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

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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 γ–γ′ involved in the formation of the intron's tertiary RNA structure. In most cases, the intron mutations showed the same effect on *in vivo* intron splicing efficiency as they did on the *in vitro* 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 γ–γ′ 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 γ and γ′ 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 aI5γ 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

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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 rI1 shows autocatalytical 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* LSUrRNA gene, or the *C.reinhardtii tscA* gene, are given according to either 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. Plasmids and corresponding *Chlamydomonas* transformants have been named according to the intron mutations which they carry. For *in vitro* splicing experiments, plasmid prI1s-s was generated by inserting a 759 bp PCR fragment from plasmid prI1s into vector pT3T7/*Eco*RV (18). prI1s-s carries the wild-type version of intron rI1, flanked by 46 bp of the 5′ exon, and 99 bp of the 3′ exon from the *tscA* gene. Subclones of all mutated derivatives from plasmid prI1s were constructed by PCR cloning, in a similar manner to plasmid prI1s-s. After cloning, all PCR fragments were completely sequenced using the dideoxy chain-termination method (25). Plasmid ptscAEE was constructed, in order to generate *in vitro* a mosaic *tscA* transcript carrying the IBS1 sequence of *S.obliquus*. Therefore, exon–exon molecules obtained from *in vitro* splicing of prI1s-s/*Xba*I transcripts were eluted from a 4% polyacrylamide–8 M urea gel (see below) and used as a template for reverse transcription, followed by PCR amplification using oligonucleotides 654 and 655. The resulting 151 bp PCR product was cloned into vector pT3T7/*Eco*RV and completely sequenced.

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

No.	Sequence	Position[a]	Probe
418	AAC AGG GTG CGA CCT GCA AA	1598–1617	*S. o.*: IBS1 and 5′ end of group II intron
419	AGT TGG ATA GGT AGG CCC TC	2192–2211	*S. o.*: 3′ end of group II intron
558	AAC AGG (A;C;T)TG CGA CCT GCA AA	1598–1617	*S. o.*: IBS1 and 5′ end of group II intron, substitution of G1
560/635/636	(C/T/G)GT TGG ATA GGT AGG CCC TC	2192–2211	*S. o.*: 3′ end of group II intron, substitution of T608 (γ′)
561	AGT TAG TAG AAC CGT GCA TGC G	2160–2176/2208–2211	*S. o.*: 3′ end of group II intron, deletion of domain VI
570	AGT TGG ATA GGT AGG CCC TCT CAG GCT TTC CCC CCA GTA GCA AGC ATG GAA ACT CTT GAA	2139–2120/2172–2211	*S. o.*: 3′ end of group II intron, deletion of domain V
641	TTT GCC CTG TC(A,C,G) TTT ACC CAC TAG	1989–2012	*S. o.:* group II intron, substitution of A398 (γ)
642	CTA GTG GGT AAA (C,G,T)GA CAG GGC AAA	1989–2012	*S. o.:* group II intron, substitution of A398 (γ)
654	TAC CCA TTT ATT TGA AGG GC	1492–1511	*C .r.*: 5′ exon of *tscA* gene
655	ATT AAA ATC GGC ATT ACT TG	1617–1636	*C. r.*: 3′ exon of *tscA* gene

[a]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 probe was an *Eco*RI–*Hin*dIII restriction fragment (635 bp) derived from plasmid pIG597.1. A *Bgl*II–*Nsi*I 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 rRNA-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 ^{35}S-labeled run off-transcripts were generated by *in vitro* transcription of plasmid prI1s-s, and its derivatives with mutated intron rI1, linearized by *Xba*I 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 $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 25 U RNase inhibitor (Boehringer, Mannheim, Germany), 500 µM ATP, CTP, GTP and UTP, 20 µCi [α-^{35}S]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 NH_4 acetate, 10 mM $MgCl_2$, 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 NH_4Cl, 40 mM Tris (pH 7.5), 60 mM $MgCl_2$ 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 prI1s-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 ptscAEE/*Xba*I were incubated for 1 h at 55°C in splicing buffer.

