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A factor related to pseudouridine synthases is required for chloroplast group II intron *trans*-splicing in *Chlamydomonas reinhardtii*

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In *Chlamydomonas reinhardtii*, the *psaA* mRNA is assembled by a process involving two steps of *trans*-splicing that remove two group II introns and give rise to the mature mRNA. The products of at least 14 nuclear genes and one chloroplast gene (*tscA*) are necessary for this process. We have cloned *Maa2*, one of the nuclear genes involved in *trans*-splicing of the second intron. *Maa2* encodes a protein with similarity to conserved domains of pseudouridine synthases, but mutagenesis of putative catalytic residues showed that this activity may not be required for *trans*-splicing of *psaA* RNA. Although it is not clear whether the pseudouridine synthase activity has been maintained in Maa2, it is possible that this enzyme was recruited during evolution as an RNA chaperone for folding or stabilizing the *psaA* intron. The Maa2 protein appears to be associated through ionic interactions with a low density membrane system in the chloroplast that also contains RNA-binding proteins involved in translation.
Keywords: *Chlamydomonas reinhardtii*/chloroplast/group II intron/*psaA*/*trans*-splicing

Introduction

Group II introns are found in bacteria, and in organelles from fungi, algae and plants. Many of these introns have been shown to be self-splicing *in vitro*, but proteins are necessary *in vivo* for splicing of some group II introns (Waldherr *et al*., 1993; Guo *et al*., 1997; Jenkins *et al*., 1997; Mills *et al*., 1997; Vogel *et al*., 1997). These introns have a characteristic secondary structure composed of six stem–loops radiating from a central core. The functions of each of these six domains and the tertiary interactions between them are the subject of intensive research (reviewed by Qin and Pyle, 1998). Group II introns may be evolutionary precursors of nuclear introns: their mechanism of splicing is similar and several snRNA components of the spliceosome have their counterparts in some of the six stem–loop domains of group II introns. Thus the snRNAs can be seen as a group II intron in pieces (Sharp, 1991; Wise, 1993).

In the green alga *Chlamydomonas reinhardtii*, the chloroplast *psaA* gene, encoding one of the two reaction centre proteins of photosystem I (PSI), is separated into three exons widely scattered around the chloroplast genome (Kück *et al*., 1987). Each of the three exons is flanked by group II intron sequences. The maturation of the *psaA* mRNA occurs via two steps of *trans*-splicing (Choquet *et al*., 1988) beginning by splicing of the transcripts of either exons 1 and 2 or exons 2 and 3. For the first *trans*-splicing reaction, a small RNA, encoded by the chloroplast *tscA* gene is necessary to complete the structure of the group II intron, which in this case is tripartite (Goldschmidt-Clermont *et al*., 1991).

Mutants affected in the *trans*-splicing of the *psaA* RNA can be grouped into three classes depending on which exon transcripts fail to be spliced. Mutants of class A are defective in the splicing of the transcripts of exons 2 and 3, mutants of class C are defective in the splicing of the transcripts of exons 1 and 2, and mutants of class B are defective in both steps of *trans*-splicing. Genetic analysis of these mutants showed that at least 14 nuclear genes and one chloroplast locus (*tscA*) are required (Goldschmidt-Clermont *et al*., 1990). Recently, one class B mutant was reported to be deficient in both *tscA* RNA maturation and in the splicing of the second intron (Hahn *et al*., 1998). Nevertheless, the mode of action of these nucleus-encoded factors is not understood at the molecular level. To understand further their role in the *trans*-splicing reactions of the *psaA* RNA, we have undertaken the cloning of their genes.

Here we report the isolation of a nuclear gene called *Maa2*, for maturation of psaA, which is required for *trans*-splicing of the second intron of the chloroplast *psaA* RNA. It encodes a polypeptide, Maa2, which displays similarity to pseudouridine synthases. However, we present evidence that this enzymatic activity is not necessary for *trans*-splicing of *psaA* RNA. The protein is enriched in a membrane fraction that may be involved in chloroplast mRNA metabolism and translation (Zerges and Rochaix, 1998).

Results

Analysis of the A18 mutant

Different *C.reinhardtii* nuclear mutants deficient in the *trans*-splicing of the chloroplast *psaA* precursor RNAs were obtained in our laboratory using insertional mutagenesis by transformation of an *arg7* mutant with the pARG7.8 plasmid containing the *Arg7* gene (Debuchy *et al*., 1989). One of these mutants, named A18, was chosen for further study. Its fluorescence transients are characteristic for a deficiency in PSI activity (data not shown). The mutant strain is unable to grow phototrophically and is highly photosensitive (unable to grow on acetate medium at 60 $\mu E/m^2s$) (Figure 1A). The PsaA protein is indetectable by immunoblot analysis (Figure 1B). Northern analysis shows that A18 is a class A mutant, affected in the *trans*-splicing of the second intron of the chloroplast *psaA* gene (Figure 1C) (Choquet *et al*., 1988). The 2.7 kb mature *psaA* mRNA is absent in A18, but the 2.6 kb RNA,

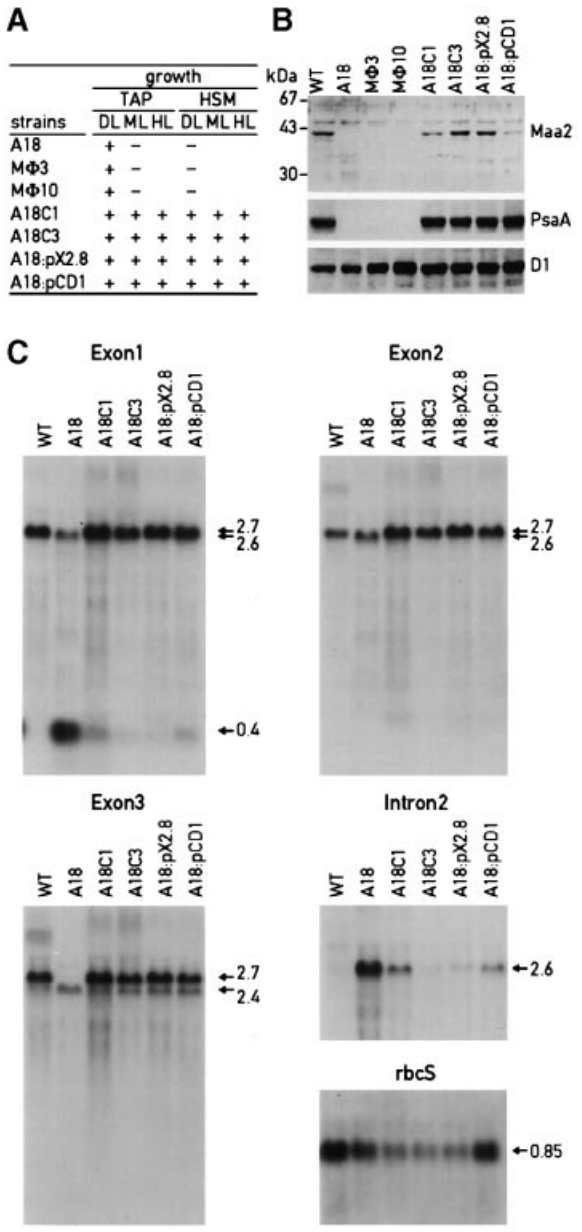


Fig. 1. Analysis of the A18 mutant and of the complemented strains. A18, Mϕ3 and Mϕ10 are the original mutant strains. A18C1 and A18C3 are two independent A18 transformants rescued with the cosmid library. A18:pX2.8 and A18:pCD1 are A18 strains transformed with the pX2.8 construct and the cDNA, respectively. (**A**) Photosynthetic growth and photosensitivity. '+' indicates normal growth, '–' indicates absence of growth. DL, dim light (~6 μE/m^{2}s); ML, medium light (~60 μE/m^{2}s); HL, high light (~600 μE/m^{2}s); TAP, Tris–acetate–phosphate medium; HSM, high-salt minimal medium. (**B**) Immunoblot analysis. Total protein (80 μg) was separated by SDS–PAGE in a 10% gel. The blot was decorated with antibodies against Maa2, PsaA or D1 (product of *psbA*) as indicated. (**C**) RNA blot probed with the three different exons, the second intron of the *psaA* gene and *rbcS* as loading control. The sizes of major transcripts are indicated: 2.7 kb corresponds to the mature *psaA* mRNA, 2.6 kb to exons 1 and 2 spliced together and retaining the 5′ part of the second intron, 0.4 kb to the exon 1 precursor, and 2.4 kb to the exon 3 precursor.

