Group II intron splicing in chloroplasts: identification of mutations determining intron stability and fate of exon RNA

Vera Holländer+ and Ulrich Kück*

Lehrstuhl für Allgemeine Botanik, Fakultät für Biologie, Ruhr-Universität Bochum, D-44780 Bochum, Germany

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ABSTRACT

In order to investigate in vivo splicing of group II introns in chloroplasts, we previously have integrated the mitochondrial intron rI1 from the green alga Scenedesmus obliquus into the Chlamydomonas chloroplast tscA gene. This construct allows a functional analysis of conserved intron sequences in vivo, since intron rI1 is correctly spliced in chloroplasts. Using site-directed mutagenesis, deletions of the conserved intron domains V and VI were performed. In another set of experiments, each possible substitution of the strictly conserved first intron nucleotide G1 was generated, as well as each possible single and double mutation of the tertiary base pairing \( \gamma-\gamma' \) involved in the formation of the intron’s tertiary RNA structure. In most cases, the intron mutations showed the same effect on in vitro intron splicing efficiency as they did on the in vivo self-splicing reaction, since catalytic activity is provided by the intron RNA itself. In vivo, all mutations have additional effects on the chimeric tscA-rI1 RNA, most probably due to the role played by trans-acting factors in intron processing. Substitutions of the \( \gamma-\gamma' \) base pair lead to an accumulation of excised intron RNA, since intron stability is increased. In sharp contrast to autocatalytic splicing, all point mutations result in a complete loss of exon RNA, although the spliced intron accumulates to high levels. Intron degradation and exon ligation only occur in double mutants with restored base pairing between the \( \gamma \) and \( \gamma' \) sites. Therefore, we conclude that intron degradation, as well as the ligation of exon–exon molecules, depends on the tertiary intron structure. Furthermore, our data suggest that intron excision proceeds in vivo independent of ligation of exon–exon molecules.

INTRODUCTION

Group II introns occur in the organelles of algae, higher plants and fungi and have also recently been discovered in some prokaryotic organisms (1,2). Group II introns are characterized by their conserved structure (Fig. 1) which participates in a characteristic splicing reaction with two transesterification steps (3). This splicing mechanism, which results in the excision of an intron lariat, is also used by nuclear pre-mRNA introns, whilst being spliced by a multisubunit ribonucleoprotein complex called the spliceosome (4). Further functional similarities between nuclear and group II introns have been found, such as corresponding RNA–RNA interactions, and conserved intron nucleotides especially participating in the lariat formation (reviewed in 5,6). These similarities, along with the detection of trans-spliced group II introns, which also depend on intermolecular interactions with trans-acting RNA molecules (7–9), have led to the assumption that group II introns and nuclear pre-mRNA introns may be functionally or even evolutionarily related (10–12).

In contrast to nuclear introns, what is generally known about contribution of cis-acting sequences during group II intron splicing, stems mainly from in vitro studies using autocatalytic intron RNAs without any associated protein factors. Compared to in vitro investigations, only a few reports show data from in vivo studies using site-directed intron mutations (13–16). In these cases the mitochondrial intron aI5 from yeast was used in a comparative analysis. The in vivo data available indicate some significant differences when identical intron point mutations are compared in vivo and in vitro, suggesting that in organelles trans-acting factors play an important role in splicing (13,16).

For chloroplasts, comparable data are not yet available, although we and others have already established in vivo splicing systems for the alga Chlamydomonas reinhardtii (17,18) and tobacco (19). In our system, we use the tscA gene from C.reinhardtii as a vehicle to introduce the mitochondrial group II intron rI1 (20) into the chloroplast genome. The tscA gene encodes a 400 nt RNA, which is involved in trans-splicing of mRNA transcribed from the discontinuous psaA gene (7,9). Using the photosynthesis-deficient mutant strain H13 from C.reinhardtii, with a deletion of the tscA gene, chimeric and non-chimeric tscA genes can be used to restore photosynthetic activity, and to select for transformants. In previous studies we have shown that the heterologous intron rI1 is correctly spliced in C.reinhardtii chloroplasts (17). Even when intron binding site 2 (IBS2) is missing, we observed unaltered splicing efficiencies of

*To whom correspondence should be addressed. Tel: +49 234 700 6212; Fax: +49 234 709 4184; Email: ulrich.kueck@ruhr-uni-bochum.de

*Present address: QIAGEN GmbH, Max-Volmer-Straße 4, D-40724 Hilden, Germany
the heterologous intron in vivo (18). Therefore, this system is suitable for in vivo investigations of mutated intron RNAs.

The tscA RNA exonic sequence can be used as an important tool for investigating mutant intron RNAs, since it tolerates insertions of heterologous sequences without losing its function during trans-splicing (7). Thus, even splicing deficient intron derivatives can be analyzed in vivo. Besides that, our system also allows a comparative investigation of mutant introns in vivo and in vitro, since H1 shows autocatalytic activities in vitro (21). As far as we know, this is the first case in which site-directed intron mutations have been analyzed in chloroplasts.

