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The Chloroplast *atpA* Gene Cluster in *Chlamydomonas reinhardtii*[1]

Functional Analysis of a Polycistronic Transcription Unit

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Most chloroplast genes in vascular plants are organized into polycistronic transcription units, which generate a complex pattern of mono-, di-, and polycistronic transcripts. In contrast, most *Chlamydomonas reinhardtii* chloroplast transcripts characterized to date have been monocistronic. This paper describes the *atpA* gene cluster in the *C. reinhardtii* chloroplast genome, which includes the *atpA*, *psbI*, *cemA*, and *atpH* genes, encoding the α-subunit of the coupling-factor-1 (CF_1) ATP synthase, a small photosystem II polypeptide, a chloroplast envelope membrane protein, and subunit III of the CF_0 ATP synthase, respectively. We show that promoters precede the *atpA*, *psbI*, and *atpH* genes, but not the *cemA* gene, and that *cemA* mRNA is present only as part of di-, tri-, or tetracistronic transcripts. Deletions introduced into the gene cluster reveal, first, that CF_1-α can be translated from di- or polycistronic transcripts, and, second, that substantial reductions in mRNA quantity have minimal effects on protein synthesis rates. We suggest that posttranscriptional mRNA processing is common in *C. reinhardtii* chloroplasts, permitting the expression of multiple genes from a single promoter.

The chloroplast genome of *Chlamydomonas reinhardtii* shares many similarities with the genomes of vascular plants. These genomes are circular DNA molecules that range in size from 120 to 200 kb and have two unique regions separated by large, inverted repeats. Although gene content is highly conserved, the distribution of genes along the chloroplast chromosome varies widely between vascular plants and *C. reinhardtii* (Sugiura, 1992).

In vascular plant chloroplasts there is substantial evidence for extensive co-transcription of genes in polycistronic operons (Sugita and Sugiura, 1996), which results in complex mRNA accumulation patterns. A striking example is the *psbB* gene cluster, which groups three *PSII* genes and two Cyt b_6/Cyt *f* complex genes into the transcription unit *psbB-psbT-psbH-petB-petD* (Barkan, 1988; Kohchi et al., 1988; Westhoff and Herrmann, 1988). Approximately 20 RNA species could be resolved for spinach and maize, each apparently resulting from processing of a primary transcript containing all five coding regions. In maize, both monocistronic and polycistronic *petB* and *petD* transcripts were shown to be engaged in translation (Barkan, 1988), but the monocistronic *petD* transcript was a substantially better template for translation than its precursor forms (Barkan et al., 1994).

The situation in *C. reinhardtii*, with most chloroplast mRNAs accumulating as monocistronic transcripts, appears to be very different from that in vascular plants. However, several instances of co-transcription of two or more genes have been documented (Rochaix, 1996). Although in most cases co-transcription was demonstrated by the accumulation of dicistronic mRNAs, the mode of *petA-petD* transcription suggests that the degree of co-transcription in *C. reinhardtii* chloroplasts may be greatly underestimated. Although only monocistronic transcripts for the *petA* and downstream *petD* genes accumulate in wild-type cells, deletion of the *petD* promoter still allowed the accumulation of wild-type levels of monocistronic *petD* mRNA as well as a small amount of a *petA-petD* co-transcript (Sturm et al., 1994). Apparently, in the absence of a functional *petD* promoter, the *petD* gene can be transcribed from the upstream *petA* promoter. In this case, a lack of transcription termination downstream of *petA* combines with efficient 5′ processing of *petD* mRNA to generate mature *petD* transcripts (Sakamoto et al., 1994). It is likely that both promoters are used in wild-type strains. Other monocistronic transcripts in *C. reinhardtii* chloroplasts may be generated similarly by a combination of co-transcription and processing.

The *atpA* gene encodes the α-subunit of the chloroplast ATP synthase (Dron et al., 1982b; Hallick, 1984; Leu et al.,

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Abbreviations: nt, nucleotides; UTR, untranslated region.