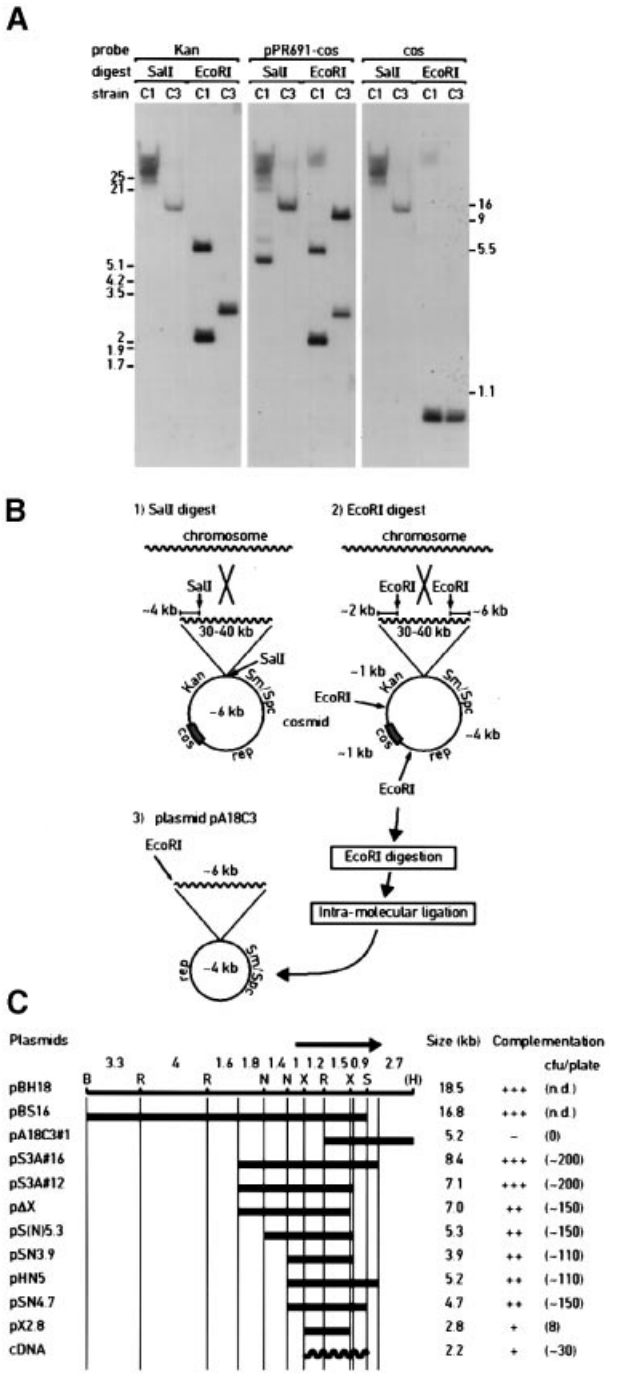
corresponding to the intermediate with spliced exon 1 and exon 2 transcripts, is detected by probing with either exon 1 or exon 2, and also with the intron 2 probe corresponding to the 5′ part of the second intron. Hybridization with intron 2 clearly shows that this transcript overaccumulates in the A18 mutant, as a consequence of the splicing defect, but is barely detectable in the wild-type strain. The exon 3 probe detects only the 2.4 kb exon 3 precursor RNA, indicating that this exon is transcribed, but remains unspliced. The large amount of unspliced exon 1 precursor that accumulates in A18 is typical of *trans*-splicing mutants of the three classes A, B and C (Choquet *et al*., 1988).

Cloning of the Maa2 gene

In order to clone the nuclear gene, *Maa2*, affected in A18, this mutant was rescued by transformation with a cosmid library (Purton and Rochaix, 1994) and selection for phototrophic growth. Two transformants, A18C1 and A18C3, were obtained that had wild-type fluorescence transients, grew on minimal medium and accumulated mature PsaA mRNA and protein (Figure 1). Nevertheless, A18C1 still accumulated the exon 1 precursor and the 2.6 kb transcript containing intron 2 (Figure 1C). This could be explained by an insufficient expression of the *Maa2* gene due to a position effect (the cosmid integrated in a DNA region with poor expression) or by the fact that the gene was not fully present in the cosmid. To estimate the number of cosmid copies inserted in A18C1 and A18C3, Southern blots were hybridized with different parts of the cosmid vector (Figure 2A). This analysis revealed that only one cosmid is inserted in the A18C3 genome because the three probes (kan, cos and pBR691-cos) hybridized independently to a fragment of ~10 kb in the *Sal*I digest of A18C3 DNA. In addition, *Eco*RI or *Sal*I digestion allowed us to determine that the vector part of the cosmid was not disrupted during integration. A similar analysis for A18C1 showed that more than one cosmid was inserted in the A18 genome or that the insertion occurred in the vector part of the cosmid.

The results of the Southern blot analysis prompted us to attempt plasmid rescue experiments with A18C3 (Figure 2B). Accordingly, total A18C3 DNA was digested with *Eco*RI and the fragments were separated by agarose gel electrophoresis. The DNA from the 10 kb region was eluted from the gel, and ligated in order to form a plasmid with a replication origin (rep), a selectable marker (Sm/Spc) and a fragment (~6 kb) of *Chlamydomonas* DNA. Transformation of the ligation product into *Escherichia coli* yielded two clones. Restriction analysis showed that one of the clones contained the expected plasmid which was named pA18C3 (Figure 2B). This plasmid, which was unable to complement the A18 mutation, was used as a probe to screen the original cosmid library. The eight independently isolated cosmids were able to complement the A18 mutation, suggesting that they carry a wild-type copy of the gene that is defective in A18. For subcloning, one of these cosmids was partially digested with *Sau*3A, and DNA fragments ranging from 5 to 10 kb were isolated and cloned into the *Bam*HI site of pBS. Two plasmids, pS3A#12 (~7 kb) and pS3A#16 (~8 kb), were found to complement the A18 mutation (Figure 2C). Restriction analysis of these two plasmids allowed us to subclone a minimal fragment of 2.8 kb (pX2.8) that is able to complement the A18 mutation (Figures 1 and 2C). A cDNA of 2.2 kb was obtained by screening a library with the insert of pS3A#12. When cloned in pKS, this cDNA (pCD1) was able to complement the A18 mutant. The

cDNA hybridizes to pX2.8, but also to the adjacent 0.9 kb *Sal*I–*Xho*I fragment (Figure 2C). This indicates that pX2.8 which is capable of complementing A18, albeit at low efficiency, contains only part of the gene. RNA blot analysis revealed the presence of the 2.7 kb mature *psaA* mRNA in all the complemented strains (Figure 1C). Nevertheless, in A18:pCD1, and to a lesser extent in A18:pX2.8, the 2.6 kb transcript containing intron 2 was still detected, as well as the 400 bp unspliced exon 1 precursor RNA, a characteristic of *psaA trans*-splicing mutants. This property could be due to insufficient expression of *Maa2* in these two transformants. Two other *psaA trans*-splicing mutants, Mϕ3 and Mϕ10, could also be complemented with pCD1 (Figure 1C), indicating that they are allelic to A18. Southern analysis of these different alleles reveals a complete deletion of the gene in A18 and Mϕ3 and a deletion of the 5′ part of the gene in Mϕ10 (Figure 3). This shows conclusively that the cDNA we obtained originates from the gene that is affected in the A18 mutant. According to the current nomenclature, this gene was named *Maa2* for maturation of psaA.