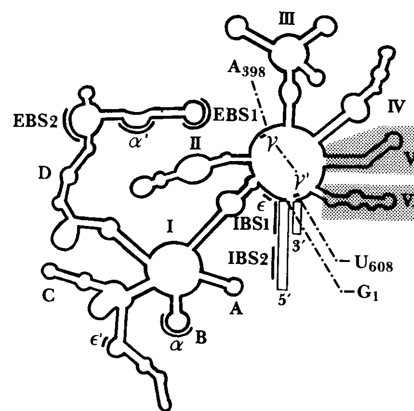

Figure 1. Schematic representation of the secondary structure of the *S.obliquus* group II intron rI1. Intron sequences are shown as a solid line, 5′ and 3′ exon sequences are represented by white boxes. Roman numerals (I–VI) denote the six structural domains of group II introns. Domain I is subdivided into sub-domains (A–D). EBS1–IBS1, EBS2–IBS2, α-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.

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 rRNA (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 TrI1s (which contains the wild-type intron rI1) were used as controls. The hybridization of transformant TrI1s 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 TΔDV, which lacks domain V, accumulates only the unspliced precursor transcript of ~1000 nt (Fig. 2A). Similarly, no splicing products were detected in transformant TΔDVI, 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

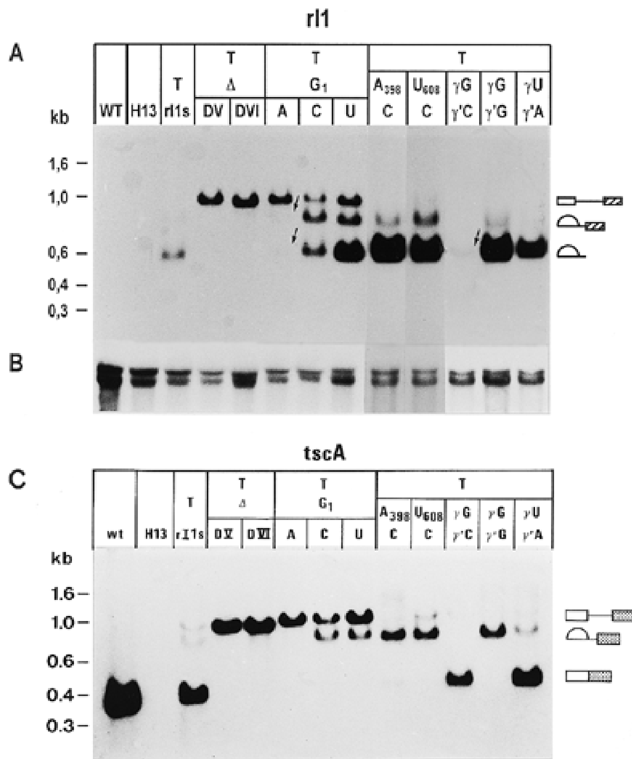

Figure 2. (**A**) *In vivo* splicing phenotypes of *C.reinhardtii* transformants carrying rI1 mutants. Total RNA, from each strain as indicated, was hybridized with an intron rI1-specific probe. The icons on the right identify the splicing products from top to bottom as: unspliced precursor transcript, intron–3′ exon splicing intermediate and intron lariat. Arrows indicate the splicing intermediate and intron lariat of transformant TG1A, and the intron lariat of TγGγ′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 rI1 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*-rI1 gene; rI1s, wild-type intron rI1; 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 γ-nucleotide A398 to C; U608C, substitution of the γ′ nucleotide U608 to C; γGγ′C, γGγ′G and γUγ′A, substitutions of both γ and γ′ positions.

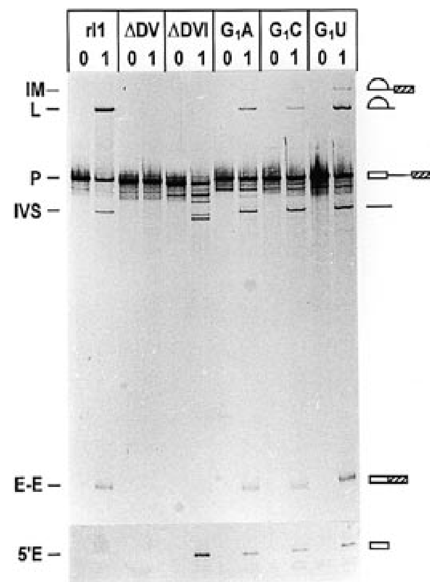

Figure 3. *In vitro* splicing of intron rI1 and its mutants. Transcripts of prI1s-s (rI1) containing either the wild-type intron, or its derivatives carrying a deletion of domain V (ΔDV) or domain VI (ΔDVI), or a substitution of the first intron nucleotide G1 (G1A, G1C, G1U), and were used in self-splicing 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.