1992). Both a monocistronic *atpA* transcript of 2.2 kb and a possible precursor form of slightly larger size have been detected in *C. reinhardtii* (Dron et al., 1982b). The relative amounts of the monocistronic *atpA* transcript and the putative precursor were found to vary in the nuclear mutants *ncc1* (Drapier et al., 1992) and *crp3* (Levy et al., 1997), suggesting that transcript maturation and/or stabilization in this region is complex and governed by at least two nuclear factors. Here we present a transcriptional analysis of the *atpA* gene cluster, the most complex analyzed to date in *C. reinhardtii*. We show that multiple promoters and mRNA-processing events together result in the accumulation of multiple, overlapping transcripts, reminiscent of transcription patterns typical in vascular plant chloroplasts.

MATERIALS AND METHODS

Strains and Growth Conditions

The wild-type *Chlamydomonas reinhardtii* strain used as a recipient for the creation of ΔatpA was P17, which was obtained by transformation of the *atpB* deletion strain CC373 to prototropy with a wild-type *atpB* gene (Stern et al., 1991). Cells were grown in Tris-acetate-phosphate medium (Harris, 1989), pH 7.2, at 25°C with 5.9 μmol photons m^{-2} s^{-1} of continuous illumination.

Plasmid Constructs

The nomenclature used for *C. reinhardtii* ctDNA restriction fragments is described by Harris (1989). Plasmid p*ΔatpA* carries a 2004-bp deletion from the left end of R7 to a *Hin*dIII site immediately downstream of *psbI*. It was constructed by subcloning the 4.1-kb *Bam*HI-*Eco*RI fragment from R15 and the 1.5-kb *Hin*dIII-*Eco*RI fragment from R7 into the *Bam*HI and *Eco*RI sites of pUC19 (Yanisch-Perron et al., 1985) after the *Eco*RI site of the 4.1-kb fragment and the *Hin*dIII site of the 1.5-kb fragment were filled in with the Klenow fragment of DNA polymerase.

Plasmid pΔ1 carries a 632-bp deletion from a *Pac*I site immediately downstream of the *atpA* stop codon to the same *Hin*dIII site, and was constructed as follows. The 4.1-kb *Bam*HI-*Eco*RI fragment from R15 was subcloned into the *Bam*HI and *Eco*RI sites of pBluescript (Promega) to generate pR15-1. The cloned R7 fragment was digested with *Pac*I, repaired with the Klenow fragment, digested with *Eco*RI, and then purified. Separately, R7 was digested with *Hin*dIII, repaired with the Klenow fragment, and digested with *Bam*HI, which is located in the multiple cloning site next to the *Eco*RI site of R7. These two purified fragments were ligated into the *Eco*RI and *Bam*HI sites of pUC19 to generate pR7-1, which carries the deletion between the *Pac*I and *Hin*dIII sites. To add upstream sequences to facilitate homologous recombination, the 2.9-kb *Eco*RI fragment of pR7-1 was inserted into the *Eco*RI site of pR15-1, yielding pΔ1.

Plasmid pΔ2 carries a 266-bp deletion from a *Hpa*I site immediately downstream of the *psbI* initiation codon to the same *Hin*dIII site and was constructed as follows. R7 was digested to completion with *Eco*RI and partially with *Hpa*I, and the 1.65-kb *Eco*RI-*Hpa*I fragment was purified. Separately, the R7 fragment was digested with *Hin*dIII, repaired with the Klenow fragment, and digested with *Bam*HI, which is located in the multiple cloning site adjacent to the *Eco*RI site of R7. These two fragments were inserted into the *Eco*RI and *Bam*HI sites of pUC19 to generate pΔ3-1. Finally, to add upstream sequences to facilitate homologous recombination, the 3.2-kb *Eco*RI fragment of pΔ3-1 was inserted into the *Eco*RI site of pR15-1, yielding pΔ2.