The Maa2 polypeptide is similar to pseudouridine synthases

The nucleotide sequence of the *Maa2* cDNA (pCD1) consists of 2111 bp with a TGTAA polyadenylation site located 12 bp upstream of the poly(A) tail. Sequence analysis revealed an open reading frame (ORF) of 410 amino acids encoding a polypeptide of 44.8 kDa. The presence of in-frame stop codons upstream of the first ATG and the match between the size of the predicted mature protein and that observed by Western blot analysis (see below) indicate that the cDNA encodes the entire protein. A putative transit sequence for import into the chloroplast is present, with an abundance of arginine and serine residues. This transit peptide could be 46 amino acids long since a possible cleavage site sequence Val–Leu–Ala is present (Franzén *et al*., 1990) (Figure 4A and B). The predicted mature protein consists of 364 amino acids with a molecular mass of 39.9 kDa. Database searches detected significant sequence similarity in two domains of pseudouridine synthases (Figure 4C). Interestingly, these enzymes are known to be poorly conserved except in these two domains, or in some cases three (Koonin, 1996). These enzymes modify uridine into pseudouridine (symbolized by Ψ), which is the most commonly modified nucleotide in RNA. It is found in tRNA and rRNA of all organisms ranging from prokaryotes to mammals, as well as in snRNA. This post-trancriptional modification is due to a breakage of the bond to the N1 of the pyrimidine ring, followed by a rotation of the uridine around the N3–C6 axis, and formation of a new carbon–carbon bond to C5. According to their amino acid sequences, pseudouridine synthases can be grouped into four families (Koonin, 1996). The Maa2 polypeptide is more closely related to the TruB family. TruB and Pus4 form Ψ in position 55 of tRNA (Nurse *et al*., 1995; Becker *et al*., 1997).

Fig. 2. Cosmid rescue strategy from A18C3 and mapping of *Maa2*. (**A**) Southern blot analysis of A18C1 and A18C3. Total DNA was digested with the indicated restriction enzymes, electrophoresed through a 0.8% agarose gel and transferred to Hybond-N+ membrane (Amersham). The same filter was hybridized sequentially with three probes, each time stripping the previous probe. Probes were obtained from pPR691, the vector used for the cosmid library. The 'kan' probe consists of the ~1 kb *Hin*dIII fragment containing the kanamycin resistance gene. The 'cos' probe is the ~1 kb *Eco*RI fragment, and 'pBR691-cos' corresponds to the remainder of the vector (~5.8 kb *Eco*RI fragment). (**B**) Integration of the cosmid in A18C3 and strategy for cosmid rescue. (1) *Sal*I digest of A18C3 DNA. A *Sal*I site is detected in the insert because the three probes (kan, cos and pBR691-cos) hybridize to a 10 kb fragment. (2) *Eco*RI digest of A18C3 DNA. Two *Eco*RI sites are detected in the cosmid insert because the 'kan' probe hybridizes to a 2 kb fragment and the 'pBR691-cos' probe hybridizes to the 2 kb fragment and to a 10 kb fragment. (3) Strategy for obtaining the plasmid pA18C3. (**C**) Mapping of *Maa2*. Black bars represent genomic DNA and the wavy line corresponds to restriction fragments that hybridize to the cDNA. Rescue efficiency is indicated by '+' signs, lack of rescue is denoted by '–', with the number of transformants per plate indicated in parentheses. H, *Hin*dIII; S, *Sal*I; X, *Xho*I; R, *Eco*RI; N, *Nsi*I; B, *Bam*HI. The *Hin*dIII site on the right is located in the polylinker of the pBR691 vector as indicated in parentheses. The arrow indicates the direction of transcription.

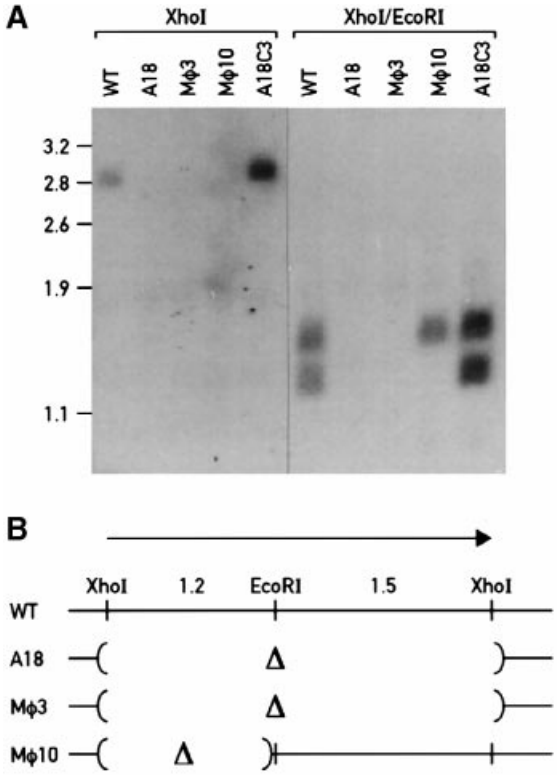


Fig. 3. DNA blot analysis of the *Maa2* mutants. (**A**) Analysis of total DNA from wild-type and the mutants digested with *Xho*I or with *Xho*I and *Eco*RI, using the *Maa2* cDNA as a probe. (**B**) Genomic map of the *Maa2* deletions. 'Δ' indicates the deleted regions, which may extend beyond the *Xho*I sites. The arrow indicates the direction of transcription.

The other pseudouridine synthases of the TruB family may form Ψ in rRNA, but could also be involved in the maturation of this RNA. The functions of Ψ in RNA are not clearly known, and the majority of pseudouridine synthase deletion mutants have no apparent phenotype.