We have investigated a set of rI1 intron derivatives with mutations in cis-acting sequences that are highly conserved in group II introns (2). These sequences are essential for both secondary and tertiary interactions within the intron RNA. Our data led us to conclude that, in general, splicing efficiency is determined by identical nucleotide mutations in vivo and in vitro. In addition, we observed in vivo-specific phenotypes, which concern, besides splicing efficiencies, mainly splicing mechanism and post-splicing metabolism. Our experiments led to the discovery of intron nucleotides and tertiary interactions which control intron stability and the fate of exon RNA in chloroplasts.

MATERIALS AND METHODS

Strains

Wild-type C. reinhardtii strain CC410, and the photosynthetically defective mutant H13 (kindly provided by Professor Bennoun, Paris, France), have been described previously (22,23).

Oligonucleotides used in this work

Relative positions of oligonucleotides complementary to either the Scenedesmus obliquus LSU RNA gene, or the C. reinhardtii tscA gene, are given according to the DNA sequence from the EMBL data library (accession no. C17375), or the sequence information published by Choquet et al. (22) (Table 1).

Plasmid construction and in vitro mutagenesis

All plasmids used for chloroplast transformations are in vitro mutated derivatives of plasmid prI1s (17). In vitro mutagenesis was performed by PCR (Table 1). The resulting amplification products containing intron rI1 together with its IBS1 were cloned into the tscA gene of plasmid pIG637.1 (17). This insertion separates the tscA gene into a 5′ and a 3′ exon of ~140 and 290 bp. Mutants of intron nucleotide A398 were generated by overlap extension as described by Ho et al. (24). Mutations at both positions 398 and 608 were obtained, using a second round of PCR mutagenesis, and template DNA taken from A398 mutants.

Chloroplast transformation

Chloroplast transformation was performed with a home-made particle gun (26) using the procedure developed by Boynton et al. (27). After transformation, the cells were incubated for ~16 h in dim light, replated on minimal medium, and placed in bright light for 4 weeks.

Table 1. DNA sequence of oligonucleotides used in this work

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequence</th>
<th>Position</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>418</td>
<td>AAC AGG GTG CGA CCT GCA AA</td>
<td>1598–1617</td>
<td>S. o.: IBS1 and 5′ end of group II intron</td>
</tr>
<tr>
<td>419</td>
<td>AGT TGG ATA AGG AGG CCC TC</td>
<td>2192–2211</td>
<td>S. o.: 3′ end of group II intron</td>
</tr>
<tr>
<td>558</td>
<td>AAC AGG (A;C;T)TG CGA CCT GCA AA</td>
<td>1598–1617</td>
<td>S. o.: IBS1 and 5′ end of group II intron, substitution of G1</td>
</tr>
<tr>
<td>560/635/636</td>
<td>(C/T)GGT TGG ATA AGG AGG CCC TC</td>
<td>2192–2211</td>
<td>S. o.: 3′ end of group II intron, substitution of T608 (γ)</td>
</tr>
<tr>
<td>561</td>
<td>AGT TAG TAG AAC GTG CGA TGC G</td>
<td>2160–2176/2208–2211</td>
<td>S. o.: 3′ end of group II intron, deletion of domain VI</td>
</tr>
<tr>
<td>570</td>
<td>AGT TGG ATA AGG AGG CCC TCT CAG GCT TTC CCC CCA GTA GCA AGC ATG GAA ACT CTT GAA</td>
<td>2139–2142/2172–2211</td>
<td>S. o.: 3′ end of group II intron, deletion of domain V</td>
</tr>
<tr>
<td>641</td>
<td>TTT GCC CTG TC(A;G)TT ACC CAC TAG</td>
<td>1989–2012</td>
<td>S. o.: group II intron, substitution of A398 (γ)</td>
</tr>
<tr>
<td>642</td>
<td>CTA GTG GGT AAA (C;G;T)GA CAG GCC AAA</td>
<td>1989–2012</td>
<td>S. o.: group II intron, substitution of A398 (γ)</td>
</tr>
<tr>
<td>654</td>
<td>TAC CCA TTT ATT TGA AGG GC</td>
<td>1492–1511</td>
<td>C. r.: 5′ exon of tscA gene</td>
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<tr>
<td>655</td>
<td>ATT AAA ATC GGC ATT ACT TG</td>
<td>1617–1636</td>
<td>C. r.: 3′ exon of tscA gene</td>
</tr>
</tbody>
</table>

*References for nucleotide sequences are given in the Materials and Methods. Abbreviations: S. o., Scenedesmus obliquus; C. r., Chlamydomonas reinhardtii.*
**Analysis of C.reinhardtii transformants**

Isolation of nucleic acids from *C.reinhardtii* was performed as described previously (9). For northern hybridization analysis, total RNA isolated from different *C.reinhardtii* transformants was separated electrophoretically, blotted onto nylon membranes, and hybridized with a radioactively labeled DNA probe, according to standard procedures (28,29). For each construct, at least two independent transformants were analyzed. The intron rI1 derived from plasmid pIG597.1. A BglIII–NsiI restriction fragment (920 bp) from plasmid pIG637.1 was used as a tscA-specific probe, and plasmid pZmc100 as a chloroplast rRNA-specific probe (17,30). The amounts of RNA applied to the gels were calibrated using the chloroplast tRNA-specific probe as an internal control. Control RNA and spliced intron RNA were quantified using BioImager (Fuji) scanning. PCR amplification of total DNA and RNA from the *C.reinhardtii* transformants was carried out as described previously (17).

