTP-labeled RNAs; hybridizations were always performed with total GTP-labeled RNA. It might be expected that some of the GTP-labeled RNAs represent intron-exon intermediates; the ~1.5- and ~1.7-kb RNAs (Figure 1) might be good candidates for intron-exon molecules because they do not correspond in size to any of the psbA introns. Clearly, further work will be required to understand all of the RNAs labeled with GTP under self-splicing conditions.

Taken together with our previous data on the rnm 23S intron (Herrin et al., 1990), these results indicate that at least four of the five known group I introns in Chlamydomonas self-splice. It may be the case that all the group I introns in Chlamydomonas self-splice. It should be mentioned, however, that not all of the cpDNA of Chlamydomonas has been characterized. In addition, the GTP-labeling assay with total RNA is dependent on accumulation of unspliced RNA in vivo to have a chance to be successful. Thus, it is possible that there are group I introns in the Chlamydomonas chloroplast genome that are as yet unknown and that do not self-splice.

**Mechanism of Splicing of psbA Introns**

The experiments presented in this report indicate some important aspects of the mechanism of splicing of psbA introns. The fact that the introns splice in the absence of proteins indicates that splicing is fundamentally RNA directed. Self-splicing may even be the primary mode of splicing for these introns in vivo. If so, this would explain
why psbA splicing mutants have not been isolated. There are other considerations, however, which make it likely that one or more proteins (or other trans-acting factors) assist in splicing these introns in vivo. The conditions for self-splicing of psbA introns are nonphysiological with respect to temperature; efficient splicing requires temperatures above 37°C. Moreover, even under optimized conditions, psbA intron 2 preRNA requires up to 1 hr for ~75 to 80% of the molecules to splice (Y. Bao and D. Herrin, unpublished results). Finally, self-splicing introns from fungal mitochondria have been shown to depend on maturases, acting in trans, for efficient splicing in vivo (Tzagoloff and Myers, 1986; Lambowitz and Perlman, 1990).

These experiments also demonstrate that magnesium ions are required for self-splicing; no activity was observed for any of the introns in the absence of Mg2+. The magnesium ions are presumably required for the RNA to fold into a catalytically active state, although some recent studies with the Tetrahymena rnr1 intron suggest that Mg2+ may have an additional, direct role in catalysis (Celander and Cech, 1991). It was also shown that efficient self-splicing of psbA introns required GTP: there was little or no splicing under these conditions in the absence of GTP. Additional studies of intron 2 self-splicing have demonstrated a strong specificity for GTP over the other nucleotides and that guanosine can effectively replace GTP (Y. Bao and D. Herrin, unpublished results). During splicing of the Tetrahymena rnr1 intron (Cech, 1987), a guanosine initiates the process by attacking the 5' splice site, resulting in cleavage of the precursor into 5'-exon and intron-3'-exon molecules; the guanosine then becomes part of an intron-exon intermediate. Using 32P-GTP labeling of in vitro synthesized RNAs, we observed the predicted intermediate molecules during splicing of introns 2 (Figure 4 and text) and 1 (Figure 7). Thus, our data suggest that sequential transesterifications, initiated by a free guanosine molecule, are involved in self-splicing of psbA introns.

RNAs of very low mobility, reminiscent of the circular molecules known to be formed by some self-splicing group I introns (Cech, 1980), were generated during self-splicing of each of the psbA introns. Recently, we used reverse transcription coupled with polymerase chain reaction (RT-PCR) and primers that anneal near the 5' and 3' ends of intron 2 to verify that this intron cyclizes (Y. Bao and D. Herrin, unpublished results); work is under way to identify the exact cyclization site.

Evolution of Chloroplast Introns

The trnL gene is the only gene identified so far in cpDNA of higher plants that contains a group I intron (reviewed by Plant and Gray, 1988). In contrast, there are a number of group I introns in the genus Chlamydomonas and in widely divergent species (Durocher et al., 1989; Turmel et al., 1989, 1991). Why has Chlamydomonas acquired a number of group I introns in its chloroplast genome? A partial answer to this question may be found in the fact that these introns are self-splicing. Insertion of an intron into a preexisting gene would presumably inactivate the gene if the intron could not be spliced out; however, self-splicing introns carry their splicing activity with them. They may also contribute to their own existence by inserting themselves into genes at the RNA level by means of a reversal of the self-splicing reactions (Woodson and Cech, 1989).

The trnL intron of higher plants is not capable of self-splicing, although its presumed blue-green algal ancestor is apparently self-splicing (Xu et al., 1990). Thus, this intron has evolved into a form that is completely dependent on trans-acting factors for splicing. This raises the question of why the group I introns in Chlamydomonas chloroplasts have not lost their self-splicing ability and become wholly dependent on trans-acting protein factors. One answer is that they probably are at least partially dependent on trans-acting factors, as discussed above. However, it could also be argued that it is more efficient and economical for the RNA to retain catalytic activity; this would require less trans-acting factors, and therefore less genes, to accomplish the task. It is also reasonable to consider that the group I introns in Chlamydomonas chloroplasts have been acquired relatively recently through intron mobility (reviewed by Dujon, 1989) or reverse self-splicing (Woodson and Cech, 1989) and have not had sufficient time to lose their self-splicing ability. Finally, the dependence of introns on trans-acting factors could provide an additional level of control of gene expression; this level of control may be more significant in higher plants.