The pseudouridine synthase activity is not required for psaA RNA trans-splicing

What is the link between pseudouridine and splicing? Because of their structural and functional similarities, nuclear introns could have evolved from group II introns (Sharp, 1991). Several of the six stem–loops in group II introns have their homologues in snRNAs of nuclear introns (Sharp, 1991; Wise, 1993). Because it is also known that some of these snRNAs contains Ψ (Patton, 1994), a distinct possibility was that Ψ formation occurs in the second intron of the *psaA* RNA, and that this modification is necessary for the *trans*-splicing activity. In order to test this hypothesis, site-directed mutagenesis on conserved residues of the Maa2 protein was performed. Recently, it was shown that a conserved aspartate is necessary for the catalytic activity of the pseudouridine synthase TruA (Huang *et al*., 1998). More recently, the essential role of this aspartic acid was confirmed for the pseudouridine synthases RsuA (Conrad *et al*., 1999), RluA (Raychaudhuri *et al*., 1999) and TruB (Ramamurthy *et al*., 1999). Mutation of this residue fully abolished the pseudouridine synthase activity. Transformation of A18 with the *Maa2* cDNA carrying a substitution in the corresponding residue (D171) (Figure 4C) by serine or glycine (D171S and D171G, respectively), or a deletion of this amino acid (D171Δ), still allows the rescue of the A18 mutant. Two other residues (D141 and K142), conserved in all pseudouridine synthases (Figure 4C), were also mutated into residues conserving the charge or the bulkiness of the side chain (D141E, D141S, K142R and K142I). None of these mutations affect the efficiency of A18 rescue. The levels of PsaA protein (Figure 5A) and the growth phenotypes (data not shown) are similar to those obtained by complementation with the wild-type cDNA. Rabbit antibodies were raised against recombinant Maa2 expressed in *E.coli* (see Materials and methods). The Maa2 protein is found at comparable levels in the strains transformed with the mutant or wild-type cDNAs, ~5% of the amount found in the wild-type (Figures 1B and 5B). This reduced level may be explained by the fact that there is no promoter in the cDNA construct used for transformation, so that expression must be driven by sequences at the insertion site. It is clear from these results, however, that the mutations introduced into the cDNA are not compensated by overexpression of the mutant Maa2 protein. The mature *psaA* mRNA is detected in all transformed strains (Figure 5C). The levels are reduced compared with the wild-type, but are similar in the strains transformed with the mutant or wild-type cDNAs. As previously noted with the wild-type cDNA (pCD1; Figure 1C), the ratio of mature mRNA (2.7 kb) to exon 3 precursor (2.4 kb) or to the *rbcS* control is reduced in the transformants (Figure 5C). Thus, *psaA* RNA *trans*-splicing in the A18 strains transformed with the mutant cDNAs is not affected compared with the control transformed with the wild-type cDNA. This indicates that the pseudouridine synthase activity of the Maa2 protein is not necessary for the *trans*-splicing of the second intron of *psaA*. However, it remains unclear whether this protein does have pseudouridine synthase activity. Attempts to detect such an activity either *in vivo* or *in vitro* were unsuccessful. Maa2 could be bifunctional, acting in the *trans*-splicing of *psaA* and also forming Ψ in another RNA.

Maa2 is associated with a membrane fraction of the chloroplast

The antiserum against Maa2 labelled a ~40 kDa protein in the wild-type and in the rescued strains, but not in A18, Mϕ3 or Mϕ10, as expected for these allelic deletion mutants (Figure 1B). The putative transit sequence of Maa2 and its role in the *trans*-splicing of the *psaA* RNA suggested that the Maa2 polypeptide is located in the chloroplast. To test this assumption, we prepared soluble and membrane protein fractions of total cells or of purified chloroplasts. The Maa2 polypeptide is present in the membrane protein fraction of total wild-type cells and absent in the soluble fraction (Figure 6A), and is undetected in the A18 mutant control. Maa2 is detected in the total chloroplast fraction and is enriched in the pellet (Figure 6B), but is not detected in the soluble fraction of the chloroplast. The purity of the chloroplasts was tested by reacting the protein blot with an antibody against the cytosolic eIF4A protein (Figure 6B). To investigate the association of Maa2 with membranes, we treated the chloroplast pellet fraction with ionic agents (NaCl and NaBr) or with an ionic and chaotropic agent (NaI). At a concentration of 1 M, each of these compounds fully releases Maa2 (Figure 6C). Treatment with a non-ionic detergent (Triton X-100), at a concentration that would

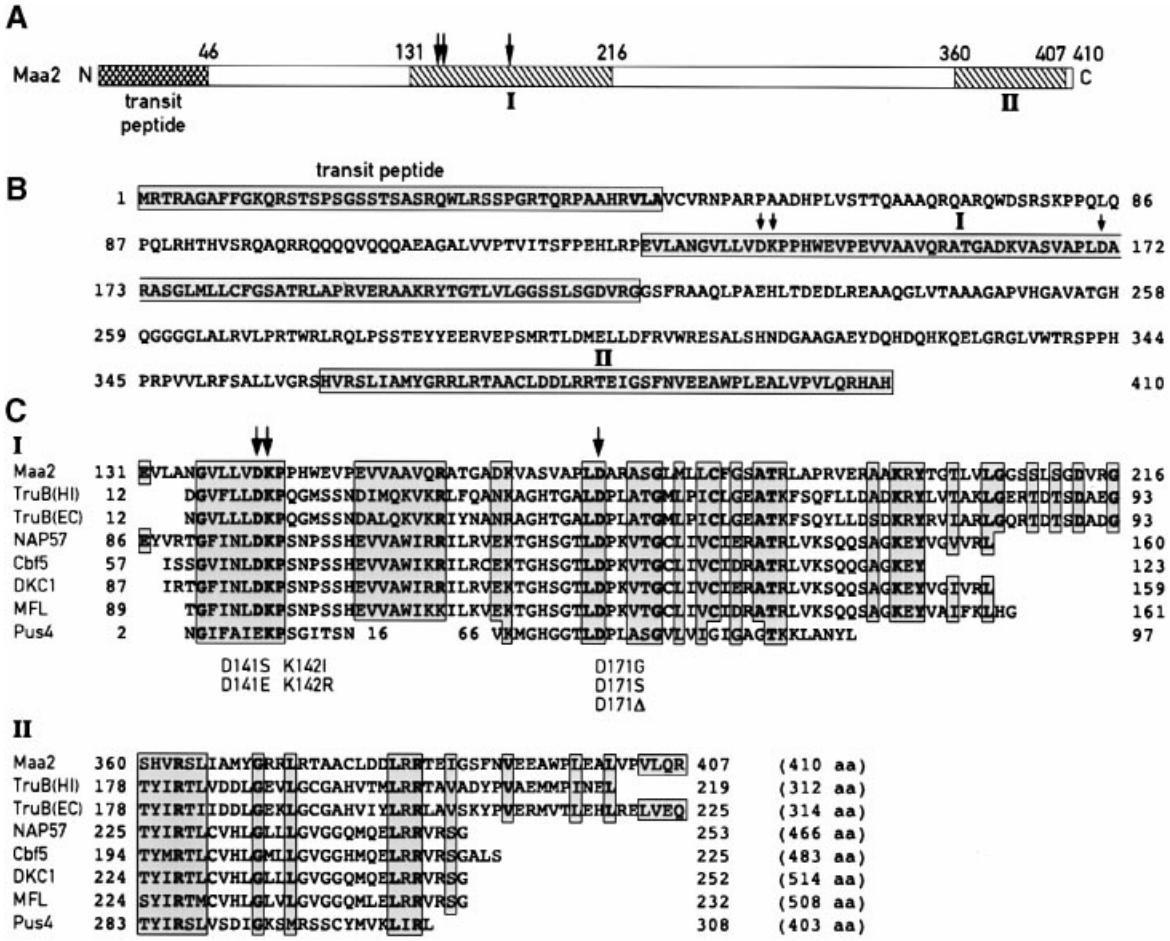


Fig. 4. Comparison of the deduced Maa2 amino acid sequence with pseudouridine synthases. (**A**) Primary structure of the Maa2 polypeptide. Domains with similarity to pseudouridine synthases are marked I and II. (**B**) Deduced amino acid sequence of the Maa2 polypeptide. The putative transit peptide and domains similar to pseudouridine synthases are shaded. The VLA residues in bold indicate the putative transit peptide cleavage site. The DDBJ/EMBL/GenBank accession No. for Maa2 is AJ243394. (**C**) Amino acid sequence comparison of the two domains similar to pseudouridine synthases. Residues conserved between the proteins are in bold, similar residues are shaded. The five proteins belong to the TruB family (Koonin, 1996). The residues that were altered by site-directed mutations in the cDNA are indicated by arrows, and the amino acid substitutions are shown under the sequences. Accession Nos for the protein sequences are: TruB(HI), P45142; TruB(EC), P09171; NAP57, P40615; CBF5, P33322; DKC1, U59152; MFL, AF089837; and Pus4, P48567.