**Run-on transcription**

Permeabilized cells for run-on transcription assays were prepared using a freeze–thaw procedure (31). The in vivo labeling was performed as described by Sakamoto et al. (32). The labeled total RNA was directly used in filter hybridizations with dot blots carrying 10 µg of various denatured plasmid DNAs.

**In vitro RNA analyses**

Uniformly 35S-labeled run-off transcripts were generated by *in vitro* transcription of plasmid pR1s-s, and its derivatives with mutated intron rI1, linearized by XhoI digestion. The reaction was performed in a final volume of 20 µl containing 1 µg plasmid DNA, 40 mM Tris–HCl (pH 8.0), 6 mM MgCl2, 2 mM spermidine, 10 mM dithiothreitol, 25 U RNase inhibitor (Boehringer, Mannheim, Germany), 500 µM ATP, CTP, GTP and UTP, 20 µCi [α-35S]UTP (Amersham, Braunschweig, Germany; 400 Ci/mmol) and 40 U T3-RNA Polymerase (Boehringer). Transcription was carried out for 1 h at 30°C to prevent self-splicing during transcription. The full-length transcripts were purified on a denaturing polyacrylamide gel and eluted from the gel by incubation for ~16 h at 4°C in 500 mM NH4 acetate, 10 mM MgCl2, 0.1 mM EDTA and 0.1% SDS. For *in vitro* splicing experiments, the eluted RNA was ethanol precipitated, resuspended in reaction buffer containing 0.5 M NH4Cl, 40 mM Tris (pH 7.5), 60 mM MgCl2 and 2 mM spermidine, and incubated for 30 min at 45°C. Reaction products were analyzed on denaturing 4% polyacrylamide–8 M urea gels. Relative splicing efficiencies were estimated by performing at least two incubations of each mutant and wild-type precursor RNA. The level of splicing products was quantified after electrophoresis and BioImager (Fuji) scanning. Relative splicing efficiencies were determined as described by Jacquier and Michel (33). In order to isolate intron lariats, *in vitro* transcription of pR1s-s and its derivatives was performed as described above. The transcripts were precipitated and resuspended in the described splicing buffer and incubated for 60 min at 45°C. The intron lariats were eluted from denaturing polyacrylamide gels. For reverse splicing, the lariats together with gel-purified *in vitro* transcripts of pscAEF/XbaI were incubated for 1 h at 55°C in splicing buffer.

**RESULTS**

**Splicing of intron rI1 mutants**

We have shown previously that rI1 is able to splice *in vitro* as well as in *C.reinhardtii* (17). Using site-directed mutagenesis, complete deletions of domains V and VI from intron rI1 were performed (Fig. 1). In addition, we generated every possible substitution of the first intron nucleotide G1, and each possible single and double mutation of positions A398 and U608, which form the conserved tertiary γ–γ′ interaction (Fig. 1). All mutated intron domains and nucleotides have been shown previously to be functional during *in vitro* splicing of yeast group II introns (3). Therefore, the corresponding changes in rI1 were chosen for a functional *in vivo* analysis. The wild-type intron and its mutated derivatives were integrated into the *C.reinhardtii* chloroplast tscA gene and transformed into *C.reinhardtii* chloroplasts.

*In vivo* splicing of chimeric tscA-rI1 RNAs was tested by RNA gel blot analysis. Figure 2A shows northern hybridizations of various intron mutants with the intron-specific probe. For comparison, all filters were rehybridized with a probe specific for chloroplast tRNA (Fig. 2B). In addition, we show the corresponding hybridization with a tscA probe (Fig. 2C). The *C.reinhardtii* wild-type strain, the recipient H13 and the transformant TrH1s (which contains the wild-type intron rI1) were used as controls. The hybridization of transformant TrH1s shows the spliced intron of ~600 nt, whereas the heterologous intron is visible in neither the *C.reinhardtii* wild-type strain nor the strain H13.

All intron mutations result in substantial alterations in splicing activity *in vivo*. Transformant TADV, which lacks domain V, accumulates only the unspliced precursor transcript of ~1000 nt (Fig. 2A). Similarly, no splicing products were detected in transformant TADVI, which lacks domain VI. In contrast, none of the G1 or γ–γ′ nucleotide substitutions completely blocks splicing *in vivo* (see Fig. 2A for examples). In each mutant, the intron-specific probe detected the unspliced precursor RNA, and the intron–3′ exon splicing intermediate, as well as variable amounts of the excised intron. The lariat structure was verified by