METHODS

Strains and Culture Conditions

Wild-type 2137 mt(+)(CC-1021) and 137c mt(+) strains (Harris, 1989), and the ac20cr1 (CC-155) and ac-u-c 2-43 (GB-1015) mutants were obtained from the Chlamydomonas Culture Center (c/o Dr. Elizabeth Harris, Botany Department, Duke University, Durham, NC). The 8-36c mutant was obtained from Laurie Mets (University of Chicago, Chicago, IL). All strains were grown in Tris-acetate-phosphate medium (Harris, 1989) under dim light (~250 lux). Cultures in logarithmic growth (~1 to 2 x 10^6 cells/mL) were used for RNA isolation, and for the isolation of cpDNA, cultures near stationary phase (~5 x 10^6 cells/mL) were used.

Plasmids and DNA Probes

The pEC23 plasmid contains EcoRl-fragment R14 (see Figure 3) in the vector pBR325 (Herrin and Michaels, 1985). Plasmid pBC1 contains the BamHl fragment B1 of Chlamydomonas cpDNA, which encodes the 3' half of the rnr23S gene including the intron (Rochaix and Malnoe, 1978), in the vector pBR313; pBC1 was a
gift of Jeffrey Palmer (Indiana University, Bloomington). To generate plasmids for in vitro transcription studies, three subclones of pEC23 were constructed in the vector pGEM3zf(+) (Promega) as follows. The ~3.2-kb EcoRI-Xbal fragment of R14 (see Figure 3) was isolated and cloned into the pGEM vector, which had been double digested with EcoRI and XbaI; the new plasmid is called pGEM.R14.1. The ~1.8-kb Xbal fragment of R14 was cloned into Xbal-digested vector, and clones in the correct orientation for transcription with T7 RNA polymerase were identified by restriction; the new plasmid is referred to as pGEM.R14.2. Intron 3 of psbA was subcloned into the Smal site of the transcription vector by isolating the ~1.8-kb Ncol-EcoRI fragment and blunt-ending it with the Klenow fragment of DNA polymerase I before ligation (Sambrook et al., 1989). Clones in the correct orientation for transcription with T7 RNA polymerase were identified by restriction; the new plasmid is called pGEM.R14.3. Before transcription, pGEM.R14.1, pGEM.R14.2, and pGEM.R14.3 were linearized 3' to the insert by digestion with XbaI, HindIII, and HindIII, respectively.

To generate an intron 1-specific probe, pGEM.R14.1 was restricted with HindIII, and the ~225-nucleotide fragment internal to the intron (Erickson et al., 1984) was purified by electrophoresis on a 2% agarose gel, followed by electroelution (Sambrook et al., 1989). Before hybridization, the gel-purified fragment was labeled with α-32P-dCTP using random primers and the Klenow fragment of Escherichia coli DNA polymerase I (Feinberg and Vogelstein, 1983).

To assay for correct splicing of intron 1, a 14-mer oligonucleotide d(5'-TACCATCGATGTCT-3'), complementary to the 5'-d(TACATC) and 3'-d(GATGCT) exon borders that flank intron 1, was used. The oligonucleotide was end labeled with T4 polynucleotide kinase and γ-32P-ATP to a specific activity of ~5 x 10⁶ cpm/μg. The conditions used for hybridization with this oligo are described below.

For primer extension analysis of intron 2 splicing products, a 21-mer oligonucleotide 5'-d(AGGCAATCGAGCGATACCC)-3', complementary to a region of exon 3 RNA, 27 nucleotides from the 3' splice site, was synthesized and labeled with polynucleotide kinase (and γ-32P-ATP) to a specific activity of ~2 x 10⁷ cpm/μg. The 5' end labeled oligo was used in primer extension cDNA synthesis as described below.

RNA Isolation and GTP Labeling

RNA was isolated as described previously (Herrin and Schmidt, 1989a, 1988b). GTP labeling was performed by using 50 μCi of α-32P-GTP and 20 to 100 μg of total RNA in 50- to 200-μL vol. The labeling mixtures also contained 30 mM MgCl₂, 50 mM NH₄SO₄, 25 mM Tris HCl, pH 7.5, and 0.3 to 0.4% SDS and were incubated for 30 or 60 min at 45°C. The reactions were terminated and prepared for electrophoresis or hybridization as described by Herrin et al. (1990).

