A multiprotein complex involved in chloroplast group II intron splicing

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ABSTRACT

The *psaA* gene of the green alga *Chlamydomonas reinhardtii* consists of three exons that are widely separated on the chloroplast genome and transcribed independently. The exons are flanked by group II intron sequences. Maturation of the *psaA* mRNA requires two steps of splicing in *trans* between the transcripts of exons 1, 2, and 3. At least 14 nuclear loci and one chloroplast gene (*tscA*) are involved in this process. Recently the genes of three of these nuclear factors have been cloned. Raa3 is involved in the first *trans*-splicing reaction, and Raa1 and Raa2 are required for the second *trans*-splicing reaction. Here we show that Raa1 and Raa2 can be coimmunoprecipitated and that they are part of a high molecular weight complex of 400–500 kD. The size and integrity of the complex are affected by mutations in other complementation groups, suggesting that the corresponding proteins may also be components of this multiprotein complex or required for its assembly. Raa1 is also associated with a larger complex.

Keywords: *Trans*-splicing; group II intron; chloroplast; *Chlamydomonas*; protein complex

INTRODUCTION

As other photosynthetic eukaryotic cells, the green alga *Chlamydomonas reinhardtii* contains three distinct genetic systems located in the nucleus, chloroplast, and mitochondria. The biogenesis of the photosynthetic apparatus depends on a close coordination between the nuclear and chloroplast systems. Some of the subunits of the photosynthetic complexes are encoded by nuclear genes, translated on cytosolic ribosomes, and imported into the chloroplast, whereas other subunits are synthesized in the chloroplast (Rochaix 1996). Genetic analysis of the assembly of the photosynthetic machinery in *Chlamydomonas* and land plants has revealed a large set of nucleus-encoded factors that are required for different posttranscriptional steps including RNA stability and processing, splicing, and translation. A striking example of the dependence of chloroplast gene expression on nucleus-encoded factors is provided by the *psaA* gene of *C. reinhardtii*. This gene consists of three exons that are dispersed on the chloroplast genome and

flanked by group II intron sequences (Kück et al. 1987). Each exon is transcribed independently (Choquet et al. 1988). Maturation of the *psaA* mRNA involves two *trans*-splicing reactions and requires at least 14 nucleus-encoded factors (Goldschmidt-Clermont et al. 1990). Mutants deficient in this *trans*-splicing process can be grouped in three different classes based on their RNA phenotype. Class A mutants are deficient in the splicing of exons 2 and 3, class C mutant are affected in the splicing of exons 1 and 2, and class B mutants are deficient in both steps of *trans*-splicing. An unusual feature of the first *psaA* intron is that its middle part is provided by another locus of the chloroplast genome, called *tscA*. It encodes a small RNA that, together with the 5'- and 3'-intron transcripts, forms a *trans*-spliced tripartite intron (Goldschmidt-Clermont et al. 1991).

Fragmented group II introns also occur in vascular plants. Maturation of the chloroplast *rps12* mRNA involves both *cis*- and *trans*-splicing, and the formation of several mitochondrial mRNAs in vascular plants requires *trans*-splicing reactions (Bonen and Vogel 2001).

Besides their importance in chloroplast and mitochondrial biogenesis, group II introns are also of special interest because it is generally believed that they represent the evolutionary precursors of nuclear introns. They are found in diverse prokaryotic organisms, and in mitochondria and chloroplasts of plants and lower eukaryotes. The classical splicing mechanism of group II introns involves two *trans*-

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esterification steps as for nuclear introns. First, the 5'-exon-intron junction is subject to nucleophilic attack by the branch-site adenosine to give rise to an intron-lariat intermediate. Subsequently, the 3'-intron-exon junction is attacked by the 3'-OH end of the cleaved exon, and the two exons are spliced together. Another pathway for group II intron splicing has been identified in which the first step occurs via hydrolysis and produces a linear excised intron (Jarrell et al. 1988; Podar et al. 1998). More recently, a third mechanism of splicing was described in which group II introns can be excised as circles (Murray et al. 2001).