**Figure 1.** Schematic representation of the secondary structure of the *S.obliquus* group II intron rI1. Introns sequences are shown as a solid line, 5′ and 3′ exon sequences are represented by white boxes. Roman numerals (I–VI) denote the six structural domains of group II introns. Domain I is subdivided into sub-domains (A–D); EBS1–IBS1, EBS2–IBS2, a, α′, γ, γ′ and ε–ε′ indicate three-dimensional base pairings. The nucleotides G1, A398 (γ and U608 (γ′), as well as the domains V and VI, which were either substituted or deleted by PCR-mediated mutagenesis, are indicated.
In vivo splicing phenotypes of C. reinhardtii transformants carrying r11 mutants. Total RNA, from each strain as indicated, was hybridized with an intron r11-specific probe. The icons on the right identify the splicing products from top to bottom as: unspliced precursor transcript, intron–3′ exon splicing intermediate and intron lariat. Arrows indicate the splicing intermediate and intron lariat of transformant TG1A, and the intron lariat of Tγ'C. (B) Calibration of total RNA in each lane by hybridization with a chloroplast rRNA probe. (C) Detection of exon RNA in C. reinhardtii transformants carrying mutant derivatives of intron r11 using a tscA-specific probe. The icons on the right identify the splicing products, from top to bottom as: unspliced precursor transcript, intron–3′ exon splicing intermediate and ligated exons. The autoradiograph was overexposed to detect even traces of exon–exon products in the mutants. WT, C. reinhardtii wild-type strain; H13, recipient strain; T, transformants carrying a chimeric tscA–r11 gene; r11s, wild-type intron r11; ΔDV, deletion of intron domain V; ΔDVI, deletion of domain VI; G1A, G1C and G1U, substitutions of the first intron nucleotide G1; A398C, substitution of the first intron nucleotide G1 to C; A508R, substitution of the γ nucleotide A398 to C; U608C, substitution of the γ nucleotide U608 to C; γC′, γ′C; γC′G and γ′C′A, substitutions of both γ and γ′ positions.

In nearly all γ–γ′ mutants, due to a reduction in efficiency at the second splicing step. Only double mutants containing a restored γ–γ′ base pair (γG–γ′C, γC–γ′G and γU–γ′A instead of the wild-type combination γA–γ′U) lack the accumulation of intermediate molecules and show splicing efficiencies comparable with the wild-type intron. Thus, the γ–γ′ tertiary interaction is an important determinant for the splicing reaction in chloroplasts, and only Watson–Crick base pairings enable an efficient splicing reaction in vivo.

In order to test whether or not the results obtained from in vivo investigations correspond with the data from in vitro splicing experiments, the wild-type intron and each intron mutant was analyzed with respect to its self-splicing activity. In addition, we want to demonstrate that r11 behaves similarly in vitro as the well studied yeast mitochondrial intron al5y(3). As shown in Figure 3, the wild-type intron r11 shows an efficient self-splicing reaction under high salt conditions, leading to the ligated exon–exon molecule and the intron lariat, as well as the linear intron RNA. As expected, the deletion of domain V completely prevents autocatalytic activity in vitro as it does in vivo. In contrast, precursor RNA lacking domain V1 performs an in vitro splicing reaction, although only the unspliced precursor RNA accumulates in vivo (Fig. 2A). This mutation abolishes branching in both cases, but in vitro it retains hydrolytic activity, leading to different linear splicing products of various lengths. The free 5′ exon has the expected size, indicating a correct 5′ but an incorrect 3′ hydrolysis reaction. As was shown previously with al5y domain V1 is the major determinant of 3′ splice site selection in vitro (34,35). All three G1 mutants and all 15 mutants with the γ–γ′ base pairing

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**Figure 2.** In vivo splicing phenotypes of C. reinhardtii transformants carrying r11 mutants. Total RNA, from each strain as indicated, was hybridized with an intron r11-specific probe. The icons on the right identify the splicing products from top to bottom as: unspliced precursor transcript, intron–3′ exon splicing intermediate and intron lariat. Arrows indicate the splicing intermediate and intron lariat of transformant TG1A, and the intron lariat of Tγ'C. (B) Calibration of total RNA in each lane by hybridization with a chloroplast rRNA probe. (C) Detection of exon RNA in C. reinhardtii transformants carrying mutant derivatives of intron r11 using a tscA-specific probe. The icons on the right identify the splicing products, from top to bottom as: unspliced precursor transcript, intron–3′ exon splicing intermediate and ligated exons. The autoradiograph was overexposed to detect even traces of exon–exon products in the mutants. WT, C. reinhardtii wild-type strain; H13, recipient strain; T, transformants carrying a chimeric tscA–r11 gene; r11s, wild-type intron r11; ΔDV, deletion of intron domain V; ΔDVI, deletion of domain VI; G1A, G1C and G1U, substitutions of the first intron nucleotide G1; A398C, substitution of the first intron nucleotide G1 to C; A508R, substitution of the γ nucleotide A398 to C; U608C, substitution of the γ nucleotide U608 to C; γC′, γ′C; γC′G and γ′C′A, substitutions of both γ and γ′ positions.