Plasmid pΔ3 carries a 313-bp deletion between two *Hpa*I sites, the first located 60 bp downstream of the *atpA* stop codon, and the second immediately downstream of the *psbI* initiation codon, and was constructed as follows. Plasmid pR7, carrying the R7 fragment in pUC19, was digested with *Hpa*I and re-ligated to generate pEcoRI12-1. To facilitate homologous recombination, the 3.2-kb *Eco*RI fragment from pEcoRI12-1 was inserted into the *Eco*RI site of pR15-1, yielding pΔ3.

To construct the *psbI-uidA* fusion gene, a 331-bp *Apo*I-*Sca*I promoter test fragment was cloned into pBluescript, excised with *Xho*I and *Sma*I, and used to replace the *petD* promoter-5′ UTR fragment of plasmid pDG2 (Sakamoto et al., 1993). The resultant plasmid was a possible transcriptional fusion of *psbI* and *uidA*, flanked by the 3′ UTR of *rbcL*. The *atpH-uidA* promoter fusion was constructed by first subcloning a 460-bp *Eco*RI-*Rsa*I fragment of R8 containing 370 bp of the 5′ noncoding region and 90 bp of the *atpH* coding region into *Eco*RI-*Sma*I-digested pBluescript, to create pHG1. A 2-kb *Bam*HI-*Sac*I fragment of pBI221 (Clontech, Palo Alto, CA) containing the *uidA*-coding region was inserted into the *Bam*HI and *Sac*I sites of pHG1 to obtain pHG2, generating a translational fusion of the *atpH* amino terminal to the entire *uidA* coding region. The *Eco*RI-*Sac*I fragment of pHG2 was inserted into *Eco*RI-*Eco*RV-digested pBluescript after blunting the *Sac*I site, yielding pHG3. A 440-bp *Hin*dIII-*Sac*I fragment containing the *rbcL* 3′ UTR was subcloned from pUC-atpX-aad (Goldschmidt-Clermont, 1991) into the *Hin*dIII and *Sac*I sites of pHG3, yielding pHG4. Then, the *Sac*I-*Kpn*I fragment of pHG4 was inserted into *Bam*HI-*Kpn*I-digested pUC19 after blunting the *Sac*I and *Bam*HI sites with the Klenow fragment of DNA polymerase, yielding pHG40. Finally, the 2.9-kb *Sal*I fragment of pHG40, carrying the expression cassette, was inserted into the *Bgl*II site of pΔ26 (Stern et al., 1991) after partially filling in the *Sal*I site with dTTP and dCTP and the *Bgl*II site with dATP and dGTP, yielding pHG5 and pHG5-R, respectively. Plasmid pHG5 carries the *atpH-uidA-rbcL* cassette in tandem with *atpB*, whereas pHG5-R has the cassette in the convergent orientation.

Plasmids 3′ *rbcL*(+) and 3′ *rbcL*(−) were constructed as follows. Plasmid pATPA-2, which was obtained from S. Ketchner (laboratory of F.-A.W.), contains a 4.37-kb *Hin*dIII-*Xba*I fragment beginning 1.25 kb upstream of the *atpA* start codon and ending within *cemA*. A 500-bp fragment containing the *rbcL* 3′ UTR and identical to that used in pUC-atpX-aad was excised from plasmid pFAR12 (Choquet et al., 1998) with *Sma*I and *Hin*dIII, and blunted with the Klenow fragment of DNA polymerase. Plasmid pATP-2 was linearized at an *Eco*47III site located approximately 120 bp downstream of the *atpA* mRNA 3′ end and 245 bp

upstream of the *cemA* start codon, and the *rbcL* 3′ UTR fragment was inserted, yielding clones with the insert in both orientations.