Although a great deal is known about the molecular mechanisms of splicing of group II introns (Bonen and Vogel 2001), less is known on the protein factors involved in this process. It is well documented that some group II introns can undergo self-splicing reactions *in vitro* via a lariat intermediate under nonphysiological conditions (Michel and Ferat 1995). However, *in vivo* splicing requires the participation of protein factors, some of which are encoded by the intron itself and are called maturases (Lambowitz and Perlman 1990). These proteins were first identified in yeast mitochondria, and some of them act both as splicing factors by promoting the folding of the intron RNA into the catalytically active structure, and as reverse transcriptases in intron mobility (Lambowitz et al. 1999). Maturase-like proteins have also been identified and characterized in plastids (Liere and Link 1995). The recent genome sequencing projects of *Arabidopsis* and rice have revealed putative proteins resembling maturases encoded by nuclear genes, which may be imported into organelles to function in group II intron splicing (Mohr and Lambowitz 2003). Other factors have an indirect effect on splicing. Thus, mutations in the *MRS2* gene that encodes a mitochondrial Mg^{2+} transporter in yeast affect the splicing of all mitochondrial group II introns (Bui et al. 1999; Gregan et al. 2001). A proper Mg^{2+} concentration is essential for the splicing process. Disruption of the *MSS116* in yeast leads to a splicing defect of mitochondrial group I and II introns (Seraphin et al. 1989). This protein contains a RNA helicase domain and appears to stimulate splicing in an ATP-dependent manner (Nierner et al. 1995). In maize, several genes involved in chloroplast group II intron splicing have been identified. *Crs1* is specifically required for the splicing of the chloroplast *atpF* intron (Till et al. 2001). *Crs2* is required for the splicing of nine chloroplast introns (Jenkins et al. 1997). The *Crs2* gene encodes a protein related to peptidyl-tRNA hydrolase. However, the protein appears to lack the hydrolase activity (Jenkins and Barkan 2001). Caf1 and Caf2 are two factors associated with *Crs2* in intron ribonucleoprotein particles with distinct intron specificities (Ostheimer et al. 2003). Together with *Crs1*, they are part of a family of proteins found in plants, eubacteria, and archaeobacteria that contain a basic repeated domain involved in RNA binding (Till et al. 2001; Ostheimer et al. 2003). In *C. reinhardtii*, the genes of three splicing factors have been

cloned and characterized, *Raa1* (M. Goldschmidt-Clermont, K. Perron, M. Rahire, and J.D. Rochaix, in prep.), *Raa2* (previously called *Maa2*; Perron et al. 1999), and *Raa3* (Rivier et al. 2001). *Raa2* is involved in splicing of the second and third *psaA* exons and displays sequence similarity with pseudouridine synthases. However, this activity is not required for the *trans*-splicing reaction. *Raa3* is required to splice the first and second *psaA* exons. It encodes a protein of 176 kD that does not show any sequence similarity to other known proteins except for a short stretch shared with pyridoxamine 5'-P oxidase.

It is apparent that several splicing factors display similarity with enzymes acting on RNA, and on tRNA in particular. *Raa2* and *Crs2*, involved in group II intron splicing, are related to pseudouridine synthases and peptidyl-tRNA hydrolase, respectively (Perron et al. 1999; Jenkins and Barkan 2001). The *CYT-18* gene in *Neurospora crassa* and the *NAM2* gene in *Saccharomyces cerevisiae* encode mitochondrial tyrosyl-tRNA synthetase and leucyl-tRNA synthetase, respectively, which are also required for splicing mitochondrial group I introns (Dujardin and Herbert 1997). It appears that the affinity for RNA of ancestral tRNA-binding proteins has been maintained during evolution and adapted for splicing (Lambowitz and Perlman 1990).

The mechanism of action of these proteins is not yet understood. It is likely that they act as RNA chaperones or splicing facilitators. Recent investigations indicate that some of them form complexes with RNA and with other protein factors. *Raa3* is found in a 1700-kD complex that contains *tsaA* RNA and the transcript of the first *psaA* exon (Rivier et al. 2001). *Crs1* and *Crs2* are also found in complexes larger than 500 kD that cosediment with their respective group II intron transcripts (Jenkins and Barkan 2001; Till et al. 2001). Two proteins of 61 and 31 kD bind specifically to domain IV of a heterologous intron inserted in the *C. reinhardtii* chloroplast genome (Bunse et al. 2001). Ribonucleoprotein complexes containing pre-tRNA with group I and II introns have also been detected in tobacco chloroplasts (Nakamura et al. 1999).