disturb hydrophobic interactions between proteins (0.05 or 0.1%), does not release Maa2 (Figure 6C). This strongly suggests that Maa2 is associated with a membrane component of the chloroplast by ionic interaction. To determine the intracellular location of Maa2 more precisely, subfractionation of intact chloroplasts was performed by sucrose density gradient centrifugation as described by Zerges and Rochaix (1998) (Figure 6D). In the presence of Mg^{2+} in the sucrose gradient, Maa2 is found associated with thylakoids. This association is abolished by loading the thylakoids on a second sucrose gradient without Mg^{2+} (Figure 6D). In this case, Maa2 is found in a less dense fraction previously described as a low density membrane (LDM) fraction. It was suggested that these membranes, which contain RNA-binding proteins, could be derived from the inner envelope and be involved in the synthesis of thylakoids proteins (Zerges and Rochaix, 1998).

Genetic evidence for an interaction between Maa2 and other splicing factors

In order to investigate whether the *Maa2* gene corresponds to one of the five complementation groups of the *psaA* class A mutants previously described (Goldschmidt-Clermont *et al*., 1990), one representative mutant of each group was transformed with the *Maa2* cDNA or genomic DNA. Wild-type prototrophic growth was restored only with the L136F and the HN22 mutants which belong to the same complementation group, indicating that *Maa2* is the gene defective in these two mutants (data not shown). Immunoblot analysis of total insoluble protein from all the known class A mutants revealed the loss of Maa2 in L136F and HN22 (Figure 7). Interestingly, another class A mutant, L121G, and two allelic class B mutants, L137H and HN31.4, show a decrease in the level of the Maa2 polypeptide (Figure 7), suggesting a possible interaction between Maa2 and the factors defective in these mutants. One possibility is that these factors are associated within a multi-protein complex.

Discussion

This study provides the first characterization of a nucleus-encoded factor involved in *trans*-splicing of the chloroplast-encoded *psaA* RNA in *C.reinhardtii*. The *Maa2* gene, required for *trans*-splicing of the second intron, is affected

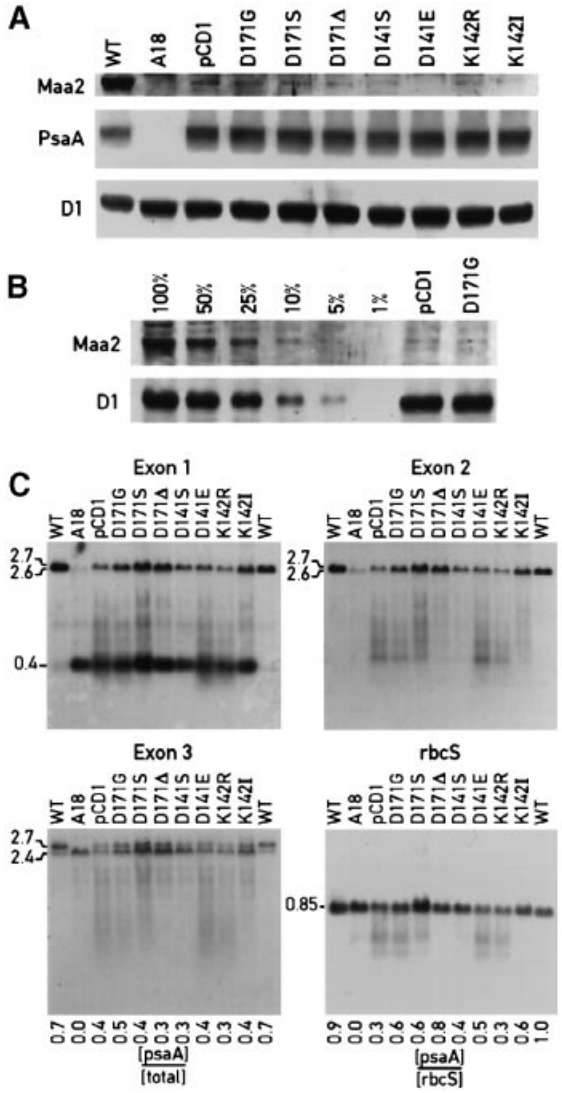


Fig. 5. Analysis of Maa2 single amino acid mutants. (**A**) Immunoblot analysis of Maa2 single amino acid mutants. A 80 μg aliquot of total protein was separated by SDS–PAGE in a 10% gel. The blot was revealed with antibodies against Maa2, PsaA or D1, as indicated. (**B**) Quantification of Maa2 abundance by immunoblot analysis in A18 expressing the wild-type *Maa2* cDNA (pCD1) or with the D171G point mutation, compared with a dilution series of the wild-type. (**C**) RNA blot analysis. The RNA was hybridized with the three different exons of *psaA*, as described in Figure 1. A18:pCD1 represents the A18 strain transformed with the wild-type cDNA. Transformants containing mutant cDNAs are indicated with the mutation. Phosphorimaging quantifications of the fraction of exon 3 transcripts that are in the form of mature mRNA are indicated below the blot probed with *psaA* exon 3. The ratio of mature mRNA (probed with *psaA* exon2) to the *rbcS* mRNA control is indicated below the blot probed with *rbcS*.

in HN22 and L136F, two previously described allelic mutants of class A (Goldschmidt-Clermont *et al*., 1990). This gene encodes Maa2, a polypeptide of 40 kDa, with similarity to pseudouridine synthases (Figure 4). These enzymes convert specific uridines into Ψs in RNAs such as tRNA, rRNA and snRNA. However, the functions of Ψ in RNA are not clearly established. Only two or three domains are conserved in these enzymes that also show similarity to dUTPase and dCTP deaminases (Koonin, 1996). The fact that Maa2 shows sequence similarity to enzymes that act on RNA is consistent with the idea that this protein could act directly on the second intron of the *psaA* RNA. A first possibility for the mechanism of action of Maa2 is that this protein forms Ψ in this group II intron, as occurs in the snRNAs of the spliceosome. For example, the terminal loop in domain I of U5 snRNA contains Ψ (Szkukalek *et al*., 1995) and this terminal loop corresponds to EBS 1 (exon-binding site) in domain I of group II introns (Wise, 1993). In order to test this hypothesis, we mutated a conserved aspartate residue known to be essential for the activity of several pseudouridine synthases (Huang *et al*., 1998; Conrad *et al*., 1999; Ramamurthy *et al*., 1999; Raychaudhuri *et al*., 1999) and also two other residues conserved among these enzymes. Because all the mutated *Maa2* cDNAs were still able to complement the A18 mutation (Figure 5), it appears that the hypothetical pseudouridine synthase activity of Maa2 is not necessary for the *trans*-splicing of *psaA* RNA.