**Figure 3.** In vitro splicing of intron r11 and its mutants. Transcripts of r11s-s (r11) containing either the wild-type intron, or its derivatives carrying a deletion of domain V (ΔDV) or domain V1 (ΔDVI), or a substitution of the first intron nucleotide G1 (G1A, G1C, G1U), and were used in self-splicing experiments (Materials and Methods). Samples were incubated for 0 (0) and 30 min (1) at 45°C and separated on a 4% polyacrylamide–8 M urea gel. Splicing products are: IM, intron–3′ exon splicing intermediate; L, intron lariat; P, unspliced precursor RNA; IVS, linear intron; E-E, ligated exons; 5′E, 5′ exon. The lower part of the autoradiogram was overexposed to show even lower molecular weight RNAs.
self-splice to some extent, producing branched intron RNA and ligated exons. As an example G1 mutants are shown in Figure 3; similar experiments with γ′-γ′ mutants provide comparable autoradiographs showing the same splicing products, including the accumulated lariat–3′ exon intermediate (data not shown). The intron–3′ exon intermediates, as well as the 5′ exon, accumulate to higher levels than those resulting from the wild-type splicing reaction, due to a reduction in splicing efficiency.

The in vitro data were determined as given in Materials and Methods and can be summarized as follows. Each substitution of G1 inhibits both self-splicing steps, to some extent with G1U being the most reactive mutant. On the other hand, none of the γ′-γ′ mutants is severely affected in the first step of the splicing reaction. In contrast, the rate of the second splicing step is markedly reduced in both single and double mutants of the γ′-γ′ base pair. Only double mutants forming a γ′-γ′ Watson–Crick base pair suppress this phenotype (data not shown). Thus, our data are in accordance with in vitro splicing data obtained with intron α5γ (15,33,36). In summary, all rI1 mutations analyzed in this contribution alter the relative splicing efficiencies in vitro as well as in vivo, and therefore the corresponding nucleotides are involved in both the autocatalytic splicing reaction and the organellar splicing process.

Loss of exon RNA in rI1 mutants

In order to detect spliced exon RNA in vivo, the tscA-specific probe was used in northern hybridizations against total RNA isolated from rI1 mutants. As can be seen in Figure 2C, the hybridization probe detected the mature tscA RNA of ∼430 nt from the wild-type strain, as well as from transformant TrI1s. In the latter, splicing of intron rI1 is a prerequisite for the formation of the mature tscA RNA. The recipient strain H13 did not show any signals in northern hybridizations, due to the deleted tscA gene (22). Only the unsuppressed precursor transcript of ∼1000 nt appeared in the non-splicing transformants TADV and TADVI. All point mutants, carrying a substitution at either the first intron nucleotide G1 or at the γ′ and γ′ sites, splice to some extent in vivo, as confirmed by hybridizations with the intron-specific probe (Fig. 2A). Thus, the tscA-specific probe detected the precursor RNA as well as varying amounts of the splicing intermediate (Fig. 2C). Even in cases where only trace amounts of the unsuppressed precursor RNA is present, RT–PCR clearly detected the tscA-rI1 precursor (data not shown). Although all point mutants accumulate the excised intron RNA, no ligated exon–exon molecules were detected in either transformants with G1 substitutions, or in transformants TA398C, TU608C and TγCy′G (Fig. 2C). In addition, no bands representing either the free 5′ exon (∼140 nt) or the 3′ exon (∼290 nt) were detected in any of the transformants carrying either the wild-type or mutated rI1 introns. Photoautotrophic growth of these transformants is guaranteed by the unsuppressed tscA-rI1 precursor.
Figure 5 summarizes in vivo accumulation of exon–exon splicing products, in all transformants carrying a mutation at the \( \gamma^{-}\gamma' \) interaction. Each substitution at the \( \gamma' \) site U608 in single or double mutants leads to a complete loss of the exon–exon RNA. In these transformants, ligated \( tscA \) exons were not detected, neither by northern hybridizations, nor by either RT–PCR or primer extension experiments (data not shown). Only those mutants with a restored \( \gamma-\gamma' \) base pairing contained amounts of spliced exon RNA comparable with amounts generated by transformant TrI1s (which carries the wild-type intron). Substitutions of the \( \gamma' \) nucleotide A398 resulted in decreased amounts of the mature \( tscA \) RNA, which were visible at least in RT–PCR experiments. In contrast, each point mutant analyzed generates exon–exon molecules during \textit{in vitro} self-splicing (data not shown, compare G1 mutants in Fig. 3). Therefore, we conclude that accumulation of ligated exon–exon molecules depends on the tertiary \( \gamma-\gamma' \) base pairing in vivo, but not \textit{in vitro}.

