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Two adjacent nuclear genes are required for functional complementation of a chloroplast *trans*-splicing mutant from *Chlamydomonas reinhardtii*

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Summary

The chloroplast *tscA* gene from *Chlamydomonas reinhardtii* encodes a co-factor RNA that is involved in *trans*-splicing of exons 1 and 2 of the *psaA* mRNA encoding a core polypeptide of photosystem I. Here we provide molecular and genetic characterization of the *trans*-splicing mutant TR72, which is defective in the 3′-end processing of the *tscA* RNA and consequently defective in splicing exons 1 and 2 of the *psaA* mRNA. Using genomic complementation, two adjacent nuclear genes were identified, *Rat1* and *Rat2*, that are able to restore the photosynthetic growth of mutant TR72. Restoration of the photosynthesis phenotype, however, was successful only with a DNA fragment containing both genes, while separate use of the two genes did not rescue the wild-type phenotype. This was further confirmed by using a set of 10 gene derivatives in complementation tests. The deduced amino acid sequence of *Rat1* shows significant sequence homology to the conserved NAD^+-binding domain of poly(ADP-ribose) polymerases of eukaryotic organisms. However, mutagenesis of conserved residues in this putative NAD^+-binding domain did not reveal any effect on restoration efficiency. Immunodetection analyses with enriched fractions of chloroplast proteins indicated that Rat1 is associated with chloroplast membranes. Using the yeast three-hybrid system, we were able to demonstrate the specific binding of *tscA* RNA by the Rat1 polypeptide. We propose that the two nuclear factors *Rat1* and *Rat2* are involved in processing of chloroplast *tscA* RNA and in subsequent splicing of *psaA* exons 1 and 2.

Keywords: *Chlamydomonas reinhardtii*, chloroplast, *psaA*, *trans*-splicing, group II intron, RNA processing.

Introduction

Group II introns have been found mainly in organellar genes from fungi, algae and higher plants, and also in eubacteria. Introns of this type are characterized by a conserved secondary structure and some are able to undergo autocatalytical splicing *in vitro*, although *in vivo* protein factors are involved in the splicing process (Bonen and Vogel, 2001; Goldschmidt-Clermont *et al.*, 1990; Holländer and Kück, 1999). Discontinuous group II introns, where intron-coding sequences are fragmented and scattered around the genome, have been discovered in chloroplasts of higher plants and algae and in plant mitochondria (Giege and Brennicke, 2001; Goldschmidt-Clermont, 1998). Fragmented group II introns are of particular interest because they are regarded as evolutionary precursors of nuclear introns. This view is based on similarities between the two splicing mechanisms and their ability to act in *trans* in the splicing reaction, like their snRNA counterparts in the spliceosome (Lambowitz *et al.*, 1999).

The unicellular green alga *Chlamydomonas reinhardtii* serves as a model system to decipher nuclear and chloroplast components involved in RNA *trans*-splicing. The independently transcribed exons of the tripartite chloroplast *psaA* gene are flanked by fragmented group II intron sequences (Kück *et al.*, 1987). Splicing of the *psaA* mRNA with its two discontinuous group II introns depends on the interaction of at least 14 nucleus-encoded factors, and is a

remarkable example of the dependence of chloroplast gene expression on nuclear-encoded factors (Goldschmidt-Clermont *et al.*, 1990). In addition, splicing of exon 1 and 2 flanking intron sequences requires an RNA co-factor, the chloroplast *tscA* RNA, providing domains I to IV of the group II intron structure (Goldschmidt-Clermont *et al.*, 1991). Although the mechanism of splicing of group II introns is well characterized, less is known about the nature and function of the protein factors involved in the splicing reaction in chloroplasts or mitochondria. To date, several protein factors involved in chloroplast group II intron splicing have been identified in maize and *C. reinhardtii*. The maize proteins CRS1 and CRS2 are required for the splicing of different subsets of group II introns in the chloroplast (Jenkins *et al.*, 1997). *Crs2* encodes a polypeptide closely related to bacterial peptidyl tRNA hydrolase, and is associated with two additional splicing factors, CAF1 and CAF2, in intron-specific ribonucleoprotein particles (Jenkins and Barkan, 2001; Ostheimer *et al.*, 2003). In *C. reinhardtii*, three splicing factors for the *psaA* gene have been cloned and characterized (Perron *et al.*, 2004). One of these factors, Raa2, shares sequence similarities with pseudouridine synthase and is involved in splicing of the second and third *psaA* exons (Perron *et al.*, 1999). Raa3, however, is required for splicing of exons 1 and 2, and displays no sequence similarity with other known proteins except for a short stretch shared with pyridoxamine 5′-P oxidase (Rivier *et al.*, 2001). The mode of action of the nucleus-encoded factors is still unknown, but it is possible that these polypeptides take part in the splicing steps either by acting as RNA chaperones, or by supporting the maintenance of the splicing complex.

Here we report on the cloning and characterization of two adjacent genes indirectly involved in the *trans*-splicing of exons 1 and 2 of the *psaA* gene. Mutant TR72 has been described as being defective in the 3′-end processing of the *tscA* RNA, and consequently as being defective in *psaA trans*-splicing (Hahn *et al.*, 1998). We applied genomic complementation to isolate two genes which are deleted in TR72. As an unusual feature, restoration of mutant TR72 was achieved only when both genes together were used in complementation experiments. Here the molecular characterization of mutant TR72 is provided and the functional role of the two isolated genes in RNA *trans*-splicing in the chloroplast of *C. reinhardtii* is discussed.

Results

Identification and characterization of mutant genes in TR72

The previously described *trans*-splicing *C. reinhardtii* mutant TR72 was generated by insertional mutagenesis with plasmid pARG7.8ϕ3 (Gumpel and Purton, 1994; Hahn *et al.*, 1998). Analysis of the thylakoid proteins of mutant TR72, a transformant with typical fluorescence induction kinetics of photosystem I mutants, revealed the lack of photosystem I polypeptides and was therefore chosen for further studies (data not shown). Transcriptional analysis demonstrated a defect in *trans*-splicing of exons 1 and 2 of the *psaA* mRNA (class C mutant). In addition, we observed the absence of a correct 3′-end-processed *tscA* RNA (Hahn *et al.*, 1998). To confirm a single insertion of pARG7.8ϕ3 into the genomic DNA of TR72, we performed a Southern hybridization with a probe specific for the 'flag'-tag of vector pARG7.8ϕ3 (Figure 1a,b). The flag-tag is a heterologous 0.4 kb *Hpa*I fragment of bacteriophage ϕX174 DNA, which was integrated into intron 7 of the *ARG7* gene from *C. reinhardtii* (Gumpel and Purton, 1994). The results obtained clearly indicate that insertion of a single copy of the vector molecule into the genome is responsible for the observed phenotype of mutant TR72. A genomic library of mutant TR72 was generated and screened for DNA inserts containing vector sequences of plasmid pARG7.8ϕ3. A short genomic DNA fragment with flanking vector sequences was identified in the library and then sequenced (probe pIG1272; Figure 1a). Oligonucleotides were synthesized based on sequence information derived from the isolated genomic DNA and used to screen pools of an indexed cosmid library by PCR, leading to a single positive cosmid (P78H3). Further subcloning of a 7.3 kb *Nco*I fragment resulted in the generation of plasmid pBA1, which is able to rescue TR72. The inserted DNA was completely sequenced (Figures 1a and 2) and, together with information from the *C. reinhardtii* database at the Joint Genome Institute, we were able to draw a map of the insertion site of plasmid pARG7.8ϕ3 in mutant TR72 (Figure 1a). Two adjacent genes with opposite direction of transcription are located at the insertion site of pARG7.8ϕ3, and were denoted *Rat1* and *Rat2*, for RNA maturation of *psaA tscA* RNA. A more precise characterization of the gene organization occurred when two independent *Rat1* cDNAs were isolated by screening a cDNA library with a genomic DNA fragment as a probe. The comparison of genomic and cDNA sequences detected eight exons in the *Rat1* gene, and the cDNAs contain an ORF of 245 amino acids encoding a polypeptide of 27.7 kDa (Figure 5). Both the existence of an upstream stop codon in frame with the ATG initiation site, and the correlation between predicted and observed protein size by Western blot analysis (see below), indicate that the cDNAs are full-length clones and encode the entire protein. Two putative CAAT-boxes are present in the 5′-flanking region and the 3′ untranslated region (UTR) contains three putative polyadenylation signals specific for *C. reinhardtii* (Pelzer-Reith *et al.*, 1995; Silflow, 1998). The transcription initiation site was mapped 125 nt upstream of the ATG initiation site by primer extension analysis (data not shown), which coincides with the position of the putative CAAT-boxes. In addition, several expressed sequence tag (EST) clones could be identified in databases corresponding to the *Rat1* gene. The *Rat2* gene was predicted using the computer