Our previous work raised the possibility that *Raa2* interacts with other *trans*-splicing factors (Perron et al. 1999). Here we show that *Raa1* and *Raa2* can be coimmunoprecipitated and that both proteins are part of a complex of 400–500 kD. As mutations in some of the other *trans*-splicing factors affect the stability or the size of this complex, it is probable that other proteins are associated with it, or involved in its assembly or stability. Moreover *Raa1* is associated with a larger complex that contains RNA.

RESULTS

Raa2 can be released from chloroplast membranes

The *Raa2* polypeptide was previously found to be associated with low-density membranes (LDMs) inside the chloroplast

(Perron et al. 1999). These membranes could be involved in chloroplast mRNA metabolism and could be derived from the chloroplast envelope (Zerges and Rochaix 1998). The association between Raa2 and these LDMs does not appear to be mediated by hydrophobic interactions because non-ionic detergents are unable to destabilize this association. Salts and ionic detergents fully solubilize Raa2, but are known to destabilize protein–protein interactions and could therefore also affect the integrity of a potential Raa2 protein complex. Ammonium sulfate at low concentration can release weakly associated proteins from membranes without affecting protein complexes. We therefore treated chloroplast membranes with different concentrations of ammonium sulfate and analyzed the insoluble and soluble fractions by immunoblotting (Fig. 1A). At 0.1 M ammonium sulfate, the majority of Raa2 was recovered in the soluble fraction, and all of it was fully released with 0.4 to 0.5 M ammonium sulfate (Fig. 1A).

Raa 2 is part of a high molecular weight complex

To test whether Raa2 is part of a protein complex, an extract prepared from total cell membranes treated with 0.5 M ammonium sulfate was loaded on a sucrose density gradient. After centrifugation, 11 fractions were recovered and subjected to immunoblot analysis. Raa2 was present mainly in fractions 6, 7, and 8 with a peak in fraction 7, corresponding to a molecular mass of 500 kD (Fig. 1B, first row). As the molecular mass of Raa2 is only 40 kD, this result indicates that the protein is present in a high molecular weight complex.

Raa2 is required for the *trans*-splicing of *psaA* exons 2 and 3. To test whether a transcript of the second intron is associated with the Raa2 complex, the same experiment was performed with a strain containing a deletion extending over the 3' part of intron 2 and exon 3 of *psaA* (Δ exon3; Fischer et al. 1996). The distribution of Raa2 from this strain was undistinguishable from the wild type after sucrose density gradient centrifugation (Fig. 1B, second row). The same profile was observed by treating the protein extract with RNase A or by inhibiting RNase activity with heparin (Fig. 1B). These results indicate that the 500-kD Raa2 complex does not contain RNA. Treatment of the extract with EDTA did not alter the complex, indicating that divalent cations are not required for its stability.

The Raa2 protein complex is affected in several mutants deficient in *trans*-splicing

It was previously shown that the level of Raa2 polypeptide is strongly reduced in several *trans*-splicing mutants belonging to other complementation groups (Perron et al. 1999). Because the stability of protein complexes is often compromised when one component is missing, this result raises the possibility that some of the other *trans*-splicing factors

FIGURE 1. Raa2 is part of a high molecular weight complex. (A) Release of Raa2 from chloroplast membranes by treatment with increasing concentrations of ammonium sulfate. (P) Insoluble pellet fraction; (S) soluble fraction. (B) Size fractionation of the Raa2 complex. Soluble protein extract obtained after 0.5 M ammonium sulfate treatment was analyzed by sucrose gradient sedimentation. In all, 11 fractions were collected (lane 1, top; lane 11, bottom) and analyzed by immunoblotting using a polyclonal antiserum against Raa2. Treatment of the protein extracts with heparin, RNaseA, or EDTA is indicated on the right. Size markers are shown at the top. (C) Analysis of the Raa2 complex in different class A and B mutants. Conditions were as in B. (D) Total proteins from the *raa1-314B* mutant (*raa1*) were analyzed by immunoblotting with antiserum against Raa2. Antiserum against D1 (a membrane protein from photosystem II) was used as the control.