primer extension experiments (data not shown). The different accumulations of transcripts and splicing products suggest alterations in the *in vivo* splicing efficiencies. An accumulation of the precursor RNA shows a reduction in efficiency of the first splicing step, whereas a decreased rate at the second splicing step is characterized by an increased amount of the splicing intermediate. The change of G1 to A, C or U markedly affects both splicing steps *in vivo*. Splicing of the G1A mutant is almost blocked at the first splicing step, whereas transformants TG1C and TG1U show a weaker reduction at both steps, resulting in simultaneous accumulation of precursor RNA, intermediate and spliced intron.

In contrast to G1 mutants, only very low amounts of the unspliced precursor *tscA*-rI1 transcript were detected in transformants with mutations at the γ–γ′ interaction. Thus, the γ–γ′ base pairing has no major effect on the first splicing step *in vivo*. On the other hand, the splicing intermediate gives a clear signal 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 rI1 behaves similarly *in vitro* as the well studied yeast mitochondrial intron aI5γ (3). As shown in Figure 3, the wild-type intron rI1 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 VI 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 aI5γ domain VI 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

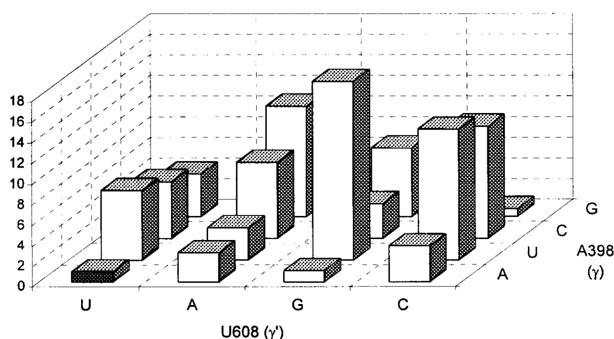

Figure 4. γ–γ′ dependent accumulation of spliced intron rI1. Intron accumulation in mutants carrying alterations at positions 398 (γ) and 608 (γ′) was determined by northern hybridizations and RNA amounts were quantified using BioImager (Fuji) scanning. All intron RNAs were standardized using chloroplast rRNA, which was quantified after hybridization and BioImager scanning. All values are relative to the wild-type intron. The accumulation of the wild-type intron (γA398–γ′U608) is indicated by a shaded bar.

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 aI5γ (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.

γ–γ′ base pairing is a determinant of intron RNA stability *in vivo*

The northern hybridizations shown in Figure 2 reveal a further significant feature for *in vivo*-processing of rI1 mutants. In transformants TA398C, TU608C, as well as in the double mutants TγGγ′G and TγUγ′A, spliced intron RNA accumulates to a much higher degree than it does in transformant TrI1s (which carries the wild-type intron). Figure 4 compares intron accumulation in all single and double mutants of the γ–γ′ base pair with TrI1s. Each substitution at position A398 and U608, as well as nearly all γ–γ′ double mutations, cause an increased abundance of excised intron rI1 up to 18 times more compared with the wild type. Only double mutant TγGγ′C, which contains a A398G and a U608C substitution (forming a Watson–Crick base pair), accumulates spliced intron RNA closed to TrI1s levels (0.8 compared to wild-type). Double mutants with a restored γ–γ′ base pairing, but with a pyrimidine at the γ site and a purine at the γ′ position (TγUγ′A and TγCγ′G), yield lower amounts of intron RNA, when compared with other γ–γ′mutants. However, they do accumulate significantly more excised intron RNA (about three times more) than TγGγ′C and TrI1s, both containing the conserved sequence, which is a purine at the γ and a pyrimidine at the γ′ position.