As the majority of mutations specifically affecting Ψ formation are without phenotypic effect, with only one recently described exception (Raychaudhuri *et al*., 1998), there is a possibility that Maa2 is a bifunctional protein acting both as a pseudouridine synthase on unknown RNAs and as a specific splicing factor of the second intron of *psaA*. There are examples of proteins that are involved in splicing and also in an additional function unrelated to splicing. The *CYT-18* gene of *Neurospora crassa* and the *NAM2* gene of *Saccharomyces cerevisiae* encode mitochondrial tyrosyl-tRNA synthetase and leucyl-tRNA synthetase, respectively, implicated in mitochondrial translation. However, these proteins are also required for the splicing of some mitochondrial group I introns (Dujardin and Herbert, 1997). These proteins have a dual function in protein synthesis and intron splicing. Maa2, with its similarity to pseudouridine synthases and its role in *psaA* splicing, may represent another case. During evolution, these proteins could have been recruited for splicing in the following way. Once introns were acquired, they would have interacted with a variety of cellular RNA-binding proteins, such as aminoacyl-tRNA synthetases or pseudouridine synthases (Lambowitz and Perlman, 1990). These interactions could have stabilized the intron structure and eventually made splicing dependent on it. In this way, it is possible that the RNA-binding activity of an ancestral pseudouridine synthase was recruited as an RNA chaperone to fold or stabilize the *psaA* intron. Whether the catalytic pseudouridine synthase activity of Maa2 has been retained is still unclear. We have not been able to demonstrate a specific interaction of Maa2 with *psaA* intron 2 RNA *in vitro*; this suggests that the interaction is only transient *in vivo*. Alternatively, the role of Maa2 may be indirect. We could not exclude the possibility that Maa2 acts on another RNA itself involved in the *trans*-splicing of the *psaA* RNA.

Some pseudouridine synthases of the TruB family also seem to be multifunctional. CBF5, NAP57, MFL and CDK1 may be involved not only in pseudouridination, but also in processing of the pre-rRNA, and it is suggested that this group of proteins could be involved in different aspects of ribosome biogenesis (Giordano *et al*., 1999). CBF5 is also required for the stability of both the RNA and protein components of the snoRNPs implicated in rRNA Ψ formation (Lafontaine *et al*., 1998). Interestingly, mutation in the dyskerin protein (DKC1), the human

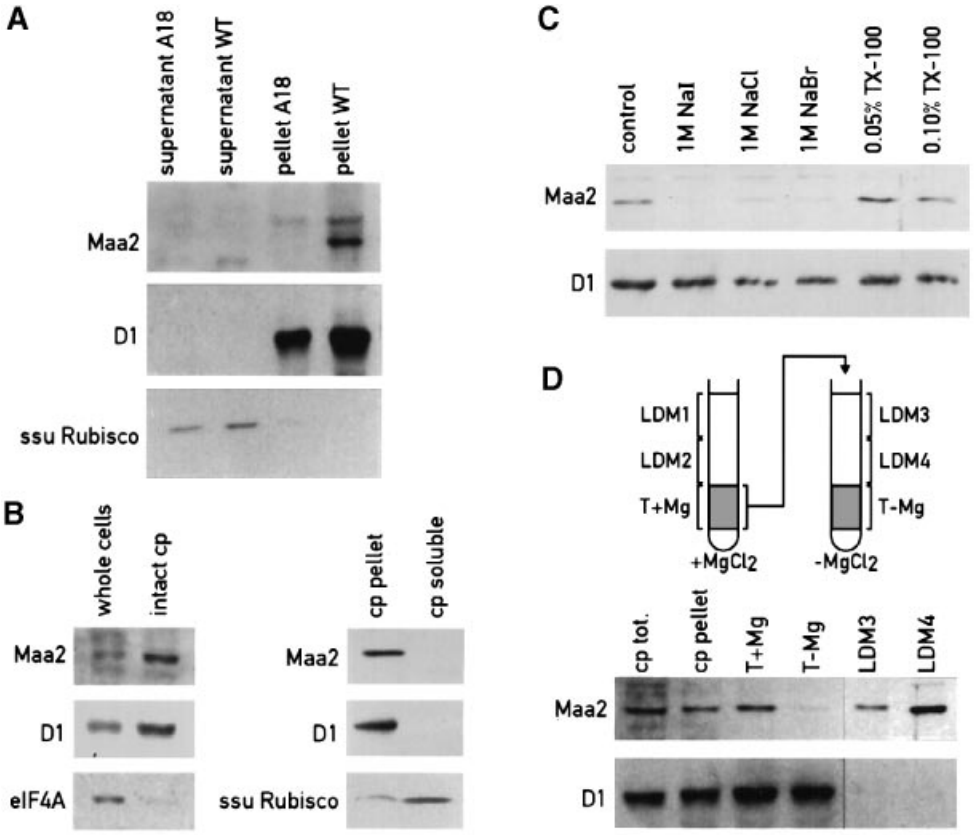


Fig. 6. Subcellular localization of the Maa2 protein. (**A**) Immunoblot analysis of soluble (supernatant) and insoluble (pellet) proteins from total cells of the A18 mutant and the wild-type. Cells were lysed by sonication and the extract was fractionated by centrifugation at 100 000 *g*. The pellet was washed as described in Materials and methods. The control antibodies are against D1 (an integral thylakoid membrane protein of PSII) and the small subunit of Rubisco (a soluble chloroplast protein). (**B**) Immunoblot analysis of whole cells and of intact chloroplasts, and of the soluble and insoluble fractions of purified wild-type chloroplasts. The controls are antibody against eIF4A (a cytoplasmic protein) and the small subunit of Rubisco. (**C**) Analysis of the insoluble chloroplast fraction after treatment of the membranes with ionic and non-ionic agents. Immunoblot analysis with antibodies against Maa2 or D1, as indicated. (**D**) Upper part: representation of the LDM and thylakoid fractions in the sucrose density gradients. Osmotically lysed chloroplasts were fractionated by sucrose density gradient centrifugation in the presence of magnesium. The thylakoid fraction was collected and resuspended in buffer without magnesium, and again fractionated in a sucrose density gradient without magnesium (Zerges and Rochaix, 1998). LDM and thylakoid fractions were collected as indicated in the scheme. LDM: low density membranes, T+Mg: thylakoid fraction from the gradient with $MgCl_2$, T-Mg: thylakoid fraction from the gradient without $MgCl_2$. Lower part: immunoblot analysis of thylakoid and LDM fractions with antibodies directed against Maa2 or D1 (integral membrane protein of PSII).

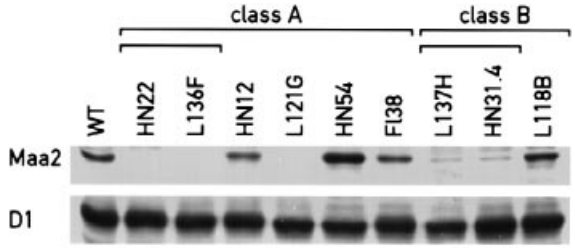


Fig. 7. Maa2 accumulation in *trans*-splicing mutants. Immunoblot analysis of the sedimentable fraction from *trans*-splicing mutants. HN22 and L136F are mutant alleles of *Maa2*. L121G, HN12, HN54 and Fl38 correspond to four other complementation groups of class A mutants (Goldschmidt-Clermont *et al*., 1990). L137H, HN31.4 and L118B are class B mutants (HN31.4 and L137H belong to the same complementation group).

homologue of NAP57 (rat), CBF5 (yeast) and MFL (*Drosophila*), was shown to be the cause of the human X-linked disease dyskeratosis congenita, a rare bone marrow disorder (Heiss *et al*., 1998). Because these proteins have a role in the processing of rRNA, it is difficult to know whether it is the absence of Ψ or a deficiency in ribosome biosynthesis that causes the phenotypes of dyskeratosis, of MFL loss-of-function mutation or of CBF5 depletion (Heiss *et al*., 1998, Lafontaine *et al*., 1998; Giordano *et al*., 1999). The only clear example of a major physiological effect due directly to a lack of Ψ was observed recently in *E.coli*, in which the absence of Ψ in the decoding centre of the 23S RNA causes a strong growth inhibition (Raychaudhuri *et al*., 1998).