could be part of the complex together with Raa2 or be required for its assembly and/or stability. To test this possibility, sucrose density gradient fractionations were performed with extracts from the recently characterized *raa1-314B* mutant deficient in exon 2–exon 3 splicing (M. Goldschmidt-Clermont, K. Perron, M. Rahire, and J.D. Rochaix, in prep.) and representative mutants of the four other class A complementation groups and of the two class B complementation groups (Fig. 1C). The Raa2 complex was missing in the *raa1-314B* mutant. Instead, Raa2 was found near the top of the sucrose gradient. Moreover, the amount of Raa2 was strongly reduced in the *raa1-314B* mutant (Fig. 1D). The Raa2 complex was also absent in the L121G mutant, and only trace amounts of Raa2 protein were found at the top of the gradient. Thus, the L121G mutation leads to the

destabilization of the complex and Raa2 can be degraded. Loss of the Raa2 complex was also observed in the class B mutant L137H, in which Raa2 was found exclusively at the top of the gradient. Interestingly, in the other class B mutant, L118B, in which Raa2 accumulates to wild-type levels, the Raa2 complex was slightly shifted toward the top of the gradient with a size of ~400 kD. The L118B factor could be a component of the complex but does not appear to be necessary for its stability.

Coimmunoprecipitation of Raa1 and Raa2

The destabilization of the Raa2 complex in the *raa1-314B* mutant raised the possibility that Raa1 and Raa2 could be part of the same complex. Raa1 was recently characterized, the protein was tagged with HA (haemagglutinin), and a Raa1 antiserum was prepared (M. Goldschmidt-Clermont, K. Perron, M. Rahire, and J.D. Rochaix, in prep.). To test the possibility that Raa1 and Raa2 are associated with the same complex, coimmunoprecipitation experiments were performed. For this purpose, the coding sequence of the HA epitope was introduced into the *Raa2* cDNA just upstream of the stop codon. The coding part of this cDNA was then inserted into an expression vector with the strong promoter, the 5'-UTR and the 3'-UTR of *PsaD* (Fischer and Rochaix 2001). This construct was used to rescue the *raa2-A18* mutant (Perron et al. 1999). In the transformants, the amount of tagged Raa2 protein was similar to or higher than the wild-type Raa2 protein expressed in its normal context (Fig. 2A). For the coimmunoprecipitation experiments, transformant 3.3 was used and named *Raa2:HA*. In this strain, the level of Raa2 polypeptide exceeds that of wild type.

The membrane fraction of *Raa2:HA* was treated with 0.5 M ammonium sulfate, and the soluble fraction was used directly for immunoprecipitation with the HA antibody. The immunoprecipitate was fractionated by SDS-PAGE and immunoblotted with either Raa2 or Raa1 antisera (M. Goldschmidt-Clermont, K. Perron, M. Rahire, and J.D. Rochaix, in prep.). In this way it was possible to detect the Raa2:HA polypeptide as well as Raa1 (Fig. 2B). Raa1 degradation products were also detected; a major band is labeled with an asterisk. No signal was detected in the *raa2-A18* mutant used as control except for the IgGs used for the immunoprecipitation. Similar immunoprecipitations were performed on extracts from the *Raa1:HA* strain containing the HA epitope (M. Goldschmidt-Clermont, K. Perron, M. Rahire, and J.D. Rochaix, in prep.) with polyclonal antibodies directed against Raa2. Immunoblotting with HA antibody revealed the presence of Raa1:HA, but its absence in the *raa1-314B* control strain (Fig. 2B, right panel). In the converse immunoprecipitations with Raa1 antiserum, we were able to coimmunoprecipitate Raa2:HA (Fig. 2C). A small amount of Raa1 protein was detectable in the *raa2* mutant strain, as seen by the presence of the major Raa1

FIGURE 2. Coimmunoprecipitation of Raa2 and Raa1. (A) Immunoblot analysis of total protein extracts from wild-type (WT), *raa2-A18* (*raa2*), and *raa2-A18* transformed with *Raa2:HA*. Three independent transformants are labeled 2.1, 2.2, and 3.3. The Raa2 and Raa2:HA proteins are marked with arrows. The blots were decorated with antisera against Raa2, HA, or D1 (control). (B) Immunoprecipitation (IP) of Raa2:HA using anti-HA (left panels) or anti-Raa2 antisera (right panel). Soluble protein extracts from the strains *raa2-A18* (*raa2*), *raa2-A18* rescued with *Raa2:HA* (*Raa2:HA*), *raa1-314B* (*raa1*), and *raa1-314B* rescued with *Raa1:HA* (*Raa1:HA*) were immunoprecipitated as indicated. The immunoprecipitates were fractionated by SDS-PAGE, blotted, and reacted (IB) with antibodies directed against Raa2, Raa1, or HA. A major Raa1 degradation product is labeled with an asterisk (*). For the separation of Raa2 and Raa1, 10% or 6% polyacrylamide gels were used, respectively. (C) Immunoprecipitation (IP) using Raa1 antiserum. The immunoprecipitates were immunoblotted with Raa1 and HA antisera as indicated.