A loss of exon–exon molecules \textit{in vivo} may be caused by an enhanced reverse splicing activity of mutated intron RNAs compared with the wild-type intron or by a hydrolytic re-opening of spliced exons. In order to assay the reverse splicing activity of wild-type and mutated rI1 \textit{in vitro}, wild-type and mutant lariats were isolated and incubated with wild-type spliced exon–exon substrate (Fig. 6). As a control, a precursor transcript carrying the wild-type intron was used for \textit{in vitro} self-splicing (data not shown; for details see Materials and Methods). As expected, our \textit{in vitro} splicing of the wild-type intron was performed, using precursor RNA derived of prI1-s/XbaI. Lane 1 shows the unspliced precursor transcript (P), lane 2 contains the reaction products obtained by \textit{in vitro} self-splicing (S). The wild-type lariat and lariats carrying a single mutation at the \( \gamma' \) site (A398G), at the \( \gamma' \) site (U608C) and a \( \gamma-\gamma' \) double mutation (\( \gamma' \)C\( \gamma' \)) were incubated together with an exon–exon transcript (lanes 3–6). In addition, each lariat as well as the exon–exon substrate was incubated separately (lanes 7–11). All reactions were incubated for 60 min at 55°C and analyzed by gel electrophoresis followed by autoradiography. The overexposure was done to detect all possible reaction products. Reaction products are: IM, intron–3’ exon splicing intermediate; L, lariat; P, unspliced precursor RNA; IVS, linear intron or broken lariat, respectively; EE, exon–exon molecule. In lanes 1 and 2, we used labeled precursor (P) RNA, while in lanes 3–11, the exon–exon as well as the lariat intron were labeled (for details see Materials and Methods).

Figure 6. Reverse splicing activity of rI1 and its mutants. As a control, \textit{in vitro} splicing of the wild-type intron was performed, using precursor RNA derived of prI1-s/XbaI. Lane 1 shows the unspliced precursor transcript (P), lane 2 contains the reaction products obtained by \textit{in vitro} self-splicing (S). The wild-type lariat and lariats carrying a single mutation at the \( \gamma' \) site (A398G), at the \( \gamma' \) site (U608C) and a \( \gamma-\gamma' \) double mutation (\( \gamma' \)C\( \gamma' \)) were incubated together with an exon–exon transcript (lanes 3–6). In addition, each lariat as well as the exon–exon substrate was incubated separately (lanes 7–11). All reactions were incubated for 60 min at 55°C and analyzed by gel electrophoresis followed by autoradiography. The overexposure was done to detect all possible reaction products. Reaction products are: IM, intron–3’ exon splicing intermediate; L, lariat; P, unspliced precursor RNA; IVS, linear intron or broken lariat, respectively; EE, exon–exon molecule. In lanes 1 and 2, we used labeled precursor (P) RNA, while in lanes 3–11, the exon–exon as well as the lariat intron were labeled (for details see Materials and Methods).

DISCUSSION

We focused on group II intron processing in chloroplasts, using the \textit{in vivo} system developed by Herdenberger \textit{et al.} (17). Site-directed intron mutations of conserved \textit{cis}-acting sequences were examined with respect to their splicing activity \textit{in vivo} and \textit{in vitro}. The results of our \textit{in vivo} studies demonstrated that intron mutations affect splicing efficiency, further post-splicing steps such as intron RNA degradation and the fate of exon RNA. To the best of our knowledge, this is the first functional analysis of group II intron mutations in chloroplasts.

Intron rI1 mutants show similar effects on their splicing activity \textit{in vitro} and \textit{in vivo}

Studies with the mitochondrial intron a15\( \gamma' \) from yeast showed that domain V, the most highly conserved domain of group II introns (2), is essential for any splicing reaction \textit{in vivo} and \textit{in vitro} (13,16,35,37). As expected, our \textit{in vitro} and \textit{in vivo} data with mutant T\( \Delta VD \) confirmed the importance of domain V for group II intron splicing, since a deletion of this domain completely prevented autocatalytic splicing as well as splicing in \textit{Chlamydomonas} chloroplasts. Because of the strict conservation of the sequence...
and structure of domain V in all known group II introns, the function of this domain most probably can be conferred to all self-splicing and non-autocatalytic group II introns. In contrast to intron domain V, domain VI is not essential for autocatalytic splicing reactions of either yeast or Scenedesmus group II introns. Domain VI contains a bulged A nucleotide, which participates in the 2′–5′ phosphodiester bonding with the first intron nucleotide G1, forming the branched lariat structure (3). Therefore, deletion of domain VI inhibits branching in vivo, although to some extent it does still allow hydrolytic splicing of both yeast introns (35,43) and intron rI1. In contrast to the autocatalytic splicing experiments, deletion of domain VI completely blocks any splicing reaction of rI1 in chloroplasts. We suppose that intron rI1 probably does not use a hydrolytic splicing pathway in vivo, although it has been shown recently that branching point mutants of aI5 perform a hydrolytic splicing reaction in the first splicing step in mitochondria, suggesting a general ability of group II introns for hydrolytic reactions in vivo (44).

In group II introns, the tertiary γ–γ′ base pair is formed between a nucleotide from the segment between domains II and III, and the last intron nucleotide (A398 and U608 in rI1) (2,22; see also Fig. 1). This study is the first analysis to investigate the significance of this interaction in vivo. All single and double mutations of the γ–γ′ interaction show only slight effects on the first splicing step. But the second splicing step is severely influenced in vitro as well as in vivo. Up until now, the γ–γ′ interaction has only been analyzed in self-splicining experiments using the yeast intron aI5γ (33) and the Podospora anserina intron COI II (45). Substitutions at the γ′ site, in both single and double rI1 mutants, as well as in aI5γ, result in a dramatic reduction in efficiency of the second splicing step (34; this work). In both introns, only those double mutants containing a restored Watson–Crick base pair between the γ and γ′ sites show splicing efficiencies comparable to the wild-type. Therefore, the formation of a γ–γ′ base pair is critical for cleavage at the 5′ exon–intron junction, in vitro, as well as in chloroplasts.