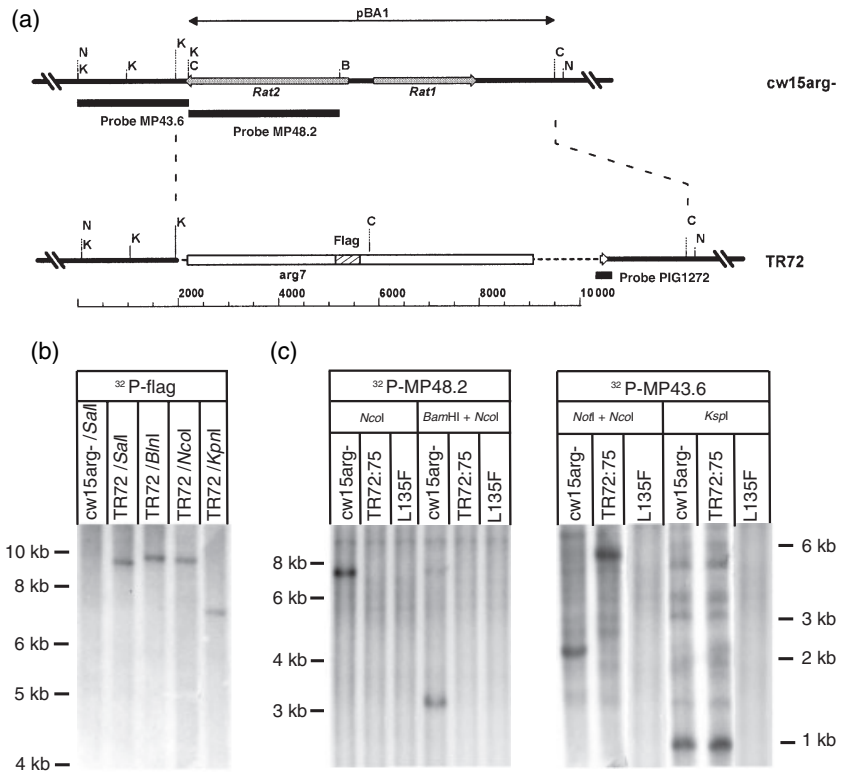

Figure 1. DNA-blot analysis of the wild-type and mutant strains TR72 and L135F.
(a) Comparative genomic map at the *Rat1–Rat2* region in wild-type and TR72 mutant strains. Insertion of plasmid pARG7.8ϕ3 leads to a genomic deletion in mutant TR72. Probes used for Southern blot experiments are shown as black bars. DNA of plasmid pARG7.8ϕ3 is shown as a dashed line containing the *ARG7* gene (white box). The DNA insert in plasmid pBA1 is indicated. Relevant restriction sites are abbreviated as: N, *Not*I; K, *Ksp*I; C, *Nco*I; B, *Bam*HI.
(b) Analysis of genomic DNA from wild-type cw15arg$^-$ and TR72 digested with the indicated restriction enzymes and probed with the flag-fragment of plasmid pARG7.8ϕ3. Size markers shown at left margin.
(c) Analysis of total DNA of the wild-type strain and mutants TR72 and L135F. The enzymes used for restriction analyses and probes are indicated. Size markers shown at margins.

program GREENGENIE at the Joint Genome Institute. The predicted sequence of Rat2 contains a putative N-terminal chloroplast target sequence with the appearance of characteristic amino acid residues for *Chlamydomonas* chloroplast import signals (data not shown). Rat2 does not share significant sequence homology with other known protein sequences in databases, except for a short glycine-rich region. Hydropathy analysis did not identify any putative membrane regions in the *Rat2* polypeptide, suggesting a chloroplast stromal localization. So far no *Rat2* EST clone has been documented in databases, and we were unable to detect a transcript by either Northern hybridization or RT-PCR. In the case of Northern analysis this could be due to a low expression rate, whereas the very high GC content of approximately 70% of the *Rat2* ORF may have inhibited initial strand synthesis in the reverse-transcription reaction. The predicted ORF contains nine exons, and analysis of both splice sites and codons revealed the appearance of splice-site consensus sequences and codon usage-specific sequences for *C. reinhardtii* (Campbell and Gowri, 1990; Silflow, 1998). Furthermore, a putative CAAT-box and a polyadenylation signal were found in the predicted 5′-flanking region and the 3′ UTR, respectively.

A hint of a deletion of genomic DNA in mutant TR72 was obtained from data from DNA-hybridization experiments using *Not*I-digested cosmid P78H3 as a probe (data not shown). The autoradiogram showed different hybridization patterns for the wild-type and mutant TR72, indicating a deletion in the nuclear DNA of TR72, which was then analysed in more detail. The right border of the inserted plasmid was analysed by sequencing a DNA fragment of a genomic library of mutant TR72 (probe pIG1272; Figure 1a). We then performed Southern hybridization to verify the left border of the integrated mutagen and to investigate deletions of genomic DNA (Figure 1c). By using genomic DNA fragments (MP43.6 and MP48.2; Figure 1a,c) and the *Rat1* cDNA (data not shown) as probes, we found a deletion of 5.8 kb genomic DNA in mutant TR72 extending from the *Ksp*I restriction site to the 3′ end of the *Rat1* gene. To expand further on our mutant analysis, another *trans*-splicing mutant, L135F (Goldschmidt-Clermont *et al.*, 1990), with a defect in *tscA* processing (M. Goldschmidt-Clermont, Department of Molecular Biology and Plant Biology, University of Geneva, Switzerland, personal communication) was used for hybridization experiments. Southern hybridization, as carried out for mutant TR72, revealed that the same locus is affected in both mutants. Moreover, the observed deletion in L135F exceeds the left- and right-hand borders detected in TR72 because fragments of L135F DNA did not hybridize to either the genomic probes MP43.6 and MP48.2 (Figure 1c) or *Rat1* cDNA (data not shown). These results provide strong evidence that the same locus is responsible for the observed phenotype in both mutants. However, complementation tests involving crossing these two mutants have not been carried out so far.

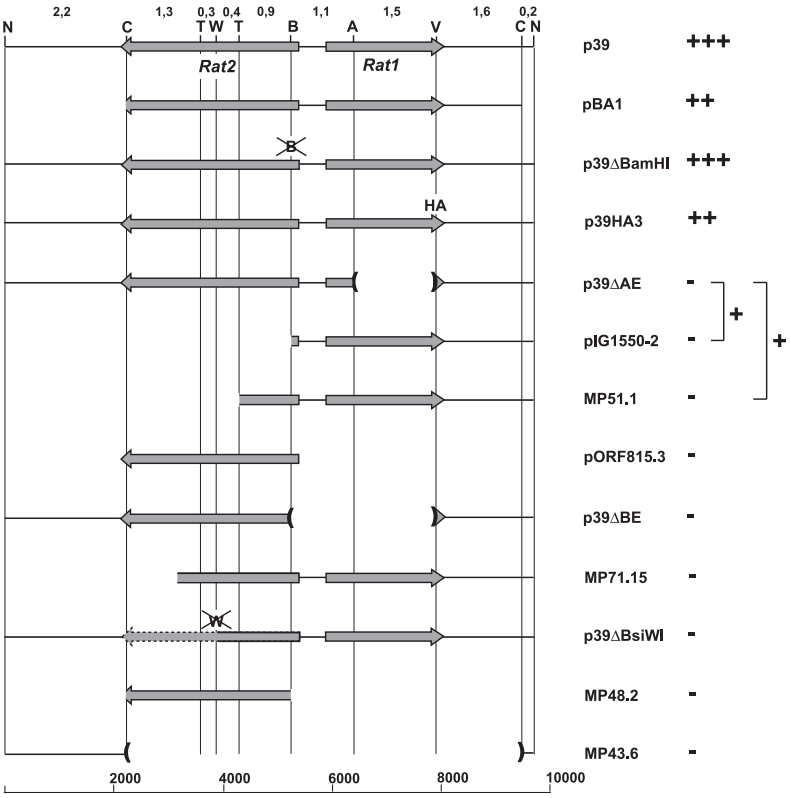

Figure 2. Genomic complementation of mutant TR72 and mapping of the genetic loci of *Rat1* and *Rat2*.
Thin lines correspond to genomic DNA. *Rat1* and *Rat2* with their respective transcription directions are shown as arrows. Plasmid designations and observed rescue efficiency: +++ (approximately three); ++ (approximately 0.7); + (approximately 0.1 transformants per μg DNA); –, lack of rescue. For construction of plasmids see Experimental procedures. Plasmids used in co-transformation experiments are shown in brackets. Scale bar for fragment length (bp) shown at bottom of figure; restriction fragment sizes (kb) shown at top. Abbreviations: N, *Not*I; C, *Nco*I; T, *Aat*II; W, *Bsi*WI; B, *Bam*HI; A, *Acc*I; V, *Eco*RV; HA, hemagglutinin epitope. Dashed line in plasmid p39ΔBsiWI shows the frame-shift mutation introduced in *Rat2* downstream of the *Bsi*WI restriction site.