degradation product marked by an asterisk, indicating that the loss of Raa2 leads to a decreased level of Raa1. No signal was detected with the HA antibody in the *raa2* strain used as control (Fig. 2C). These results clearly show that it is possible to coimmunoprecipitate Raa1 and Raa2.

Cosedimentation of Raa1 and Raa2

The results of Figure 1C indicate that Raa2 is part of a complex and that its integrity depends on several other factors including Raa1. Attempts to rescue representative mutants of the five class A complementation groups by transformation with *Raa1* failed, indicating that the *raa1-314B* mutant belongs to another complementation group. The *Raa1* gene encodes a protein of 205 kD (M. Goldschmidt-Clermont, K. Perron, M. Rahire, and J.D. Rochaix, in prep.). To further test whether Raa1 is part of the Raa2 complex, a strain carrying the *Raa1* gene tagged with an HA epitope was used (*Raa1:HA*; M. Goldschmidt-Clermont, K. Perron, M. Rahire, and J.D. Rochaix, in prep.). Extracts obtained with 0.5 M ammonium sulfate from this strain were fractionated by sucrose density gradient centrifugation. Under the conditions used, which were different from those in Figure 1C (see Materials and Methods), Raa1 was found in the same fractions as Raa2, corresponding to a molecular mass close to 400 kD, slightly smaller than that estimated in Figure 1C (Fig. 3A). It is possible that the HA tag induces changes in the complex and thus affects its sedimentation. Indeed, a small amount of Raa2 was found near the top of the gradient, suggesting that some of the Raa2 proteins cannot integrate into the complex in the presence of the HA-tagged Raa1 protein, or that this complex is less stable and tends to dissociate. The facts that HA-tagged Raa1 and Raa2 cosediment in the sucrose gradient and that sedimentation of Raa2 is affected in the absence of Raa1 (Fig. 1C) strongly suggests that they are part of the same 400–500-kD protein complex.

Although a large portion of the Raa1 polypeptide was found together with Raa2 in fractions 4 and 5 of the gradient (Fig. 3), smaller amounts of Raa1 were also detected in the higher molecular weight fractions. This raised the possibility that

larger complexes were destabilized because of the high salt extraction (0.5 M ammonium sulfate). Therefore, the release of the complex from the membrane was performed with 0.2 M ammonium sulfate, and the soluble fraction was centrifuged in a sucrose gradient. Under these milder conditions, Raa1 and Raa2 were found in the same lower molecular weight fractions (Fig. 3, fractions 4 and 5). However, a portion of Raa1 was observed in a higher molecular weight complex (Fig. 3, fractions 8 and 9). This complex is sensitive to RNase treatment, indicating that it contains RNA (M. Goldschmidt-Clermont, K. Perron, M. Rahire, and J.D. Rochaix, in prep.). Raa2 was undetectable in this larger complex.

DISCUSSION

The maturation of the chloroplast *psaA* mRNA involves two *trans*-splicing reactions and the participation of at least 14 nucleus-encoded factors, of which seven are required for the splicing of exons 1 and 2 (class C), five are required for the splicing of exons 2 and 3 (class A), and two are required for both *trans*-splicing reactions (class B). The Raa1 and Raa2 factors both belong to class A (Perron et al. 1999; M. Goldschmidt-Clermont, K. Perron, M. Rahire, and J.D. Rochaix, in prep.). The results presented here clearly show that Raa1 and Raa2 are part of a multiprotein complex of 400–500 kD. This is based on the cofractionation of these two proteins during sucrose density gradient centrifugation, on the reduced accumulation of Raa2 in the *raa1-314B* mutant and the diminished levels of Raa1 in the *raa2-A18* mutant, and on the reciprocal coimmunoprecipitations, which show that either protein can be coimmunoprecipitated with the other. It is not yet clear whether Raa1 and Raa2 interact directly or whether they interact indirectly through other components of the complex. Moreover, milder extraction of the complexes from the membrane reveal that Raa1 is associated with a larger molecular weight complex containing RNA (Fig. 3; M. Goldschmidt-Clermont, K. Perron, M. Rahire, and J.D. Rochaix, in prep.). It is possible that these different complexes result from a dynamic exchange of factors as observed during spliceosome formation in eukaryotic cells.