Maa2 is a chloroplast protein based on its enrichment in isolated chloroplasts (Figure 6) and has a putative transit sequence for import into the chloroplast. It co-fractionates with an insoluble fraction and can be released by salt treatment but not by treatment with a non-ionic detergent (Triton X-100) at a concentration that disturbs hydrophobic interactions between proteins (Figure 6C). This strongly suggests that Maa2 is associated with a membrane fraction of the chloroplast through ionic interactions. Because no transmembrane helices are predicted in the Maa2 polypeptide, we postulate that this interaction could be mediated by binding of Maa2 to a membrane protein. Maa2 is found in the LDM fraction of the chloroplast, which has a lipid composition similar to that of the inner envelope (Zerges and Rochaix, 1998; Figure 6D). This compartment was identified by the

Table I. Description of primers used in this work

Primer	Sequence (5′–3′)	Complementary to
D171G	GTGGCGCCACTG**GGC**GCCCGCGCGAGCG	702–729[a]
D171Δ	GGTGGCGCCACTG...GCCCGCGCGAGCGG	701–730[a]
D171S	GGTGGCGCCACTG**AGC**GCCCGCGCGAGCGG	701–730[a]
D141E	CCAATGGCGTGCTGCTGGTT**GAG**AAGCCGCCGCATTGGGAG	604–644[a]
D141S	CCAATGGCGTGCTGCTGGTT**TCC**AAGCCGCCGCATTGGGAG	604–644[a]
K142R	CCAATGGCGTGCTGCTGGTTGAC**CGG**CCGCCGCATTGGGAG	604–644[a]
K142I	CCAATGGCGTGCTGCTGGTTGAC**ATT**CCGCCGCATTGGGAG	604–644[a]
pA18/NruI	CGACGGCACCGTGCA	966–952[a]
pA18/rbcS	GGACGCGGGCAGGGG	208–222[a]
F	CAAATCAATTGAATTGCTATA	563–543[b]
C	CAATTATGTCTAAAAAGACAG	161–181[b]

[a]Numbered according to the *Maa2* cDNA sequence; mutant codons are indicated in bold.
[b]Numbered according to the intron 2 sequence.

presence of proteins (80, 60, 47, 46, 32 and 31 kDa) that can be UV cross-linked with the chloroplast *psbC* 5′-untranslated region RNA. In the presence of Mg^{2+}, this RNA-binding activity is found associated with the thylakoids after sucrose density gradient centrifugation, as in the case of Maa2. However, when Mg^{2+} is removed, these RNA-binding activities, as well as Maa2, band in less dense fractions of the sucrose density gradient. Interestingly, a nucleus-encoded poly(A)-binding protein (RB47) which is involved in *psbA* mRNA translation was also found in this fraction (Yohn *et al*., 1998; Zerges and Rochaix, 1998). This LDM fraction can be separated from thylakoids and may represent a distinct compartment that may play a role in chloroplast mRNA metabolism, as suggested by the presence of the RNA-binding proteins and of Maa2.

There are precedents for the membrane association of proteins implicated in RNA processing. For example, in the yeast *S.cerevisiae*, proteins involved in mitochondrial RNA processing and turnover are associated with the inner mitochondrial membrane (Dake *et al*., 1988; Wiesenberger and Fox, 1997). Activator proteins, encoded by nuclear genes, are required to mediate interaction between mRNA and mitochondrial ribosomes at the surface of the inner membrane and allow translation (Fox, 1996). In plants, the chloroplast envelopes are the site of many biochemical processes (for a review, see Joyard *et al.*, 1998). The plastid genome is found associated with the PEND protein located in the inner envelope membrane in young pea leaves (Sato *et al*., 1993), and a topoisomerase II is detected in the inner envelope membrane of wheat plastids (Marisson and Leech, 1992). Factors implicated in mRNA translation are also found associated with envelope membranes (Rolland *et al*., 1997).

The LDM could allow a compartmentalization of RNA maturation and targeting of photosynthetic polypeptides. *Trans*-splicing of *psaA* mRNA could occur at the membrane, allowing the PsaA protein to be translated and inserted directly into the membrane as an early step in thylakoid biogenesis. There is evidence for vesicular trafficking between the inner chloroplast envelope and the thylakoid membranes (Hugueney *et al*., 1995; Hoober *et al*., 1998).

In addition to the steps necessary for *cis*-splicing of group II introns, *trans*-splicing of *psaA* requires a prior step: recognition and assembly of the separate precursors of the split introns. Maa2 could be implicated in the recognition and assembly of the two precursor transcripts (*trans* recognition activity) or be required for the splicing reaction (splicing activity). By analogy with splicing of nuclear introns, these different steps could be spatially and temporally coordinated. Thus, Maa2 could be part of a multi-protein complex that would be the group II intron counterpart of the spliceosome. The fact that two other mutants affected in the *trans*-splicing of the *psaA* RNA, and belonging to other complementation groups, showed a decrease in the level of Maa2 polypeptide (Figure 7) is compatible with the existence of a multi-protein complex formed by different nucleus-encoded factors. The cloning and characterization of Maa2 provides a basis for the investigation of such a complex by biochemical means and for the further investigation of the group II intron splicing machinery.

Materials and methods

Strains and media

Chlamydomonas reinhardtii wild-type and mutant strains were grown as described (Harris, 1989). TAP (Tris–acetate–phosphate) and HSM (high-salt minimal) media were solidified with 2% Bacto agar (Difco) for plate cultures. The strains CC620 and CC621 were obtained from the Chlamydomonas genetics center collection (Duke, USA). The mutants Mϕ3 and Mϕ10 were obtained from Dr Saul Purton. The mutant A18 was obtained from Dr Andrea Auchincloss.

Nucleic acid techniques

Procedures for standard molecular techniques were performed as described (Sambrook *et al*., 1989). The bacterial host was *E.coli* DH5α. Total *C.reinhardtii* DNA and RNA were isolated according to Rochaix *et al.* (1988) and Goldschmidt-Clermont *et al.* (1990), respectively. For Northern blot experiments, 3 μg of total RNA was electrophoresed through a 1.2% agarose gel containing formaldehyde and MOPS buffer, transferred to Hybond-N+ membrane (Amersham) and probed with double-stranded DNA labelled by random priming with [α^{32}-P]dATP. For *psaA* exon 1, the probe was the 400 bp *Hin*dIII fragment of R21, for exon 2, the 140 bp *Ava*I–*Kpn*I fragment, and for exon 3 the 750 bp *Bst*XI fragment in the middle of this exon. The intron 2 probe was ~400 bp of the second intron (5′ part) obtained by PCR amplification with primers F and C on the pMVS251 plasmid (F.Dürrenberger, personal communication). The genomic cosmid library contains inserts of 30–40 kb cloned into pPR691 (Purton and Rochaix, 1994). The cDNA library was constructed by Dr Hans Sommer (Max Planck Institute, Köln, Germany) and contains double-stranded cDNA fragments with *Eco*RI–*Not*I adaptors (Pharmacia) cloned into the *Eco*RI site of λ NM1149. The insert of pS3A#12 was used to screen the cDNA library. Two positive cDNAs, of 2.2 and 3.5 kb, were obtained from 300 000 phages. When cloned into pKS, these two cDNAs were able to comple-

ment the A18 mutation. The smallest one gave a transformation yield (30 colonies per plates) eight times higher than the larger one (four colonies per plate). This 3.5 kb cDNA could result from a cloning artefact since two fused cDNAs ligated in the same λ phage have been detected previously in this library. For these reasons, only the 2.2 kb cDNA was retained and cloned into the *Eco*RI site of pKS (Stratagene) resulting in pCD1. Sequencing was performed with a DNA sequencer (ABI prism, 377 DNA sequencer, Perkin Elmer).