Isolation of cpDNA and Hybridization to GTP-Labelled RNA

cpDNA was isolated from 13°C mt(+) cells (wild type) as described by Herrin and Worley (1990). For DNA gel blot hybridization, ~1.5 μg of cpDNA was digested to completion with EcoRI or BamH1, electrophoresed in 0.7% agarose gels, and bated onto nylon membranes (Reed and Mann, 1985). The blots were probed with ~50 μg of GTP-labeled RNA, prepared as indicated above, by incubation for 4 hr in 50% formamide, 0.75 M NaCl, 400 μg/mL denatured sperm DNA, 1 mM EDTA, 50 mM Tris HCl, pH 7.5, and 1% SDS at 42°C. The blots were washed at 55°C in 0.5 x SSPE (1 x SSPE = 0.15 M NaCl, 10 mM NaH₂PO₄, pH 7.4, 1 mM EDTA), 1% SDS for 45 min. Similar conditions of blotting, hybridization, and washing were used during the probing of cpDNA clones with total GTP-labeled RNA.

In Vitro Transcription, Purification, and Self-Splicing

Plasmid DNA was transcribed with T7 RNA polymerase (U.S. Biochemical) as described by Latham et al. (1990). In some reactions, α-32P-GTP (3000 Ci/mmol) was added to 2 μCi/μL. Transcription was allowed to proceed for 1 hr at 28°C or 37 to 38°C, and then phenol-extracted and precipitated with ethanol. When desired, transcripts were isolated from polyacrylamide/urea gels (Latham et al., 1990) or, for the intron 1 precursor, from 1.5% agarose/10 mM methylmercuryhydroxide gels (Sambrook et al., 1989). For purifying unlabeled transcripts, the RNAs were localized by UV shadowing or by coelectrophoresing using the same 32P-labeled transcripts in adjacent lanes; the RNA was excised and eluted as described (Latham et al., 1990).

The standard reaction for splicing of in vitro synthesized α-32P-transcripts was the same as for GTP labeling of cellular RNA described above, except nonradioactive GTP was added to a final concentration of 100 μM. Nonradioactive precursor transcripts were spliced with α-32P-GTP (~3000 Ci/mmol) substituted for GTP at a final concentration of 0.33 μM. Splicing reactions were typically performed at 45°C for 30 min unless otherwise specified and terminated as described in Herrin et al. (1990). Other changes to the splicing conditions are described in the figure legends or in the text.

RNA Electrophoresis, Blotting, and Hybridization

Electrophoresis of RNA on denaturing agarose/formaldehyde gels, diffusion blotting of the RNA to nylon membranes, and hybridization with random primer-labeled DNA were performed as described (Herrin and Schmidt, 1987; Herrin et al., 1990). Molecular size markers for denaturing agarose gels were E. coli rRNA (Pharmacia) and rRNAs of Chlamydomonas. Hybridization with the 14-mer oligonucleotide was in 6 x SSPE, 1% SDS, 400 μg/mL denatured sperm DNA at 42°C, which corresponds to a stringency equal to the predicted Tₘ (using the program of Rychlik and Rhoads, 1989). The blot was washed at room temperature for 15 min, then at 37 to 40°C for 15 min in 5 x SSPE, 0.2% SDS.

Unless specified, PAGE was performed on a sequencing gel apparatus, using 19:1 acrylamide:bisacrylamide, 7 to 8 M urea, and 1 x TBE (90 mM Tris, 83 mM boric acid, 1 mM EDTA, pH 8.3); the gels were prewarmed and electrophoresed at ~50 to 55°C. All samples for PAGE were denatured by heating to 80 to 90°C in formamide (Sambrook et al., 1989). For size markers, pUC18 digested with TaqI and phage φX174 RNA restricted with Haell1 (New England Biolabs) were used; the fragments were end labeled using the exchange reaction of T4 kinase (Sambrook et al., 1989). To extend the range of DNA markers above 1500
nucleotides and increase the number of fragments, a construct consisting of a portion of λ phage DNA cloned into pUC18 was used. This construct was made by cutting circular λ DNA with EcoRI and ligating it to pUC18 digested with EcoRI; a clone containing the −24-kb annealed end fragments of λ was identified and called pAT-1. To prepare end-labeled markers, pAT-1 was cut with Hincll and labeled with T4 kinase using the exchange reaction (Sambrook et al., 1989). This provided a large number of fragments between 5000 and 250 nucleotides, whose sizes were verified by comparison to other commercially available molecular size markers.

Primer Extension and cDNA Sequencing

Primer extension of the transcription/splicing products of pgEM.R14.2 (intron 2) with the 21-mer oligonucleotide (see above) was carried out as described by Boorstein and Craig (1989) in the presence of 75 μg/mL actinomycin D. Their procedure was modified by performing the annealing step at 42°C for 30 min and the RNA was not removed prior to electrophoresis. After electrophoresis, the −158-nucleotide cDNA was eluted from the gel (Latham et al., 1990) and sequenced by using Maxam-Gilbert reactions (Sambrook et al., 1989).

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REFERENCES


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