The first intron nucleotide G1 is highly conserved in group II introns and nuclear pre-mRNA introns. It interacts with the bulged A of domain VI to form the branched structure (2). Nevertheless, substitutions of G1 in the rI1 intron to A, C or U, support excision of the intron lariat in vitro, as well as in Chlamydomonas chloroplasts, as was verified by primer extension analyses (data not shown). Therefore, the identity of the 5′-terminal intron nucleotide is not essential for branching, although all G1 mutations result in a marked decrease in the efficiency of splicing, at both splicing steps. Similar results have been obtained with G1 mutants of intron aI5γ, which also splice in vitro and in yeast mitochondria, with reduced rates at both reaction steps (15,36). The efficiency of the first splicing step is decreased in G1 mutants, since guanine seems to be the optimal factor for binding and stabilizing the catalytically active intron structure. This putative factor recognizes at least the positions and identities of the first (G1) and last intron nucleotide (γ′), as well as the three-dimensional structure built up by the γ–γ′ interaction. In this way, the intermediate complex is stabilized and the 3′ splice site is fixed near the 5′ exon. Thus, exon ligation and simultaneous excision of the intron RNA through transesterification is possible. A mutation in one of these nucleotides results in either a destabilization of the intermediate complex (which consists of the intron–3′ exon lariat and the free 5′ exon), and retain the splice sites inside the catalytic center (reviewed in 3). In vivo, it may be supposed that a trans-acting factor is required for binding and stabilizing the catalytically active intron structure. This putative factor recognizes at least the positions and identities of the first (G1) and last intron nucleotide (γ′), as well as the three-dimensional structure built up by the γ–γ′ interaction. In this way, the intermediate complex is stabilized and the 3′ splice site is fixed near the 5′ exon. Therefore, a trans-acting factor is most probably required for correct second step splicing.

In vitro, several cis-acting elements stabilize the intermediate complex (which consists of the intron–3′ exon lariat and the free 5′ exon), and retain the splice sites inside the catalytic center (reviewed in 3). In vivo, it may be supposed that a trans-acting factor is required for binding and stabilizing the catalytically active intron structure. This putative factor recognizes at least the positions and identities of the first (G1) and last intron nucleotide (γ′), as well as the three-dimensional structure built up by the γ–γ′ interaction. In this way, the intermediate complex is stabilized and the 3′ splice site is fixed near the 5′ exon. Thus, exon ligation and simultaneous excision of the intron RNA through transesterification is possible. A mutation in one of these nucleotides results in either a destabilization of the intermediate complex (which consists of the intron–3′ exon lariat and the free 5′ exon), and retain the splice sites inside the catalytic center (reviewed in 3). In vivo, it may be supposed that a trans-acting factor is required for binding and stabilizing the catalytically active intron structure. This putative factor recognizes at least the positions and identities of the first (G1) and last intron nucleotide (γ′), as well as the three-dimensional structure built up by the γ–γ′ interaction. In this way, the intermediate complex is stabilized and the 3′ splice site is fixed near the 5′ exon. Thus, exon ligation and simultaneous excision of the intron RNA through transesterification is possible. A mutation in one of these nucleotides results in either a destabilization of the intermediate complex (which consists of the intron–3′ exon lariat and the free 5′ exon), and retain the splice sites inside the catalytic center (reviewed in 3). In vivo, it may be supposed that a trans-acting factor is required for binding and stabilizing the catalytically active intron structure. This putative factor recognizes at least the positions and identities of the first (G1) and last intron nucleotide (γ′), as well as the three-dimensional structure built up by the γ–γ′ interaction. In this way, the intermediate complex is stabilized and the 3′ splice site is fixed near the 5′ exon. Thus, exon ligation and simultaneous excision of the intron RNA through transesterification is possible.

Mutations in either G1 or γ–γ′ nucleotides support in vivo intron excision, whilst exon ligation is completely blocked (exon–exon molecules were neither detected by northern hybridization nor by primer extension or RT–PCR). Only single mutants of A398 (γ) and double mutants with restored γ–γ′ interaction, exhibit significant amounts of spliced tscA RNA. In contrast, in both rI1 and aI5γ (33), in vitro splicing of G1 and γ–γ′ mutants leads to reduced splicing efficiencies, and production of the expected exon–exon RNAs. In addition, the intron lariat is also formed, since intron excision and exon ligation occur simultaneously through transesterification.

A possible explanation for the lack of exon–exon molecules might be a reverse reaction of excised intron RNAs by either hydrolysis or transesterification leading to a reduction of spliced exons in vivo (35,47). Reverse splicing experiments using various rI1 lariats show that intron mutants, which lack ligated exons in vivo, do not exhibit increased reverse splicing or spliced exon re-opening activities compared to the wild-type intron. Therefore, it is more likely that, instead of exon re-opening, no ligated exons are formed in the presence of mutant introns. Since no comparable phenotype is visible in vitro, we suggest that the rI1 mutations inhibit the binding of one or more splicing factors. Although branching is not affected during the first splicing step in vivo, the ligation of the exons during the second splicing step is prevented. Therefore, a trans-acting factor is most probably required for correct second step splicing.