Chloroplast Transformation

Strain ΔatpA was obtained by co-transformation of the wild-type strain P17 with pΔ*atpA* and pCrBH4.8, which contains a version of the *rrn16* gene conferring spectinomycin resistance, as described previously (Chen et al., 1993). ΔatpA was determined to be homoplasmic by DNA-filter hybridizations and by its inability to grow on medium lacking acetate. Strains Δ1, Δ2, and Δ3 were obtained by bombarding Δ*atpA* with the corresponding plasmids and selecting for growth on minimal medium. Homoplasmicity was verified by DNA-filter hybridizations. The *psbI* and *atpH* promoter test constructs were introduced into the *atpB* deletion-mutant strain CC373 (Shepherd et al., 1979), and transformants were selected on medium lacking acetate. Integration of the chimeric *uidA* genes described above was verified by PCR and DNA-filter hybridizations. Strains 3′ *rbcL*(+) and 3′ *rbcL*(−) were created by bombarding Δ*atpA* cells as described previously (Kuras and Wollman, 1994), and selecting for photosynthetic growth on minimal medium under bright light.

RNA Analysis

Total RNAs were extracted from 20-mL cultures at a density of approximately 2×10^6 cells mL^{-1} following a method described for *Saccharomyces cerevisiae* (Schmitt et al., 1990). Cells were pelleted and resuspended in 400 μL of 50 mM $NaC_2H_3O_2$, pH 4.8, 10 mM EDTA, and 100 μM aurintricarboxylic acid. The cells were transferred to 1.5-mL microcentrifuge tubes, and SDS was added to a final concentration of 2%. Then, 1 volume of phenol equilibrated at pH 4.8 was added and the mixture was incubated for 4 min at 65°C and quick frozen in liquid N. After thawing, the aqueous phase was recovered by centrifugation and RNA was precipitated with ethanol. RNA samples were fractionated in formaldehyde-agarose gels, transferred to nylon membranes under vacuum, and hybridized with ^{32}P-labeled probes made by random priming, as described previously (Drapier et al., 1992). Gels were analyzed using a phosphor imager (Molecular Dynamics, Sunnyvale, CA). Probes used were as follows, unless designated otherwise: for *atpA*, the 947-bp *Eco*RI-*Pst*I fragment of R7; for *psbI*, the 215-bp *Rsa*I-*Nsi*I fragment of R7; for *cemA*, the 683-bp *Xba*I-*Eco*RI fragment of R7; for *atpH*, the 291-bp *Afl*III-*Cla*I fragment of R8; and for *rbcL*, the fragment R15-4 of Dron et al. (1982a).

The probe for S1 nuclease protection of the *cemA* and *atpH* 3′ ends was labeled by Klenow fill-in at the *Eco*RI site between R7 and R8, and then isolated from an agarose gel after digestion with *Bgl*I. S1 nuclease protection followed a published protocol (Ausubel et al., 1990) with slight modifications. Total RNA was co-precipitated with the radiolabeled probe and resuspended in 16 μL of formamide and 4 μL of 5× hybridization buffer containing 200 mM Pipes, pH 6.4, 2 M NaCl, and 5 mM EDTA. This mixture was denatured at 65°C for 10 min and annealed at 30°C overnight. S1 nuclease buffer (300 μL) containing 5 or 10 units of S1 nuclease μg^{-1} RNA was added to the annealed mixture and incubated for 1 h at 30°C. The products were collected by ethanol precipitation and analyzed by alkaline gel electrophoresis using a γ-^{32}P-labeled 1-kb ladder (GIBCO-BRL) for molecular mass standards. Primer extension (Sturm et al., 1994) and RNase protection using uniformly labeled antisense RNA probes (Levy et al., 1997) were carried out as described previously. The probe for RNase protection of the *atpA* 3′ end was made from plasmid p22ApS, a 331-bp *Apo*I-*Sca*I fragment extending from 26 bp upstream of the *atpA* translation termination codon to 23 bp downstream of the *psbI* 5′ end. Run-on transcription assays were carried out as described by Gagne and Guertin (1992) with modifications (Stern and Kindle, 1993).