Restoration of photosynthesis

To learn which of the two deleted genes in TR72 is responsible for the photosynthesis-deficient phenotype, we performed complementation transformations. Starting with a 9.7 kb *Not*I subfragment of P78H3 in plasmid p39, which complements the mutant phenotype and contains both genes, several subclones or deletion derivatives were generated and tested for their ability to rescue the mutant (Figure 2). Plasmid pBA1 contains a 7.3 kb *Nco*I subfragment of plasmid p39 and was able to complement, albeit with lower efficiency than p39 (Figure 2). Plasmid pBA1 contains one intact gene (*Rat1*) and a second gene (*Rat2*) truncated at the extreme 3′ end. No smaller fragment (pIG1550-2, MP51.1, pORF815.3, MP71.15, MP48.2) or deletion construct (p39ΔAE, p39ΔBE, MP43.6) containing one entire gene, or none, was able to restore the wild-type phenotype (Figure 2). These results indicate that both genes are functional and necessary for complementation. To test this assumption two additional plasmids, p39ΔBamHI and p39ΔBsiWI, were constructed. Plasmid p39ΔBamHI contains a filled-in *Bam*HI restriction site located in a deduced intron sequence of *Rat2* and is still able to restore the wild-type phenotype. Plasmid p39ΔBsiWI contains a filled-in *Bsi*WI restriction site in a deduced exon sequence of *Rat2* and is unable to complement, probably due to a generated frame-shift mutation. In addition, co-transformation of plasmid p39ΔAE containing intact *Rat2* together with plasmid pIG1550-2 or MP51.1, which both contain the intact *Rat1* gene, led to complementation of the mutant.

To address the question whether both genes are needed for correct *tscA* processing, co-transformation experiments were performed with plasmid pSP124S, which contains the modified BLE^R gene (Lumbreras *et al.*, 1998) and p39ΔAE or pIG1550-2. The resulting transformants were analysed by PCR for the presence of the inserted *Rat1* or *Rat2* gene, and the expression of *Rat1* was verified by RT-PCR (Figure 3c). Transformants harbouring either the *Rat1* or *Rat2* gene were non-photosynthetic (data not shown) and were assayed for *tscA* processing (Figure 3a). Northern blot analysis with a *tscA* specific probe revealed that correct *tscA* processing was restored in all complemented transformants. No mature 0.45 kb *tscA* RNA was detectable in mutant TR72 and transformants harbouring either the *Rat1* or *Rat2* gene, while wild-type levels of processed RNA accumulated in transformants complemented with plasmid p39 (T34.1 and T56.1; Figure 3a). In mutant TR72, larger transcripts preferably accumulate representing *tscA* precursor transcripts. The appearance of wild-type processed *tscA* RNA in complemented transformants is accompanied by expression of the *Rat1* gene, as verified by RT-PCR analysis. The *Rat1* transcript is present in the wild-type and in transformants T34.1 and T56.1, whereas this transcript is missing in mutant

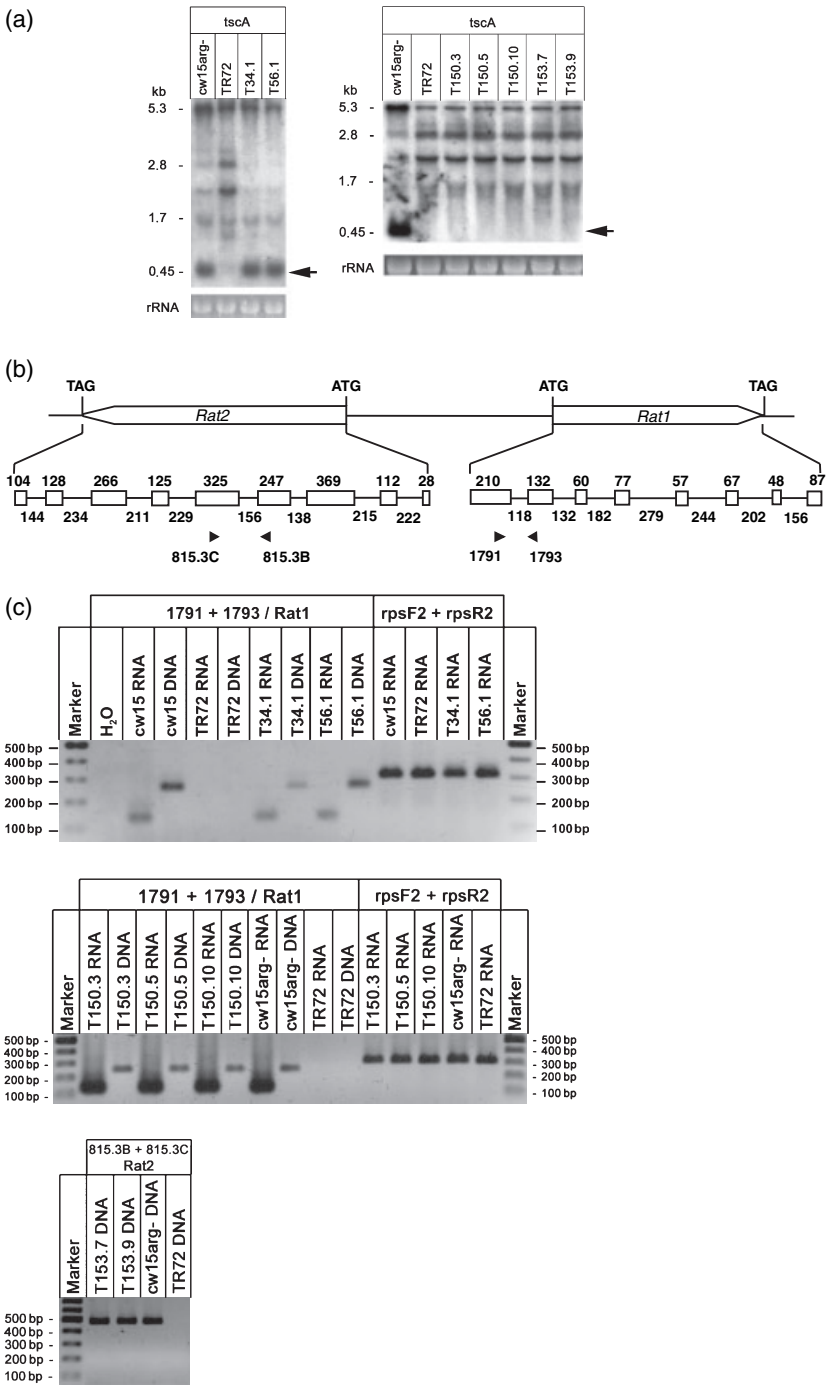

Figure 3. Analysis of *tscA* RNA processing in mutant TR72 and complemented transformants.
(a) RNA gel-blot analysis of wild-type (cw15arg$^-$) and mutant TR72 transformants complemented with plasmid p39 (T34.1, T56.1, left panel) and non-photosynthetic transformants (T150.3, T150.5, T150.10, T153.7, T153.9, right panel). 30 μg total RNA was separated on a 1.5% agarose/formaldehyde gel, transferred to a nylon membrane and probed with a *tscA*-specific radioactive DNA fragment. Arrows indicate mature *tscA* RNA. The intensity of rRNA was used as a loading control; *tscA* transcript sizes shown at left margin.
(b) Organization of *Rat1* and *Rat2* genes. Exons and introns represented by grey boxes and thin lines, respectively, with their corresponding length (bp). Arrow shows transcriptional direction. Oligonucleotides used for RT-PCR analysis (815.3C, 815.3B, 1791 and 1793) are shown as arrowheads.
(c) Upper panel: RT-PCR analysis of *Rat1* transcripts in wild-type (cw15), mutant TR72, and transformants T34.1 and T56.1. Middle panel: RT-PCR analysis of *Rat1* transcripts in non-photosynthetic transformants. RT-PCR was carried out by using either poly(A) RNA (RNA) or genomic DNA (DNA) as template. Oligonucleotides used for RT-PCR are shown whereby oligonucleotides rpsF2 and rpsR2 specifically amplify a fragment of the *Rps18* (ribosomal polypeptide) transcript and serve as a control for the cDNA preparation. Lower panel: PCR analysis of non-photosynthetic transformants harbouring the *Rat2* gene.

TR72 due to the observed genomic deletion (Figure 1c). The results obtained suggest that both genes function co-operatively in processing the *tscA* RNA, which is a co-factor of the *psaA trans*-splicing process.