Enhanced intron accumulation in γ–γ′ mutants may be caused by mutated intron RNA having a higher stability. Alternatively, it could be explained by an increased transcription rate of *tscA*-rI1 genes with mutated introns, leading to greater amounts of splicing products. In order to distinguish between these two possibilities, run-on transcription experiments were performed. Transformant TrI1s (carrying the wild-type intron) and transformant TU608C (with a U to C substitution at the γ′ site) were chosen for use in run-on transcription experiments, since they had previously been shown to accumulate the spliced intron RNA at different levels (Fig. 2A). Radioactively labeled transcripts from both transformants were used to probe immobilized chloroplast DNA fragments. Positive and negative controls as well as the chimeric *tscA*-rI1 gene gave similar signals with both transformants analyzed (data not shown), although transformant TU608C did accumulate the excised intron RNA to a much higher level than transformant TrI1s (Fig. 2). Since the relative transcription activities were similar in both cases, we conclude that the intron mutation does not significantly alter the *tscA*-rI1 transcription rate, and therefore the differences in intron RNA accumulation are most probably due to different intron RNA stabilities. Thus, the post-splicing metabolism of this intron RNA is affected by the γ–γ′ tertiary interaction, with only purines at the γ site and pyrimidines at the γ′ position being able to support efficient intron degradation.

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 unspliced precursor transcript of ~1000 nt appeared in the non-splicing transformants TΔDV and TΔDVI. 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 unspliced 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 TγGγ′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 unspliced *tscA*-rI1 precursor.

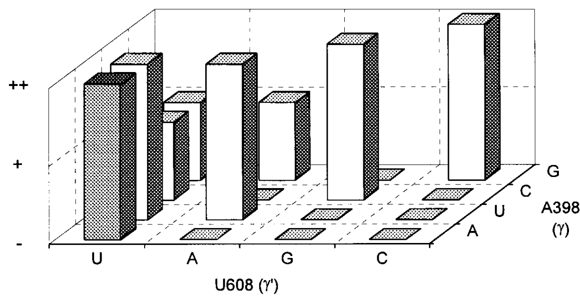

Figure 5. γ–γ′-dependent occurrence of exon–exon molecules during *in vivo* splicing. Mutants with substitutions at positions 398 (γ) and 608 (γ′) were analyzed by northern hybridization (Fig. 2C) and RT–PCR (data not shown). ++, spliced exon–exon molecules were visible in northern hybridizations and RT–PCR (e.g. TrI1s); +, ligated exons were only detected in RT–PCR assays (e.g. TA398C); –, exon–exon molecules were not detected (e.g. TU608C). The exon–exon accumulation in transformant TrI1s carrying the wild-type intron (γA398–γ′U608) is indicated by a shaded bar.

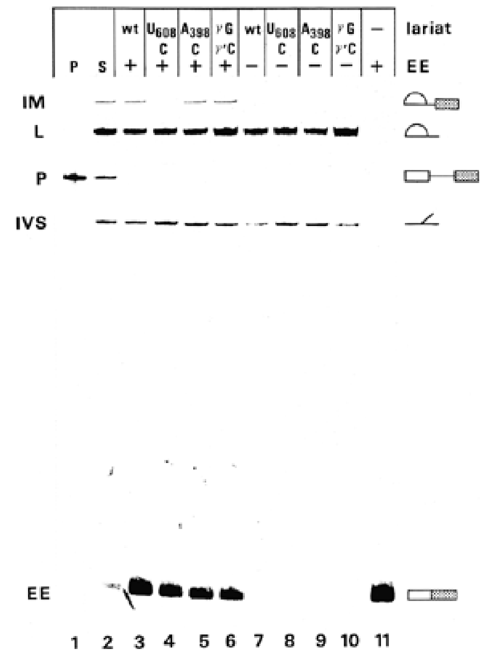

Figure 6. Reverse splicing activity of rI1 and its mutants. As a control, *in vitro* splicing of the wild-type intron was performed, using precursor RNA derived of prI1s-s/*Xba*I. Lane 1 shows the unspliced precursor transcript (P), lane 2 contains the reaction products obtained by *in vitro* self-splicing (S). The wild-type lariat and lariats carrying a single mutation at the γ site (A398G), at the γ′ site (U608C) and a γ–γ′ double mutation (γGγ′C) 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, intron lariat; P, unspliced precursor RNA; IVS, linear intron or broken lariat, respectively; E E, 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 5 summarizes *in vivo* accumulation of exon–exon splicing products, in all transformants carrying a mutation at the γ–γ′ interaction. Each substitution at the γ′ 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 γ–γ′ base pairing contained amounts of spliced exon RNA comparable with amounts generated by transformant TrI1s (which carries the wild-type intron). Substitutions of the γ 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 *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 γ–γ′ base pairing *in vivo*, but not *in vitro*.