Protein Analysis

Pulse-labeling experiments were carried out as described previously (Drapier et al., 1992) in the presence of an inhibitor of cytoplasmic translation (6.6 μg mL^{-1} cycloheximide). Proteins of solubilized cells were separated in urea/SDS-polyacrylamide gels (Piccioni et al., 1981), and radioactive polypeptides were detected using a phosphor imager. For immunoblots, proteins from unlabeled, solubilized cells were separated as described above, transferred to nitrocellulose, incubated with specific polyclonal antibodies followed by ^{125}I-protein A, as described previously (de Vitry et al., 1989). Detection and quantification of labeling were performed using a phosphor imager. Chloroplast F_1F_0 ATP synthase anti-α- and anti-β-subunit sera were kindly provided by C. Lemaire (Centre de Génétique Moléculaire, Gif-sur-Yvette, France). Antiserum against OEE2 was obtained in our laboratory.

RESULTS

Tetracistronic Transcription Unit in the *atpA* Region of the Chloroplast Genome

Three genes are found in the region immediately downstream of the *atpA* gene, as shown in Figure 1A: *psbI*, which encodes a small PSII subunit (Boudreau et al., 1994; Kunstner et al., 1995); *cemA*, which encodes a putative envelope membrane protein involved in C uptake (Rolland et al., 1997); and *atpH*, which encodes subunit III of the chloroplast ATP synthase (Lemaire and Wollman, 1989b). When total RNA was extracted from a wild-type strain and analyzed by RNA-filter hybridization with an intragenic *atpA* DNA fragment, two major transcripts of 2.1 and 2.5 kb were identified, as well as two less-abundant transcripts of 4.6 and 5.3 kb (Fig. 1B, *atpA* probe). The 2.1-kb band (transcript 4) has the size expected for monocistronic *atpA* mRNA, and represents approximately 90% of the total signal. Transcript 3 accounts for approximately 10% of the signal, and transcripts 1 and 2 accounts for less than 1% each.

The sizes of the four *atpA*-containing transcripts strongly suggested that the four genes clustered in the *atpA* region