The accumulation of the 400–500-kD Raa2 complex is drastically reduced in the L121G mutant that belongs to class A. Interestingly, the complex is also affected in the two class B mutants L137H and L118B. The complex is absent in the former and altered in size in the latter. One possibility is that these mutations affect the genes of other components of the Raa2 complex: loss of some of the subunits could lead to destabilization and degradation of the complex. It is not entirely surprising that class B mutations, which affect *trans*-splicing of both introns, affect a complex that appears to be specifically involved in *trans*-splicing of the second intron. Some of the class B factors could be shared between ribonucleoprotein complexes specific for either the first or second *trans*-splicing reaction. The L118B mutation gives rise to a smaller complex, but Raa2 is not degraded. This

FIGURE 3. Raa2 and Raa1 complexes. Size fractionation of HA-tagged Raa1 and Raa2. Soluble protein extracts obtained after ammonium sulfate treatment from the *raa1-314B* strain rescued with *Raa1:HA* were fractionated by sucrose gradient centrifugation (see Materials and Methods). Fractions 1 and 10 correspond to the *top* and *bottom* fractions of the gradient, respectively. Size markers are indicated in kilodaltons at the *top* of the figure. Fractions were analyzed by immunoblotting using monoclonal HA antibodies (HA) or polyclonal Raa2 antibodies (Raa2). Raa1::HA and Raa2 proteins are indicated by wedges. The lower molecular weight band reacting with Raa2 antibodies is most likely due to a nonspecific interaction.

could be explained if the L118B factor is not necessary for the stability of the complex but is required for the splicing of both *psaA* introns. It could represent a protein specific of group II introns analogous to the eight Sm proteins that are common to the U1, U2, U4, and U5 snRNPs of the nuclear spliceosome (Luhrmann et al. 1990). A factor, Crs2, required for splicing of nine group II introns has also been characterized in maize (Jenkins et al. 1997; Jenkins and Barkan 2001).

A hypothetical scheme of the 400–500-kD Raa2 complex is shown in Figure 4. It could represent a chloroplast pre-spliceosomal complex of group II introns. The complex contains Raa1 and Raa2, but the stoichiometry of the components remains to be established. The three genetically defined factors pL121G, pL118G, and pL137H could also be part of the Raa2 complex. However, another possibility is that some of these factors are required for the assembly and/or stability of the complex without being part of the mature complex. It is also possible that the effect of the mutations is indirect, especially for the class B mutants. In the case of the class B mutant HN31, the factor affected by the mutation appears to be a bifunctional protein. It is involved both in the maturation of the *tscA* RNA required for splicing of exons 1 and 2, and also in the second *trans*-splicing reaction (Hahn et al. 1998).

An RNA protein complex could be identified for Raa1 (M. Goldschmidt-Clermont, K. Perron, M. Rahire, and J.D. Rochaix, in prep.) but not for Raa2. We have been unable to detect any RNA-binding activity of Raa2 using UV cross-linking experiments with solubilized chloroplast extracts

and transcripts of the second *psaA* intron. It is, however, likely that Raa2, which is required for splicing and is related to pseudouridine synthases, interacts in some way with RNA, perhaps only transiently. A different complex of 1700 kD required for the splicing of exons 1 and 2 of *psaA* contains the Raa3 protein, *tscA* RNA, and the transcript of *psaA* exon 1 with the 5' part of intron 1 (Fig. 4; Rivier et al. 2001). This ribonucleoprotein complex is found in the soluble fraction of the chloroplast, unlike the Raa2/Raa1 complex, which is associated with chloroplast membranes and can be released from the membranes by treatment with ammonium sulfate.