Maa2 mutagenesis

Mutagenesis of the D171 residue of Maa2 was performed by PCR (*Pfu* DNA polymerase, Stratagene) with a mutagenic primer (D171S, D171G or D171Δ, see Table I) and a primer (pA18/NruI) located ~200 bp downstream. The amplified fragment (~250 bp) was purified by agarose gel electrophoresis and used as a megaprimer in a PCR (Sarkar and Sommer, 1990) with another primer (pA18/rbcS) located at the beginning of the ORF. The amplified fragment was digested with *Msc*I and *Eco*47III. The fragment of ~190 bp was purified by agarose gel electrophoresis and inserted in place of the wild-type fragment in the pCD1 plasmid. Mutagenesis of the Maa2 D141 and K142 residues was performed by PCR with a primer (D141E, D141S, K142I or K142R) carrying the mutation and half of the *Msc*I site and a second primer (pA18/NruI) located ~350 bp downstream. The amplified fragment was digested with *Eco*47III, purified by agarose gel electrophoresis and inserted as described for the D171 mutations. All the constructions were verified by DNA sequencing.

Nuclear transformation

The nuclear transformation was performed as described (Stevens *et al*., 1996) with the following modifications. Cell wall-deficient cells (*cw15*) were collected by centrifugation and resuspended in HSM at a concentration of 10^8 cells/ml. Aliquots (0.3 ml) of the cell suspension with 300 mg of glass beads (0.4 mm, Thomas scientific), 2 μg of transforming DNA and 0.1 ml of 20% PEG (polyethylene glycol 8000) were vortexed (maximal speed) for 15 s. After addition of 0.3 ml of HSM, the contents of the tube were spread on an HSM plate and incubated in the light (60 $\mu E/m^2s$).

For nuclear transformation of cells with wild-type cell walls, autolysin treatment was performed as follows. Autolysin was prepared as described (Buchanan and Snell, 1988) by mating concentrated suspensions (~3×10^8 cells) of the CC620 and CC621 strains for 3.5 h. Cells were removed by centrifugation and the supernatant containing soluble autolysin was sterilized by filtration (Schleicher & Schuell, 0.22 μm) and stored at –70°C until used. For transformation, a culture in exponential growth phase was concentrated 4-fold and autolysin was added to the medium in a 1/50 dilution. Cells were treated for 1 h with agitation, and then diluted 10 times. Cells were then collected by centrifugation and resuspended in HSM at a concentration of 10^8 cells/ml and transformed as described for cell wall-deficient cells.

Fluorescence transients

Fluorescence transients were measured according to Bennoun and Delepelaire (1982) and Fenton and Crofts (1990). Cells were grown on TAP plates in the dark for 7 days.

Antiserum production

The 2.1 kb *Acc*I–*Pst*I fragment of the cDNA (encoding from amino acid 15 to the end of the ORF) was blunted and cloned into the blunted *Nde*I site of the pET15b vector, and the plasmid was introduced into *E.coli* BL21(DE3)pLysS (Studier *et al*., 1990). For overexpression, transformed cells were grown at 37°C to an A_{600} of 0.8 in LB medium supplemented with ampicillin (100 μg/ml) and chloramphenicol (20 μg/ml). Isopropyl-β-D-thiogalactopyranoside (IPTG; 1mM) was added and the culture continued for 2 h. Under these conditions, the majority of recombinant protein was found in inclusion bodies. This insoluble fraction was purified (according to pET System Manual, Novagen, 1992) and loaded onto a nickel column (Ni-NTA superflow, Qiagen) under denaturing conditions according to the Qiagen protocol. The majority of the recombinant protein eluted at pH 4.5. Fractions containing the recombinant protein were pooled and purified by SDS–PAGE in a 10% gel. Polyclonal antiserum was raised in rabbit by injection of 100 μg of purified recombinant protein every 3 weeks, nine injections in total. The antiserum was purified by affinity chromatography on Maa2–Sepharose prepared by binding recombinant protein (purified and renatured on a Ni-NTA column according to the Qiagen protocol) to cyanogen bromide-activated Sepharose.

Protein extracts

For total protein extract, a cell pellet was resuspended in lysis buffer containing protease inhibitors (50 mM Tris pH 6.8, 2% SDS, 10 mM EDTA, 5 mM ε-amino caproic acid, 1 mM benzamidine HCl, 25 μg/ml pepstatin A, 10 μg/ml leupeptin), incubated at room temperature for 1 h and then centrifuged for 5 min in a microfuge. The resulting supernatant was used as total protein extract.

For soluble and insoluble fractions, the pellet was resuspended in lysis buffer without SDS, sonicated on ice and centrifuged at 100 000 *g* for 30 min at 4°C; the resulting supernatant was the soluble fraction. The pellet was washed with 1 ml of STN solution (0.4 M sucrose, 100 mM Tris pH 8.0, 10 mM NaCl) and centrifuged again for 15 min at 50 000 *g*, 4°C to remove soluble contaminant proteins. The pellet was then resuspended in lysis buffer and proteins from the insoluble fraction were extracted as described for the total protein extract.

Chloroplast and LDM purifications were performed as described by Zerges and Rochaix (1998) with addition of protease inhibitors (5 mM ε-amino caproic acid, 1 mM benzamidine HCl, 25 μg/ml pepstatin A, 10 μg/ml leupeptin) in all solutions. Briefly, *cw15* cells were broken in a Bionebulizer (Gascol Appartus Co., Terre Haute, IN) at 22 bar. Broken cells were loaded onto a discontinuous 75/45% Percoll gradient. Chloroplast were collected at the 45–75% interface, washed once and lysed osmotically. For LDM preparation, lysed chloroplasts were loaded on a continuous sucrose gradient (0.3–1.8 M) containing 5 mM $MgCl_2$. The thylakoid fraction was isolated, washed and harshly resuspended by pipetting and vortexing in a buffer without $MgCl_2$ but containing 10 mM EDTA. This fraction was then submitted to a second centrifugation in a continuous sucrose gradient without $MgCl_2$. Thylakoid and LDM fractions were isolated as described in Figure 6D.

Ionic and detergent treatments of membranes were performed as follows: total chloroplast membranes solubilized in chloroplast lysis buffer (10 mM Tricine pH 7.8, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol) were incubated with either 1 M NaCl, 1 M NaBr, 1 M NaI, 0.1 or 0.05% Triton X-100 for 20 min on ice. Then 1 ml of STN solution was added and samples were centrifuged for 15 min at 50 000 *g*, 4°C. The pellet was washed again in STN solution and centrifuged again as before. Protein concentration was determined with the bicinchoninic acid assay (Smith *et al*., 1995).

Immunoblot analysis

For Western blot analysis, proteins were separated by SDS–PAGE using a 10% (w/v) acrylamide gel and electroblotted to nitrocellulose membranes (Protran, 0.45 μm; Schleicher & Schuell, Inc., Keen, NH). Filters were incubated with specific antibodies, and visualized by the enhanced chemiluminescence technique (Supersignal, Pierce).

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