Functional analysis of the Rat1 *gene*

Database searches using the amino acid sequence encoded by the *Rat1* gene detected significant sequence similarity to the NAD$^+$-binding domain of poly(ADP-ribose) polymerases (PARP) of eukaryotic organisms (Figure 4). These enzymes are involved in the repair of UV-damaged DNA, and show a modular construction with domains specific for DNA binding, autoregulation and a C-terminal NAD$^+$-binding domain. The latter is regarded as an RNA-binding domain (Nagy *et al.*, 2000). To date, no poly(ADP-ribose) polymerase could be detected in *C. reinhardtii*. Site-directed mutageneses were performed to elucidate the function of this putative

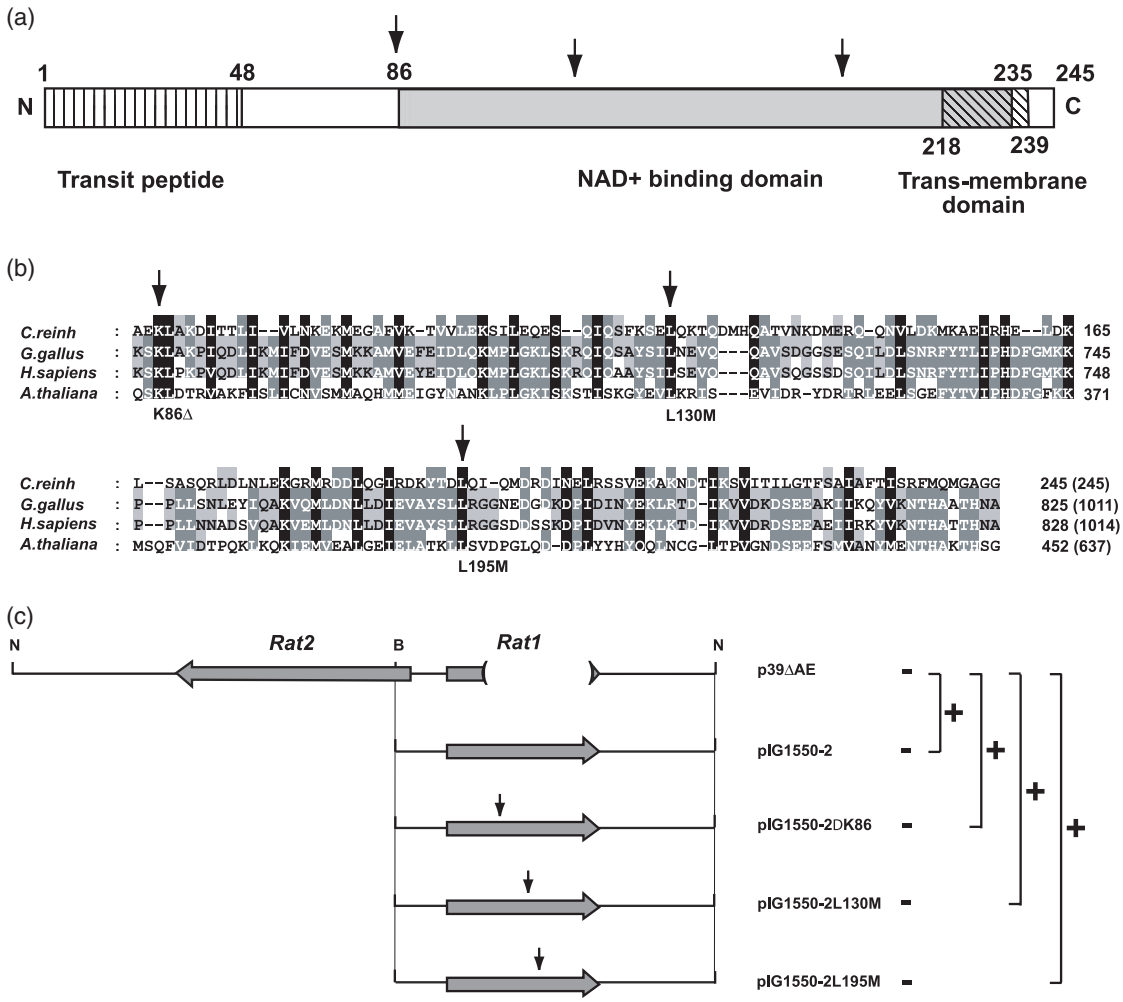

Figure 4. Structure and comparison of the deduced Rat1 sequence.
(a) Scheme of primary structure of the Rat1 polypeptide. The putative transit peptide, the domain with similarities to the NAD^+-binding domains of poly(ADP-ribose) polymerases (PARP), and a putative *trans*-membrane domain are shown.
(b) Amino acid sequence comparison of the *Chlamydomonas reinhardtii* Rat1 domain similar to the NAD^+-binding domains of eukaryotic PARP proteins. Deletion or substitution of the corresponding amino acid residues are shown under the sequence. The following protein sequences are used for comparison: *Gallus gallus*, P26446; *Homo sapiens*, P09874; *Arabidopsis thaliana*, Q11207.
(c) Complementation analysis of constructs with site-directed mutations in the *Rat1* gene. Corresponding rescue efficiency of specified constructs and abbreviations as in Figure 2. Residues that were altered by site-directed mutagenesis shown by arrows (a–c).

NAD^+-binding domain in *Rat1* for *trans*-splicing of the *psaA* gene. Co-transformation of plasmid p39ΔAE and derivatives of pIG1550-2 carrying a deletion of residue K86 (pIG1550-2ΔK86), or substitutions of residues L130 (pIG1550-2L130M) or L195 (pIG1550-2L195M) by methionine, still allowed the rescue of mutant TR72 (Figure 4). The deletion of residue K86 should influence the structure of the putative NAD^+-binding domain because this residue is located at the boundary of a helical region and a loop structure in the homologous PARP domain. The two substitutions might alter the structure of the side chain and thus change the chemical properties of the residues. The observation that the mutations do not change complementation efficiency, compared with the non-mutated construct, suggests that the conserved residues are not necessary for functioning of the Rat1 polypeptide. However, it remains to be elucidated whether the Rat1 polypeptide possesses NAD^+-binding activity.

Cellular localization of the Rat1 polypeptide

As a protein involved in 3′-end processing of the chloroplast *tscA* RNA, the Rat1 polypeptide might be expected to be localized in the chloroplast. To analyse the molecular mass and subcellular localization of the Rat1 polypeptide, a triple-hemagglutinin (HA) epitope was inserted into the coding sequence of the *Rat1* gene. Mutant cells were transformed with this construct (p39HA3, Figure 2). A monoclonal HA antibody detected a protein of approximately 27 kDa in

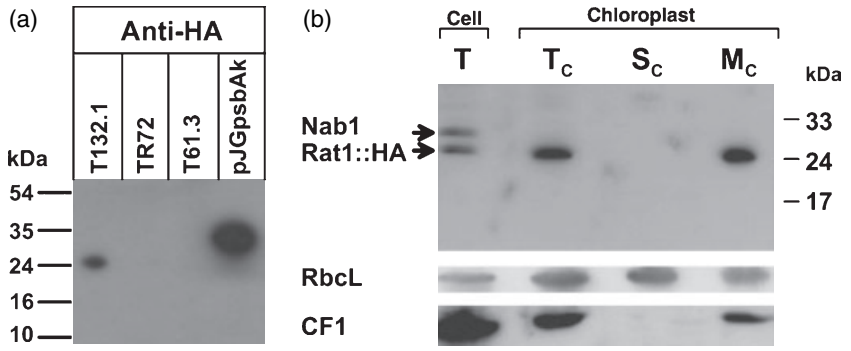

Figure 5. Detection and subcellular localization of Rat1 polypeptide.
(a) Immunoblot analysis of Rat1 in total cell extracts from mutant TR72 and from transformants carrying the Rat1–HA construct (T132.1) or the non-tagged *Rat1* gene (T61.3). To detect the Rat1::HA polypeptide, 50 μg protein was fractionated on a 12% polyacrylamide gel, transferred to a PVDF membrane and decorated with αHA as described under Experimental procedures. A total protein extract of yeast strain pJGpsbAk expressing an HA-tagged version of truncated PsbA polypeptide was used as control.
(b) Immunoblot analysis of total protein extracts from cell fractions of transformant T132.1. Total protein extracts (T), total chloroplast extract (T_C), and soluble (S_C) and insoluble (M_C) fractions of chloroplast were prepared as described in Experimental procedures. PVDF membranes were decorated with the antibodies indicated; the membrane in the upper part of the figure was first incubated with Nab1 antibodies followed by a washing step, then decorated with αHA to detect the specific signals of both antibodies. Abbreviations: Nab1, nucleic acid-binding protein 1, cytoplasmic marker protein; RbcL, large subunit of Rubisco, chloroplast soluble marker protein; CF1, coupling factor 1 of chloroplast ATP synthase, chloroplast membrane marker protein.

the extract from transformant T132.1, which carries the HA-tagged *Rat1* gene, but not in the wild type or in transformant T61.3 carrying a non-tagged *Rat1* gene (Figure 5a). The observed molecular mass of approximately 27 kDa is in agreement with the molecular mass of the predicted mature Rat1 polypeptide containing the additional amino acids of the HA tag. To investigate the subcellular localization of the polypeptide, chloroplasts from transformant T132.1 were isolated by centrifugation on Percoll gradients, and soluble and membrane fractions were prepared from these by high-speed centrifugation. The separated proteins were then probed with antisera raised against several marker proteins (Figure 5b). In addition to the signal in the whole-cell extract, the HA antibody detected the tagged Rat1 polypeptide in the total chloroplast extract and in the membrane fraction of the chloroplast. To control the quality of the fractionation process, we also analysed the distribution of the cytoplasmic Nab1 protein (J.H. Mussgnug, M. Hamilton, I. Elles, A. Fink, A. Kapazoglon, C.W. Mullineaux, C.W. Hippler, JN, P.J. Nixon & O. Kruse, unpublished data); the soluble chloroplast protein RbcL (large subunit of Rubisco); and the chloroplast membrane protein CF1 (coupling factor 1 of chloroplast ATP synthase) (Figure 5b). While Nab1 was detectable only in the whole-cell extract, RbcL was detected in all fractions of the chloroplast, with slightly smaller amounts in the insoluble compared with the soluble fraction. In contrast, the chloroplast membrane marker protein is found only in the membrane fraction, demonstrating that the soluble fraction is not contaminated with insoluble proteins. Co-fractionation of the Rat1 polypeptide and the chloroplast membrane protein indicates the membrane association of Rat1. These findings are supported by *in silico* studies using PSORT (http://psort.nibb.ac.jp) or PREDOTAR (http://genoplante-info.infobiogen.fr/predotar/predotar.html), as well as by hydropathy analysis (Kyte and Doolittle, 1982) discovering a putative N-terminal transit peptide of 48 amino acids and a putative C-terminal *trans*-membrane span between residues 218 and 239 (Figure 4a).