In vitro, several cis-acting elements stabilize the intermediate complex (which consists of the intron–3′ exon lariat and the free 5′ exon), and retain the splice sites inside the catalytic center (reviewed in 3). In vivo, it may be supposed that a trans-acting factor is required for binding and stabilizing the catalytically active intron structure. This putative factor recognizes at least the positions and identities of the first (G1) and last intron nucleotide (γ′), as well as the three-dimensional structure built up by the γ–γ′ interaction. In this way, the intermediate complex is stabilized and the 3′ splice site is fixed near the 5′ exon. Thus, exon ligation and simultaneous excision of the intron RNA through transesterification is possible.
Since rI1 does not contain an open reading frame, all trans-acting factors involved in the splicing reaction must be encoded in C.reinhardtii. So far, only a few mitochondrial trans-splicing factors have been described for group II introns (reviewed in 52). In contrast, no chloroplast trans-acting factor able to bind group II introns is yet known. However, several mutants which affect chloroplast group II intron splicing have been reported; including two nuclear mutations in maize, which result in splicing deficiencies in chloroplasts (53). Although the plastome of C.reinhardtii does not contain continuous group II introns, there are two split introns (9). These introns are spliced in trans by forming the typical group II intron structure intermolecularly. At least 14 different nuclear products are required for trans-splicing of these introns (7,23,54,55). Some of these trans-splicing factors are probably also involved in cis-splicing of the heterologous intron rI1.

**Intron RNA stability depends on the γ–γ′ interaction in vivo**

Mutations of the γ–γ′ base pair, and to a lesser extent of the first intron nucleotide G1, result in an accumulation of very high levels of spliced intron RNA (when compared with the wild-type intron rI1). This accumulation of the wild-type intron in C.reinhardtii chloroplasts corresponds to the accumulation observed in *S.obliquus* mitochondria (56).

Since run-on transcription assays revealed that the rate of chimeric tscA-rI1 gene transcription remains unaltered in the mutants, accumulation of the excised intron must be due to increased intron stability. Similarly, in yeast mitochondria, several mutations of domains V and VI of α5γ alter the accumulation of spliced intron (15,16). However, in contrast to our findings, the excised α5γ intron barely accumulated. The mutants analyzed alter intron structure and splicing in a way that promotes the degradation of the excised intron RNA (44).

Intron mutations can also inhibit intron degradation, as shown for the first time with intron rI1. The altered sequence and structure of the intron RNA probably impedes either efficient recognition, or hydrolysis by ribonucleases. Alternatively, changes to the intron’s structure could strengthen binding between the RNA and trans-acting proteins, providing the RNA with protection against RNases. This mechanism has previously been suggested for group I introns by Margossian and Butow (57). The nuclear-encoded SUV3 protein of yeast shows homology to helicases and is a part of the mitochondrial exonexcision complexes ‘mtEXO’ (58,59). Mutation of the *sun3* gene leads to an increased accumulation of spliced group I introns in mitochondria, since SUV3 most probably releases intron-bound splicing proteins via its helicase function and thereby enables degradation of the naked RNA by the exonucleobase complex (57,60). An efficient degradation of excised intron RNAs is important, since free introns can cause toxicity by interactions with other cellular RNAs or by exon reopening reactions (58,61).

The point mutations analyzed in this paper show dramatic effects not only on splicing efficiency, but also on exon ligation and intron stability. The conserved sequence of the γ–γ′ interaction, which in all group II introns has a purine at the γ site and a pyrimidine at the γ′ position, might be a compromise between these three functions. It has been conserved through evolution, since at least intron degradation depends not only on the γ–γ′ base pairing but also on the purine–pyrimidine distribution between these sites.

In most cases, all of the cis-acting elements mentioned above show similar effects on the relative splicing efficiency of rI1 both in vitro and in vivo as well as an intron α5γ (15,34,35). However, further comparisons between autocatalytic splicing and in vivo processing of rI1 and other group II introns have revealed significant differences, mainly in phenotypes including post-splicing mechanisms. Since protein–RNA interactions determine splicing in vivo, intron-, species- and organelle-specific differences in binding between the intron RNA and trans-factors are likely. The co-evolution of introns and splicing factors has led to specific changes in intron sequences, structural elements and specific processing reactions. These changes have resulted in a complete loss of autocatalytic activity, as is the case for most group II introns (3,62). Still, a high similarity between group II introns is guaranteed, since the catalysis is driven by the RNA itself, and alteration of the intron RNA is therefore limited. This conservation enables a horizontal transfer of relatively ancient introns, which do not require specific splicing factors for processing. This is proven by the successful transfer of rI1 between mitochondria and chloroplasts of different green algae as well as into *Escherichia coli* (63).

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35случайно чисел, приведенных в тексте.