A loss of exon–exon molecules *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 *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 *in vitro* self-splicing, leading to the formation of ligated exons, linear and lariat intron as well as of the intron–3′ exon splicing intermediate. After incubation of the wild-type lariat together with spliced exon substrate, the intron–3′ exon splicing intermediate is visible, suggesting that the second splicing step is easily reversible (Fig. 6). This intermediate is not detected after separate incubations of the lariat or the exon–exon substrate. Similarly, mutants A398C and γGγ′C produced the intron–3′ exon intermediate to similar levels compared to the wild-type lariat (Fig. 6). These intron mutations do not cause a loss of exon–exon molecules *in vivo* (Figs 2C and 5). In contrast, mutant U608C, which does not accumulate spliced exons *in vivo*, formed only trace amounts of the intermediate molecule. These data show that reverse splicing reactions of the excised intron RNA should not be responsible for the loss of exon RNAs. Instead, our data suggest that intron excision may proceed independently of exon ligation *in vivo*.

DISCUSSION

We focused on group II intron processing in chloroplasts, using the *in vivo* system developed by Herdenberger *et al.* (17). Site-directed intron mutations of conserved *cis*-acting sequences were examined with respect to their splicing activity *in vivo* and *in vitro*. The results of our *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 *in vitro* and *in vivo*

Studies with the mitochondrial intron aI5γ from yeast showed that domain V, the most highly conserved domain of group II introns (2), is essential for any splicing reaction *in vivo* and *in vitro* (13,16,35,37). As expected, our *in vitro* and *in vivo* data with mutant TΔDV 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 *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′ posphodiester bonding with the first intron nucleotide G1, forming the branched lariat structure (3). Therefore, deletion of domain VI inhibits branching *in vitro*, 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 aI5γ 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 pairing is formed between a nucleotide from the segment between domains II and III, and the last intron nucleotide (A398 and U608 in rI1) (2,21; 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-splicing experiments using the yeast intron aI5γ (33) and the *Podospora anserina* intron COI I1 (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 3′ 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 substrate for transesterification (36). After branching *in vitro*, G1 from aI5γ interacts by a non-Watson–Crick base pairing with the penultimate intron nucleotide and thus contributes to the conformational change proceeding the second splicing reaction (36,46). Whereas the penultimate nucleotide is an adenosine in most group II introns, including aI5γ, in rI1 a cytosine (C607) is located at this position (2,20). In aI5γ, the substitution of the penultimate A to a C leads to a significant reduction of the second splicing step efficiency (36). It is possible that in rI1 the non-optimal interaction of G1 and C607 is further weakened by mutations at the first intron nucleotide, leading to a further decrease in the splicing rate.

Our data, as well as recent reports for the yeast intron aI5γ (13–16), demonstrate a general agreement between the *in vitro* and *in vivo* splicing efficiencies of intron mutants. This confirms that in both cases splicing is catalyzed by the intron RNA itself, and depends very much on similar secondary and tertiary intron structures.

Intron excision occurs independently from exon–exon ligation during *in vivo* splicing

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 either 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 (15,33), *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. A mutation in one of these nucleotides results in either a weakened interaction with the putative *trans*-factor (A398, γ′), or in a complete loss of binding (G1 and U608, γ′). This leads to either a destabilization of the intermediate complex, or to formation of an inactive conformation. In this case, the intron can be excised by hydrolytic cleavage. This assumption is supported by the detection in yeast mitochondria of a aI5γ 5′ hydrolysis reaction (44). Since no transesterification occurs, the 5′ and 3′ exons are released separately. The non-ligated exons are undetectable *in vivo*. They are rapidly degraded, since they lack the typical end structures necessary to stabilize them in chloroplasts (48–51).

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 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 aI5γ alter the accumulation of spliced intron (15,16). However, in contrast to our findings, the excised aI5γ 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 part of the mitochondrial exoribonuclease complexes 'mtEXO' (58,59). Mutation of the *suv3* 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 exoribonuclease 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 aI5γ (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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