The Rat1 polypeptide binds specifically to tscA RNA

The above data led to the assumption that the Rat1 polypeptide is directly involved in 3′-end processing of the *tscA* RNA. Therefore we used the yeast three-hybrid system (Jaeger *et al.*, 2004) to test the *tscA*-binding properties of Rat1. Using the system developed by Putz *et al.* (1996), the cDNA sequence of the N-terminal truncated Rat1 polypeptide (lacking residues 1 to 19) was linked to the transcription activation domain of GAL4 (AD–Rat1), leading to plasmid pADTR72ΔN19 (Figure 6). In the second chimeric protein of this system, the GAL4 DNA-binding domain is fused to the RNA-binding protein RevM10 (DB-RevM10), which specifically binds the rev responsive element (RRE) RNA. The sequence of the mature *tscA* RNA was recombined with the RRE RNA sequence, leading to a hybrid RNA. Two further hybrid RNAs were generated carrying sequences of domains 4 to 6 of intron rI1 (Kück *et al.*, 1990) and a fragment of the 3′ UTR of the *Lhcbm6* transcript, together with the RRE sequence, respectively, to serve as controls for the specificity of the potential RNA protein interactions (Figure 6). Expression of the reporter genes in the yeast three-hybrid system was initiated by interaction of the fusion proteins with the hybrid RNA molecule, reconstituting a functional transcription-activating factor. As shown in Figure 6, activity of the reporter β-galactosidase is measured only in strains that contain the RRE–*tscA* hybrid RNA together with the AD–

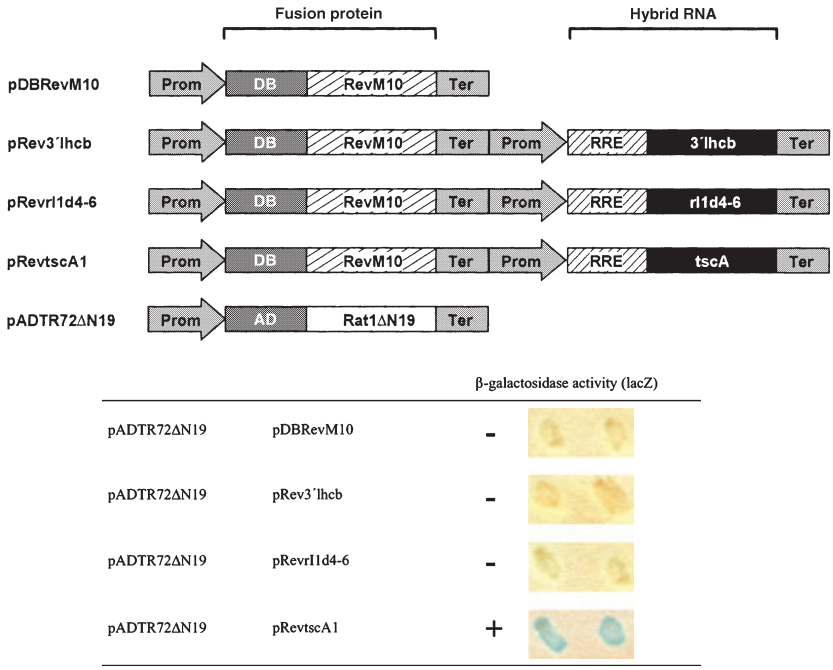

Figure 6. Yeast three-hybrid analysis to demonstrate Rat1–*tscA* RNA binding properties.
Upper part of figure, schematic overview of constructs used in the three-hybrid analysis. The transcription units generating fusion proteins and hybrid RNAs are under control of ADH promoters (Prom) and terminators (Ter). Expression of the reporter gene β-galactosidase (LacZ) in transformants can be recognized by the blue colour of yeast colonies and is denoted by – and + signs, respectively. Abbreviations: DB, GAL4 DNA-binding domain; AD, GAL4-activation domain; RevM10, RNA-binding protein RevM10; Rat1ΔN19, N-terminal truncated Rat1 polypeptide; RRE, RRE RNA which is specifically bound by RevM10; 3′lhcb, fragment of 3′ UTR of the *Lhcb* transcript; rI1d4-6, domains 4 to 6 of intron rI1; tscA, processed *tscA* RNA.

Rat1 fusion protein. The observed enzyme activity indicates an interaction of the Rat1 polypeptide and the *tscA* RNA. No transcriptional activation of the reporter gene is observed using hybrid RNAs with other target RNA sequences (domains 4 to 6 of intron rI1 and 3′ *Lhcb*). To exclude transcriptional activation mediated by the fusion proteins without RNA interaction, we tested the activation potential of these proteins in separate experiments. Neither the AD–Rat1 fusion protein itself, nor the combination of the fusion proteins AD–Rat1 and DB-RevM10, was sufficient to initiate expression of the reporter genes (Figure 6). This *in vivo* test system clearly demonstrates the interaction of the Rat1 polypeptide with the *tscA* RNA.

Discussion

To date, few group II intron-splicing factors have been described and characterized at the molecular level. The results presented here extend the existing data by providing the analysis of two nuclear genes from *C. reinhardtii*, which appear to function co-operatively in the 3′-end processing of *tscA* RNA, a co-factor of *trans*-splicing of the *psaA* transcript.

Two adjacent nuclear genes are part of a functional unit for chloroplast RNA processing

Mutants TR72 and L135F were generated in independent screens for photosynthesis-deficient mutants of *C. reinhardtii*. While L135F (Goldschmidt-Clermont *et al.*, 1990) was mutagenized with UV light, TR72 was obtained by insertional mutagenesis. Despite different mutagenesis procedures, the same genomic locus is affected in both mutants, which led to an identical *tscA*-processing defect. As a consequence, both mutants fail to splice exons 1 and 2 of *psaA* mRNA. Transformation experiments with derivatives of cosmid P78H3 suggest that both genes participate in the complementation of mutant TR72. The *Rat1* gene is expressed functionally, as verified by RT-PCR, primer-extension experiments and immunodetection. The failure to detect any *Rat2* transcripts was unexpected, but could be explained by a very low expression rate together with the extremely high GC content of *Rat2*, which prevents detection by Northern hybridization or RT-PCR, respectively.

The structure of the *Rat1–Rat2* locus is remarkable in that it is composed of two adjacent functionally related genes with inverse direction of transcription. This arrangement, both functional and structural, has been found in other eukaryotic organisms. In a screen for genes necessary for photoproduction of H_2 in *Chlamydomonas*, two adjacent genes, *HydEF* and *HydG*, were characterized (Posewitz *et al.*, 2004). These genes encode polypeptides which function in the assembly of [Fe] hydrogenase, and are arranged in an order suggestive of divergent expression from the same promoter region. Consequently the similar arrangement of *Rat1* and *Rat2*, which is believed to be transcribed from an intermediate promoter sequence, is comparable with the structure of the *HydEF–HydG* functional unit. Cenkci *et al.* (2003) identified a gene (*Rex1*) in *Chlamydomonas*, which is involved in repairing UV light-induced DNA damage. The *Rex1* gene is predicted to encode two different proteins: a small protein of about 8.8 kDa (Rex1-S) and another of about 31.8 kDa (Rex1-B). Complementation experiments with various genomic constructs revealed that total

complementation depended on both ORFs, whereas partial complementation was achieved using *Rex1-S* in genomic transformation. These findings resemble our data with the *Rat1–Rat2* locus, which promotes restoration of mutant TR72 only when both genes are used for complementation. Another example for neighbouring genes, which form a functional unit, comes from yeast where two adjacent nuclear genes participate in the suppression of a mitochondrial splicing deficiency (Altamura *et al.*, 1994).

RNA maturation of polycistronic precursor transcripts in the chloroplasts of land plants and algae is a process with functional significance for the expression of plastome-encoded photosynthesis genes. For instance, impaired processing of a pentacistronic precursor RNA in the Arabidopsis mutant *hcf107* and a similar defect in the *C. reinhardtii* mutant *mbb1* led to a defective photosystem II (Felder *et al.*, 2001; Vaistij *et al.*, 2000). Similarly, maturation of the *C. reinhardtii tscA* precursor transcript is a complex process leading to the generation of a set of different molecule species. The *tscA* gene is co-transcribed with *chlN* in a dicistronic transcription unit (Rochaix, 1996) and then extensively processed, resulting in the observed multiple hybridization pattern (Figure 3; Hahn *et al.*, 1998). With respect to the complex maturation pattern of the *tscA–chlN* co-transcript, it appears likely that several factors play a part in the processing steps of the precursor molecule. In the non-allelic *trans*-splicing mutant HN31, a single locus mediates both *trans*-splicing of *psaA* precursors and *tscA* processing (Hahn *et al.*, 1998). Taken together, maturation of *tscA* RNA in the chloroplast of *C. reinhardtii* relies on at least three different nuclear factors: the *Rat1*, *Rat2* and HN31 products.