The fact that these two complexes have different chloroplast localizations could indicate that they act in different ways or in different steps of the *psaA* splicing pathway. It will be interesting to test whether the L118B mutation, which affects both *trans*-splicing reactions, alters the size of the 1700-kD complex in the same way as that of the Raa2 complex. It is possible that the two *trans*-splicing reactions are spatially restricted, with the first reaction occurring in the stromal phase and the second reaction on the membrane. Membrane association could provide a link to translation. It is noteworthy that translation factors of *C. reinhardtii* have also been localized to plastid low-density membranes (LDMs), which could represent an intermediate during thylakoid membrane biogenesis (Zerges and Rochaix 1998). The *trans*-splicing reaction of the second *psaA* intron could occur on these membranes and could be coupled to the translation and integration of the PsaA protein into chloroplast membranes at an early step of photosystem I biosynthesis. Another interesting difference between these two complexes is that no other known class C or B *trans*-splicing mutation affects the level of Raa3 protein. This is in contrast to Raa2, which is strongly reduced in one mutant of class A (L121G) and one of class B (L137H).

Finally, it is possible that the 400–500-kD Raa2 complex is involved in the storage of *trans*-splicing factors. In mammalian cells, splicing factors are stored in structures called speckles and are released at transcription and splicing sites (Misteli et al. 1997). The Raa2 complex could represent a protein storage complex of this sort that does not contain RNA. The existence of such a group II intron complex together with larger RNA-containing complexes would then suggest a dynamic partitioning of inactive and active splicing factors. Although their exact role remains to be elucidated, our work describes novel multiprotein complexes involved in chloroplast group II intron splicing and provides a basis for the biochemical isolation and characterization of other *trans*-splicing factors and the study of their functional role in the splicing process.

MATERIALS AND METHODS

Strains and media

Chlamydomonas reinhardtii wild-type and mutant strains were grown as described (Harris 1989). *Trans*-splicing mutants were

FIGURE 4. Chloroplast *psaA* splicing complexes. The Raa3 complex required for the first *psaA* *trans*-splicing reaction is present in the stroma, and consists of Raa3, *tscA* RNA, the *psaA* exon 1 precursor transcript with the 5' part of intron 1, and other unknown factors (Rivier et al. 2001). Although our data show that Raa1 and Raa2 are part of the 400–500-kD complex associated with chloroplast membranes (drawn as a lipid bilayer), the presence of the other factors rests on indirect arguments (see text). Another possibility is that these other factors are not constituents of the complex but are required for its assembly and/or stability. Raa1 is also associated with a large molecular weight complex that contains RNA. The complexes are drawn according to their relative size. The *psaA* exons and the split group II introns are shown in the lower part.

described previously (Perron et al. 1999). The *raa1-314B* mutant was isolated and characterized (M. Goldschmidt-Clermont, K. Perron, M. Rahire, and J.D. Rochaix, in prep.).

Nucleic acid techniques

To insert the triple HA epitope, an *StuI* restriction site was created by PCR just upstream of the stop codon of the *Raa2* ORF in plasmid pCD1. A 180-bp fragment containing the 3'-end of the *Raa2* coding sequence was amplified by PCR with oligonucleotides A18/PmlI (5'-GTGCGATCGCTCATT-3') and NheI/*StuI* containing the *StuI* restriction site (5'-GGCGCTAGCGCCGAGTGGTCAAGGCCTATGCGCGTGCCGTTGCAGCAC-3'). After NheI digestion, this fragment was introduced in the *Raa2* cDNA to replace the original PmlI/NheI fragment. A triple HA epitope was introduced and the construction verified by DNA sequencing (ABI prism, 377 DNA sequencer; Perkin Elmer). The full *Raa2:HA* ORF was amplified by PCR with oligonucleotides A18/RI, containing the EcoRI restriction site (5'-GGAATTCTCAAGGACTGCTAGCGGC-3'), and A18/NdeI, containing the NdeI restriction site (5'-ACCACAGCCATATGCGGACGCGGGCAGGG-3'). This amplified ORF was digested with EcoRI and NdeI and inserted at the NdeI and EcoRI sites of the pGenD2 vector containing the *PsaD* flanking regions (Fischer and Rochaix 2001).

Nuclear transformation

Nuclear transformation of the class A mutants A18, HN12, L121G, HN54, and F138 were performed as described previously (Perron et al. 1999). The mutants were treated with autolysin to remove the cell wall and transformed with 2 µg of the construct 518 (M. Goldschmidt-Clermont, K. Perron, M. Rahire, and J.D. Rochaix, in prep.), containing the *Raa1* gene, or with 2 µg of pJN4, conferring emetine resistance (Nelson et al. 1994), as a positive control.

Raa2 solubilization and immunoblot analysis

Immunoblot analysis and Raa2 solubilization were described previously (Perron et al. 1999). Briefly, to test Raa2 solubilization, chloroplast membranes were resuspended in chloroplast lysis buffer (10 mM Tricine at pH 7.8, 5 mM MgCl₂, 5 mM β-mercaptoethanol) containing protease inhibitors (PI: 5 mM ε-amino caproic acid, 1 mM benzamidine HCl, 25 µg/mL pepstatin A, 10 µg/mL leupeptine) and one of the agents to be tested. The extract was incubated for 20 min on ice and centrifuged for 15 min at 50,000g at 4°C. The supernatant containing solubilized Raa2 and the pellet containing the nonsolubilized part were analyzed by immunoblotting.

Coimmunoprecipitation

Protein extract (1 mg), prepared as described for sucrose gradient analysis, was preincubated in 500 µL of TNX100 (Tris-HCl 50 mM at pH 7.4, 100 mM NaCl, 0.5% [w/v] Triton X-100) with 25 µL of protein G agarose (Boehringer Mannheim) during 6 h on a rolling wheel at 4°C and then centrifuged (5 min in a microfuge). The

supernatant was transferred to a new tube and incubated for 16 h in the same conditions with primary antibody directed against Raa1, Raa2, or HA epitope (HA-11; Eurogentec). Protein G agarose (25 µL) was added, and the incubation was continued for 3 h at 4°C. After a 5-min microfuge centrifugation, the pellet was washed five times in TNX100 and twice in 50 mM Tris-HCl (pH 7.4; 15-sec microfuge centrifugations between each wash). The pellet was resuspended in SDS gel loading buffer, boiled for 5 min, and centrifuged (microfuge for 5 min), and the supernatant was analyzed by SDS-PAGE gel and immunoblotting.

Sucrose density gradient centrifugation

Protein extracts for sedimentation on sucrose density gradients were prepared as follows: a pellet containing 5×10^8 cells was resuspended in 10 mL of HKM (20 mM HEPES-KOH at pH 7.8, 50 mM KCl, 10 mM MgCl₂) containing protease inhibitor (PI). Cells were broken by freezing and thawing and centrifuged for 20 min at 10,000 rpm (HB6; Sorvall) at 4°C. The pellet was resuspended in 2 mL of HKM + PI containing 0.5 M ammonium sulfate and incubated for 20 min on ice. Alternatively, 0.2 M ammonium sulfate was used to preserve the larger complexes (Fig. 3). The extract was then centrifuged at 100,000g for 20 min, and the supernatant was loaded on a sucrose gradient. For the experiment shown in Figures 1 and 3, heparin (0.5 mg/mL) or RNase A (0.5 mg/mL) was added in HKM buffer for breaking the cells. For EDTA treatment, HK buffer (HMK without Mg²⁺) containing 10 mM EDTA was used.

Extract (1 mL) was loaded on a 10-mL linear sucrose gradient (0.1–1.3 M) in HKM buffer (or in HK plus 10 mM EDTA) and centrifuged at 145,000g (SW40; Beckmann) for 16 h at 4°C (Fig. 1). Fractions (1 mL) were collected and numbered from 1 (top) to 11 (bottom), sample number 1 corresponding to the volume of protein extract (1 mL) loaded on the gradient. Molecular weight standards from Sigma were used for calibration (Sigma Aldrich MW-GF-70 kit). Each fraction was precipitated by adding 100 µL of trichloroacetic acid (75%) and incubated for 30 min on ice. After a 15-min microfuge centrifugation, the pellet was washed once with ice cold acetone, dried in air, and resuspended in protein SDS gel loading buffer. The acrylamide concentration for SDS-PAGE was 10% (for Raa2 detection) or 6% (for Raa1 detection). The conditions used for the experiments shown in Figure 3 were similar except that the extract was centrifuged at 145,000g for 17 h. In these conditions, the Raa2 complex was found in fractions corresponding to a molecular mass near 400 kD rather than 500 kD.

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