The Rat1 polypeptide contains a putative RNA-binding domain

As a processing factor of *tscA*, the Rat1 polypeptide is expected to interact with this RNA molecule. Direct, specific binding of *tscA* by Rat1 was demonstrated with the yeast three-hybrid system. This *in vivo* test system has been used successfully in many cases to decipher RNA protein-binding properties. Interactions have been characterized mostly between RNA-binding proteins and their RNA target molecules localized in the nucleo/cytoplasmic compartment (including viral RNA–protein interactions). However, the three-hybrid approach is also suitable for verifying RNA-binding properties of organellar polypeptides (Jaeger *et al.*, 2004). For instance, Rho and Martinis (2000) and Rho *et al.* (2002) analysed different proteins which are involved in splicing of the fourth intron (bI4) of the yeast mitochondrial *cob* gene. Using the three-hybrid system they were able to demonstrate the RNA-binding capacity of a nuclear-encoded leucyl-tRNA synthetase (LeuRS) as well as a bI4 intron-encoded maturase from the *cob* gene. Thus this system is also suitable for verifying interactions of organellar proteins with their respective target RNAs in the nuclear background of yeast cells.

Rat1 contains a domain of about 150 amino acids with similarity to the NAD^+-binding domain of eukaryotic poly (ADP-ribose) polymerase. NAD^+-binding domains are thought to be ancestors of RNA-binding motifs, and to some extent exhibit sequence-specific RNA-binding properties. For instance, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was identified to bind AU-rich elements of lymphokine mRNA 3′-untranslated regions mediated by the NAD^+-binding region of the enzyme (Nagy and Rigby, 1995). Furthermore, the glycolytic enzyme lactate dehydrogenase exhibits similar RNA-binding activity which is also dependent on the NAD^+-binding domain, as validated by competition analysis using NAD^+ (Pioli *et al.*, 2002). Hammerhead ribozymes, as well as several group I and group II introns, are naturally occurring ribozymes with autocatalytical activity acting via transesterification (Doudna and Cech, 2002). It was shown that the NAD^+-binding domain of GAPDH facilitated an up to 25-fold increase of the *in vitro* cleavage rates of hammerhead ribozymes, probably due to the unfolding activity of GAPDH (Sioud and Jespersen, 1996). The putative NAD^+-binding domain of Rat1 probably functions in a similar manner. Rat1 might be responsible for the formation of a structure of *tscA* RNA, which is then processed to the mature form of the molecule by additional factors or by the protein itself. The latter assumption for the Rat1 function is supported by the observation that proteins with NAD^+-binding domains exhibit RNA-cleavage activity. For instance, using *in vitro* cleavage assays, the RNase active centre of the Asd-1 protein from the hyperthermophilic archaeon *Sulfolobus solfataricus* was shown to be localized within the N-terminal mononucleotide-binding domain of the protein (Evguenieva-Hackenberg *et al.*, 2002).

The direct demonstration of RNA-binding activity localized in the NAD^+-binding region of different enzymes supports the general concept that enzymes containing this domain may exhibit specific RNA-binding activity and play additional roles in nucleic acid metabolism. A similar scenario is discussed for several splicing factors of group II introns, which have been characterized molecularly. These splicing factors often exhibit similarities to proteins involved in RNA metabolism. For instance, the maize splicing factor CRS2 is related to peptidyl-tRNA hydrolase, and this polypeptide is required for the splicing of nine group II introns in the chloroplast (Jenkins and Barkan, 2001). The CRS2-associated factors CAF1 and CAF2 contain a basic repeated domain involved in RNA binding, which is also found in CRS1 (Ostheimer *et al.*, 2003; Till *et al.*, 2001). The *Chlamydomonas* Raa2 protein takes part in the *trans*-splicing of the second intron of the chloroplast *psaA* gene, and displays sequence similarities to pseudouridine

synthase (Perron *et al.*, 1999). It was shown for CRS2 and Raa2, respectively, that the polypeptides lack the enzymatic activity, and that this activity is not required for the splicing reaction (Jenkins and Barkan, 2001; Perron *et al.*, 1999). Similar results were obtained for the single-domain protein Rat1. Mutagenesis of conserved residues in the putative NAD^+-binding region did not alter the function in *trans*-splicing, suggesting that proper NAD^+-binding is not a prerequisite for splicing. However, it remains unclear whether or not Rat1 possesses NAD^+-binding activity.

Rat1 is a candidate protein for a chloroplast spliceosome

Recent data derived from biochemical and genetic approaches suggest the existence of ribonucleoprotein (RNP) particles in the chloroplast, which facilitate excision of group II introns from their precursor molecules and are therefore suggested to be the chloroplast counterparts of the nuclear spliceosome. For instance, both the maize CRS1 and CRS2 splicing factors are found in large stromal RNP complexes together with their target intron RNAs (Jenkins and Barkan, 2001; Till *et al.*, 2001). CRS2 is required for the splicing of nine group II introns in the chloroplast and forms complexes with either CAF1 or CAF2, but not with both simultaneously (Ostheimer *et al.*, 2003). CRS2-associated factors appear to recruit CRS2 to different intron subsets and appear to be responsible for intron specificity of the RNP complex *in vivo*. In *C. reinhardtii*, at least 14 nuclear factors are required for the *trans*-splicing of the chloroplast *psaA* mRNA (Goldschmidt-Clermont *et al.*, 1990). The splicing factor Raa3 is part of a high molecular-weight complex in the chloroplast stroma containing *tscA* RNA and *psaA* exon 1 transcript (Rivier *et al.*, 2001). Raa1 appears to be part of two different complexes associated with chloroplast membranes: one complex contains RNA and additional, as yet uncharacterized factors; the other complex is made up of Raa2 and other genetically defined factors (Perron *et al.*, 2004). Furthermore, the specific binding of a 61- and a 31-kDa protein to domain IV of a heterologous group II intron in *Chlamydomonas* chloroplasts has been reported (Bunse *et al.*, 2001). The model for the *psaA trans*-splicing emerged from the existing data and proposes two spatially restricted protein complexes: an Raa3-containing complex which is located in the stroma and facilitates splicing of exons 1 and 2; and an Raa1-containing complex which is bound to chloroplast membranes and is required for splicing of exons 2 and 3 (Perron *et al.*, 2004). We have used cell fractionation and immunological methods to show that the Rat1 polypeptide is located in the chloroplast membrane fraction. The protein is probably anchored in a chloroplast membrane, as a C-terminal *trans*-membrane domain was identified by hydropathy analysis. It is remarkable that the processing factor Rat1 binds the mature form of the *tscA* RNA, suggesting a function that extends beyond the processing reaction. Rat1 could merge the stromal Raa3 complex, which also contains *tscA* RNA and the membrane-associated Raa1 complex. This putative dual function of the RNA-processing factor Rat1 is reminiscent of the yeast mitochondrial splicing factor Mss116p, which is believed to be required for some RNA end-processing reactions in addition to splicing (Huang *et al.*, 2005). If Rat1 connects the Raa3 and Raa1 complexes, both splicing reactions would occur at the chloroplast membrane and are potentially coupled to translation, as proposed by Perron *et al.* (2004), as plastid translation factors are localized at the low-density membrane in *Chlamydomonas* (Zerges and Rochaix, 1998). However, the exact localization of Rat1 remains vague and needs to be determined by more precise fractionation of the chloroplast membrane system. Co-fractionation experiments are expected to solve the question as to whether Rat1, together with other *tscA*-processing factors (Rat2, HN31) and/or other splicing factors, forms part of a common complex. Experimental data from such an approach should provide a better and more detailed understanding of the composition of a putative chloroplast spliceosome.

Experimental procedures

Strains and culture conditions

Chlamydomonas reinhardtii cw15, mt-(CC3491) was obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC, USA). Mutant strains TR72 and L135F have been described previously (Goldschmidt-Clermont *et al.*, 1990; Hahn *et al.*, 1998). Wild-type and mutant strains were grown as described on Tris-acetate-phosphate (TAP) and high-salt minimal media (Harris, 1989).

Nucleic acid techniques

Procedures for standard molecular techniques were performed as described by Sambrook and Russel (2001). *Chlamydomonas reinhardtii* total DNA and RNA were prepared by phenol extraction and selective LiCl precipitation, as described previously (Kück *et al.*, 1987). For Southern blot hybridizations the DNA was transferred to a positively charged nylon membrane (GeneScreen® ; Perkin-Elmer, Zaventem, Belgium) and hybridized with radiolabelled dsDNA probes. Total RNA was transferred to nylon membranes (Nytran$^+$; Schleicher & Schuell, Dassel, Germany) and hybridized with a radioactively labelled *tscA*-specific probe (Herdenberger *et al.*, 1994). Poly(A) RNA was prepared using the PolyA Tract® poly(A)-RNA Isolation System IV (Promega, Mannheim, Germany) according to the manufacturer's protocols. RT-PCR was carried out using ImProm-II® Reverse Transcriptase (Promega) for first-strand synthesis by random and oligo d(T) priming. For the PCR reaction, Taq polymerase (Eppendorf, Hamburg, Germany) and the Expand Long Template PCR System (Roche, Mannheim, Germany) were used.

Nuclear transformation of C. reinhardtii

Transformation of mutant TR72 was carried out using the glass bead method (Kindle, 1990). The transformation mixture contained

2–6 × 10^8 cells ml^{-1}, 7 μg supercoiled plasmid DNA and 0.3 g glass beads (0.5 mm diameter). After transformation (15 sec in a vortex at top speed), cells were spread on solid TAP medium and incubated overnight in dim light, and transformants were then selected for photoautotrophic growth in medium-intensity light (approximately 60 μE m^{-2} sec^{-1}).

Complementation and cloning of Rat1 *and* Rat2 *genes*

Chlamydomonas reinhardtii mutant TR72 was generated by insertional mutagenesis using plasmid pARG7.8ϕ3 (Hahn *et al.*, 1998). Using vector sequences as probes, a short *Nru*I–*Eco*RI fragment containing genomic and vector DNA was identified in a DNA library of mutant TR72 and cloned, leading to plasmid pIG1272. Based on sequence information of this genomic DNA fragment flanking the integration site of pARG7.8ϕ3 in mutant TR72, PCR primers were designed to screen pooled DNA from an indexed wild-type cosmid library (Zhang *et al.*, 1994), leading to cosmid P78H3. The insert of pIG1272 was used to screen a wild-type cDNA library of *C. reinhardtii* (Boudreau *et al.*, 2000). Two independent cDNAs encoding the *Rat1* gene were obtained (p126.1 and p119.20). To identify the complementing region of cosmid P78H3, the insert DNA was digested with several restriction enzymes and cloned into different vectors.

Rat1 *mutagenesis*

Mutagenesis of the *Rat1* sequence was carried out using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, the Netherlands) according to the manufacturer's protocols. Plasmid pIG1550-2 was used as template in the PCR reactions, giving rise to plasmids pIG1550-2ΔK86 (deletion of K86 residue of Rat1); pIG1550-L130M (L130 residue of Rat1 mutated into M); and pIG1550-2L195M (L195 residue of Rat1 mutated into M). In all cases the constructs were verified by DNA sequencing.

Epitope tagging of Rat1

A triple-HA epitope encoded by annealed oligonucleotides was inserted into the singular *Eco*RV site of plasmid p39 (Figure 2), giving rise to plasmid p39HA3. The HA epitope containing plasmid p39HA3 was transformed into mutant TR72. The transformants were first selected for restoration of photosynthesis, then screened for expression of the HA epitope by immunoblotting.

Cellular fractionation

To obtain total protein extracts, cell pellets of 500 ml cultures were treated as described (Bunse *et al.*, 2001). Chloroplasts were prepared as described previously (Zerges and Rochaix, 1998), and further separation into a soluble and insoluble fraction was carried out according to Auchincloss *et al.* (2002).

Immunoblot analysis

Protein samples were boiled for 5 min at a final concentration of 10% glycerol, 300 mM 2-mercaptoethanol, 2% SDS, 30 mM Tris pH 6.8, prior to loading on polyacrylamide gels. Proteins were fractionated by electrophoresis and transferred to PVDF Western blotting membranes (Roche). The filters were blocked in a Tris-buffered salt solution containing 1% blocking reagent (Roche) for 1 h, incubated with primary antisera (α-Rubisco, 1:5000; α-NAB1, 1:20 000; α-CF_1, 1:5000) or an HA monoclonal antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, California, USA) at 4°C overnight, and reacted with peroxidase-linked anti-rabbit Ig for 1 h. Signals were visualized by enhanced chemiluminescence (Durrant, 1990).

The yeast three-hybrid system

The yeast strain CG1945 and plasmid pGAD424 were obtained from Clontech (Heidelberg, Germany) and the plasmids pDBRevM10 and pPGKRRE (Putz *et al.*, 1996) were generous gifts from U. Putz (University of Hamburg). The plasmid pADTR72ΔN19 was constructed as follows. Oligonucleotides 2421 and 2422 were used in a PCR reaction with plasmid p126.1 as template to amplify the *Rat1* cDNA lacking the first 19 codons of the ORF from the 5′ end. Oligonucleotides 2421 and 2422 contain *Eco*RI and *Bam*HI restriction sites, respectively, at their 5′ ends. The amplified PCR fragment was digested with *Eco*RI and *Bam*HI and cloned in the likewise restricted vector pGAD424, and the construct was verified by sequencing. For the construction of plasmid pRevrl1d4-6, PCR was used to amplify a DNA fragment from plasmid pIG597.1 carrying intron rI1 and flanking exon sequences from the mitochondrial LSUrRNA gene of *Scenedesmus obliquus* (Herdenberger *et al.*, 1994). Oligonucleotides 1054 and 1055 contain *Mlu*I restriction sites at their 5′ end, and were used to amplify domains IV, V and VI of intron rI1. The PCR fragment was cloned into pBIIKS+ (Stratagene), the resulting plasmid prI1d4–6MluI was digested with *Mlu*I and the intron DNA fragment was cloned into the unique *Mlu*I restriction site of plasmid pPGKRRE, leading to a fusion of the RRE RNA and the intron domains in plasmid pPGKrI1d4-6. To construct plasmid pRevrI1d4-6, the RNA expression cassette with the PGK promoter and terminator was cloned as a *Xho*I–*Sal*I fragment into the unique *Sal*I restriction site of plasmid pDBRevM10. To create plasmids pRevtscA1 and pRev3′Ihcb, respectively, PCRs were carried out with oligonucleotides 1056 and 1057 with pIG637.1 (Herdenberger *et al.*, 1994) as template, and with oligonucleotides 1058 and 1059 with λ65/3-1 (DH, unpublished data) as template. These oligonucleotides added *Mlu*I restriction sites to the PCR fragments, and the subsequent cloning strategy was the same as for plasmid pRevrI1d4-6. The fusion RNA in plasmid pRevtsca1 carries sequences of the mature *tscA* RNA, and plasmid pRev3′Ihcb contains a part of the 3′ UTR of the *Lhcb* gene of *Chlamydomonas*. All constructs were verified by custom-directed DNA sequencing. Plasmids pDBRevM10, pRevtscA1, pRevrI1d4-6 and pRev3′Ihcb were introduced into the yeast strain CG1945 by transformation, and Trp^+ transformants containing hybrid RNAs were screened for expression of the fusion RNAs using an RRE RNA-specific 220 nt *Eco*RI fragment of plasmid pPGKRRE as a probe for Northern hybridization. Transformants expressing the different RNAs were then transformed with plasmid pADTR72ΔN19 and plated on media lacking tryptophan and leucine. Resulting Trp^+ and Leu^+ prototrophs were tested further for the expression of the reporter genes by streaking on media lacking histidine and by using a colony-lift filter assay.

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References

Altamura, N., Dujardin, G., Groudinsky, O. and Slonimski, P.P. (1994) Two adjacent nuclear genes, ISF1 and NAM7/UPF1, cooperatively participate in mitochondrial functions in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **242**, 49–56.

Auchincloss, A.H., Zerges, W., Perron, K., Girard-Bascou, J. and Rochalx, J.D. (2002) Characterization of TbcZ, a nucleus-encoded factor specifically required for translation of the chloroplast psbC mRNA in *Chlamydomonas reinhardtii*. *J. Cell. Biol.* **157**, 953–962.

Bonen, L. and Vogel, J. (2001) The ins and outs of group II introns. *Trends Genet.* **17**, 322–331.

Boudreau, E., Nickelsen, J., Lemaire, S.D., Ossenbühl, F. and Rochaix, J.D. (2000) The *Nac2* gene of *Chlamydomonas* encodes a chloroplast TPR-like protein involved in *psbD* mRNA stability. *EMBO J.* **19**, 3366–3376.

Bunse, A.A., Nickelsen, J. and Kück, U. (2001) Intron-specific RNA binding proteins in the chloroplast of the green alga *Chlamydomonas reinhardtii*. *Biochim. Biophys. Acta*, **1519**, 46–54.

Campbell, W.H. and Gowri, G. (1990) Codon usage in higher plants, green algae and cyanobacteria. *Plant Physiol.* **92**, 1–11.

Cenkci, B., Petersen, J.L. and Small, G.D. (2003) REX1, a novel gene required for DNA repair. *J. Biol. Chem.* **278**, 22574–22577.

Doudna, J.A. and Cech, T.R. (2002) The chemical repertoire of natural ribozymes. *Nature*, **418**, 222–228.

Durrant, I. (1990) Light-based detection of biomolecules. *Nature*, **346**, 297–298.

Evguenieva-Hackenberg, E., Schiltz, E. and Klug, G. (2002) Dehydrogenases from all three domains of life cleave RNA. *J. Biol. Chem.* **277**, 46145–46150.

Felder, S., Meierhoff, K., Sane, A.P., Meurer, J., Driemel, C., Plücken, H., Klaff, P., Stein, B., Bechtold, N. and Westhoff, P. (2001) The nucleus-encoded *HCF107* gene of Arabidopsis provides a link between intercistronic RNA processing and the accumulation of translation-competent *psbH* transcripts in chloroplasts. *Plant Cell*, **13**, 2127–2141.

Giege, P. and Brennicke, A. (2001) From gene to protein in higher plant mitochondria. *C. R. Acad. Sci. III*, **324**, 209–217.

Goldschmidt-Clermont, M. (1998) Coordination of nuclear and chloroplast gene expression in plant cells. *Int. Rev. Cytol.* **177**, 115–180.

Goldschmidt-Clermont, M., Girard-Bascou, J., Choquet, Y. and Rochaix, J.D. (1990) *Trans*-splicing mutants of *Chlamydomonas reinhardtii*. *Mol. Gen. Genet.* **223**, 417–425.

Goldschmidt-Clermont, M., Choquet, Y., Girard-Bascou, J., Michel, F., Schirmer-Rahire, M. and Rochaix, J.D. (1991) A small chloroplast RNA may be required for *trans*-splicing in *Chlamydomonas reinhardtii*. *Cell*, **65**, 135–143.

Gumpel, N.J. and Purton, S. (1994) Playing tag with *Chlamydomonas*. *Trends Cell Biol.* **4**, 299–301.

Hahn, D., Nickelsen, J., Hackert, A. and Kück, U. (1998) A single nuclear locus is involved in both chloroplast RNA *trans*-splicing and 3′ end processing. *Plant J.* **15**, 575–581.

Harris, E.H. (1989) *The Chlamydomonas Sourcebook, A Comprehensive Guide to Biology and Laboratory Use*. San Diego, CA, USA: Academic Press.

Herdenberger, F., Holländer, V. and Kück, U. (1994) Correct *in vivo* RNA splicing of a mitochondrial intron in algal chloroplasts. *Nucleic Acids Res.* **22**, 2869–2875.

Holländer, V. and Kück, U. (1999) Group II intron splicing in chloroplasts: identification of mutations determining intron stability and fate of exon RNA. *Nucleic Acids Res.* **27**, 2345–2353.

Huang, H.-R., Rowe, C.E., Mohr, S., Jiang, Y., Lambowitz, A.M. and Perlman, P.S. (2005) The splicing of the yeast mitochondrial group I and group II introns requires a DEAD-box protein with RNA chaperone function. *Proc. Natl Acad. Sci. USA*, **102**, 163–168.

Jaeger, S., Eriani, G. and Martin, F. (2004) Results and prospects of the yeast three-hybrid system. *FEBS Lett.* **556**, 7–12.

Jenkins, B.D. and Barkan, A. (2001) Recruitment of a peptidyl-tRNA hydrolase as a facilitator of group II intron splicing in chloroplasts. *EMBO J.* **20**, 872–879.

Jenkins, B.D., Kulhanek, D.J. and Barkan, A. (1997) Nuclear mutations that block group II RNA splicing in maize chloroplasts reveal several intron classes with distinct requirements for splicing factors. *Plant Cell*, **9**, 283–296.

Kindle, K.L. (1990) High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. *Proc. Natl Acad. Sci. USA*, **87**, 1228–1232.

Kück, U., Choquet, Y., Schneider, M., Dron, M. and Bennoun, P. (1987) Structural and transcriptional analysis of two homologous genes for the P700 chlorophyll *a*-apoproteins in *Chlamydomonas reinhardtii*: evidence for *in vivo trans*-splicing. *EMBO J.* **6**, 2185–2195.

Kück, U., Godehardt, I. and Schmidt, U. (1990) A self-splicing group II intron in the mitochondrial large subunit rRNA (LSUrRNA) gene of the eukaryotic alga *Scenedesmus obliquus*. *Nucleic Acids Res.* **18**, 2691–2697.

Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105–132.

Lambowitz, A.M., Caprara, M., Zimmerly, S. and Perlman, P.S. (1999) Group I and group II ribozymes as RNPs: clues to the past and guides to the future. In *The RNA World*, 2nd edn (Gesteland, R.F., Cech, T.R. and Atkins, J.F., eds). Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press, pp. 451–485.

Lumbreras, V., Stevens, D.R. and Purton, S. (1998) Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. *Plant J.* **14**, 441–447.

Nagy, E. and Rigby, W.F. (1995) Glyceraldehyde-3-phosphate dehydrogenase selectively binds AU-rich RNA in the NAD(+)-binding region (Rossmann fold). *J. Biol. Chem.* **270**, 2755–2763.

Nagy, E., Henics, T., Eckert, M., Miseta, A., Lightowlers, R.N. and Kellermayer, M. (2000) Identification of the NAD(+)-binding fold of glyceraldehyde-3-phosphate dehydrogenase as a novel RNA-binding domain. *Biochem. Biophys. Res. Commun.* **275**, 253–260.

Ostheimer, G.J., Williams-Carrier, R., Belcher, S., Osborne, E., Gierke, J. and Barkan, A. (2003) Group II intron splicing factors derived by diversification of an ancient RNA-binding domain. *EMBO J.* **22**, 3919–3929.

Pelzer-Reith, B., Freund, S., Schnarrenberger, C., Yatsuki, H. and Hori, K. (1995) The plastid aldolase gene from *Chlamydomonas reinhardtii*: intron/exon organization, evolution, and promoter structure. *Mol. Gen. Genet.* **248**, 481–486.

Perron, K., Goldschmidt-Clermont, M. and Rochaix, J.D. (1999) A factor related to pseudouridine synthases is required for chloroplast group II intron *trans*-splicing in *Chlamydomonas reinhardtii*. *EMBO J.* **18**, 6481–6490.

Perron, K., Goldschmidt-Clermont, M. and Rochaix, J.D. (2004) A multiprotein complex involved in chloroplast group II intron splicing. *RNA*, **10**, 704–711.

Pioli, P.A., Hamilton, B.J., Connolly, J.E., Brewer, G. and Rigby, W.F. (2002) Lactate dehydrogenase is an AU-rich element-binding protein that directly interacts with AUF1. *J. Biol. Chem.* **277**, 35738–35745.

Posewitz, M.C., King, P.W., Smolinski, S.L., Zhang, L., Seibert, M. and Ghirardi, M.L. (2004) Discovery of two novel radical S-adenosylmethionine proteins required for the assembly of an active [Fe] hydrogenase. *J. Biol. Chem.* **279**, 25711–25720.

Putz, U., Skehel, P. and Kuhl, D. (1996) A tri-hybrid system for the analysis and detection of RNA–protein interactions. *Nucleic Acids Res.* **24**, 4838–4840.

Rho, S.B. and Martinis, S.A. (2000) The bI4 group I intron binds directly to both its protein splicing partners, a tRNA synthetase and maturase, to facilitate RNA splicing activity. *RNA*, **6**, 1882–1894.

Rho, S.B., Lincecum, T.L. and Martinis, S.A. (2002) An inserted region of leucyl-tRNA synthetase plays a critical role in group I intron splicing. *EMBO J.* **21**, 6874–6881.

Rivier, C., Goldschmidt-Clermont, M. and Rochaix, J.D. (2001) Identification of an RNA–protein complex involved in chloroplast group II intron *trans*-splicing in *Chlamydomonas reinhardtii*. *EMBO J.* **20**, 1765–1773.

Rochaix, J.D. (1996) Post-transcriptional regulation of chloroplast gene expression in *Chlamydomonas reinhardtii*. *Plant Mol. Biol.* **32**, 327–341.

Sambrook, J. and Russel, D.W. (2001) *Molecular Cloning – A Laboratory Manual*. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

Silflow, C.D. (1998) Organization of the nuclear genome. In *The Molecular Biology of Chloroplast and Mitochondria in Chlamydomonas* (Rochaix, J.D., Goldschmidt-Clermont, M. and Merchant, S., eds). Dordrecht, The Netherlands: Kluwer, pp. 25–40.

Sioud, M. and Jespersen, L. (1996) Enhancement of hammerhead ribozyme catalysis by glyceraldehyde-3-phosphate dehydrogenase. *J. Mol. Biol.* **257**, 775–789.

Till, B., Schmitz-Linneweber, C., Williams-Carrier, R. and Barkan, A. (2001) CRS1 is a novel group II intron splicing factor that was derived from a domain of ancient origin. *RNA*, **7**, 1227–1238.

Vaistij, F.E., Boudreau, E., Lemaire, S.D., Goldschmidt-Clermont, M. and Rochaix, J.D. (2000) Characterization of Mbb1, a nucleus-encoded tetratricopeptide-like repeat protein required for expression of the chloroplast *psbB/psbT/psbH* gene cluster in *Chlamydomonas reinhardtii*. *Proc. Natl Acad. Sci. USA*, **97**, 14813–14818.

Zerges, W. and Rochaix, J.D. (1998) Low density membranes are associated with RNA-binding proteins and thylakoids in the chloroplast of *Chlamydomonas reinhardtii*. *J. Cell Biol.* **140**, 101–110.

Zhang, H., Herman, P.L. and Weeks, D.P. (1994) Gene isolation through genomic complementation using an indexed library of *Chlamydomonas reinhardtii* DNA. *Plant. Mol. Biol.* **24**, 663–672.