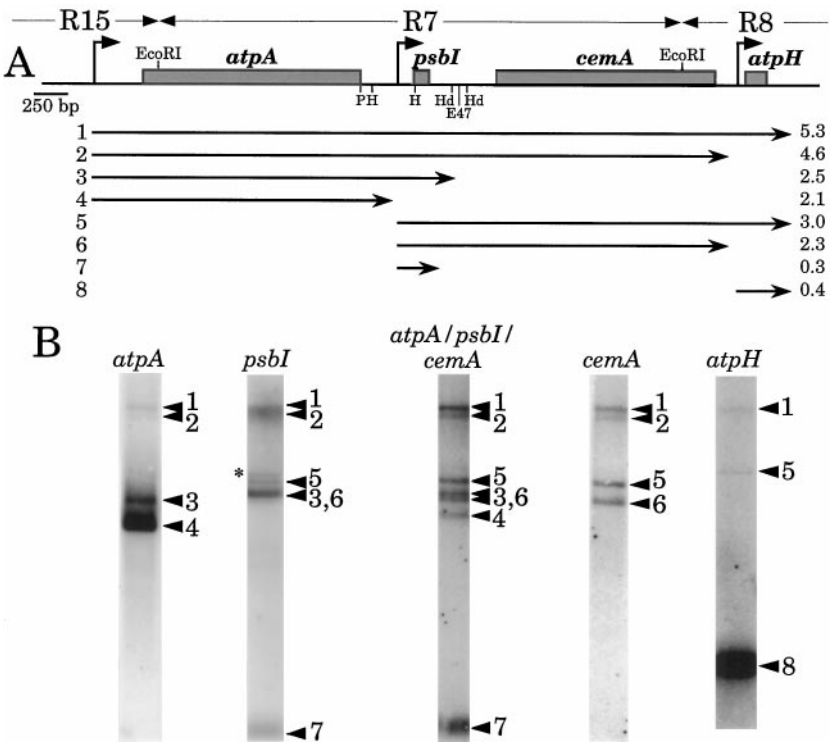

Figure 1. Transcription of the *C. reinhardtii* chloroplast *atpA* gene cluster. A, Map of the *atpA* region of the chloroplast genome. R15, R7, and R8 are *Eco*RI restriction fragments (Rochaix, 1980). Other restriction sites are: P, *Pac*I; H, *Hpa*I; Hd, *Hin*dIII; and E47, *Eco*47III. The three promoters are indicated by bent arrows. The extents of transcripts are shown as numbered arrows, with estimated sizes in kilobase pairs shown at the right. B, RNA accumulation in wild-type cells. Blots of total RNA were hybridized with the probes shown at the top of each lane as described in "Materials and Methods." The *atpA-psbI-cemA* probe was the 513-bp *Sca*I-*Dra*II fragment of R7. Not all blots are from the same gel, so the relative migration of some species varies slightly. The asterisk in the *psbI* lane indicates a transcript of unknown origin, which is not seen when a larger probe is used (third lane).

were co-transcribed as a tetracistronic transcript. Support for the hypothesis of co-transcription was obtained by hybridizing similar RNA blots with probes for *psbI*, *cemA*, and *atpH*, as shown in Figure 1B; the deduced extents of the transcripts from this region are shown as numbered arrows in Figure 1A. The results show that *atpA* is present as part of tetra-, tri-, di-, and monocistronic transcripts 1 through 4. Hybridization with *psbI* revealed three additional mRNAs: transcript 5 (*psbI* + *cemA* + *atpH*), transcript 6 (*psbI* + *cemA*), and transcript 7 (monocistronic *psbI*). The *atpH* probe revealed one additional transcript, transcript 8, which corresponds to monocistronic *atpH*. The *cemA* probe failed to detect a monocistronic *cemA* transcript. This probe labeled only transcripts 1, 2, 5, and 6, each of which carries at least one other coding region upstream of *cemA*. Transcripts 1 through 8 accumulated in widely different amounts, the most abundant being the monocistronic *atpA* and *atpH* mRNAs.

Promoters in the *atpA* Gene Cluster

The transcripts shown in Figure 1 could be transcribed from a single *atpA*-proximal promoter, with RNA processing generating the remainder of the transcripts. Alternatively, functional promoters could also lie immediately upstream of *psbI* and *atpH*, where they might be required for expression of their respective genes or may be redundant with the *atpA*-proximal promoter.

To localize promoters within this region, we first mapped mRNA 5′ ends upstream of *atpA*, *psbI*, and *atpH* by performing primer-extension experiments. As shown in Figure 2, the *atpA* 5′ end mapped to two consecutive thymidines located 390 and 391 nt upstream of the AUG initiation codon. These termini are 35 nt upstream of those estimated by an S1 nuclease protection assay (Dron et al., 1982b), and are within a putative promoter element (Dron et al., 1982b). Single 5′ ends were mapped for *psbI* and *atpH*. The *atpH* terminus is preceded by a sequence that matches the palindromic TATAAT(AT) consensus sequence previously observed in *C. reinhardtii* chloroplast promoters (Klein et al., 1992); this sequence starts at position −13 relative to the mature 5′ end. A- and T-rich sequences are also found surrounding or immediately upstream of the *atpA* and *psbI* 5′ ends, although neither matches the consensus. Given the A- and T-rich nature of ctDNA intergenic regions, these similarities may be fortuitous.

One reliable assay for promoter activity is to fuse putative promoters upstream of reporter genes, and to introduce these chimeric genes into chloroplasts by biolistic

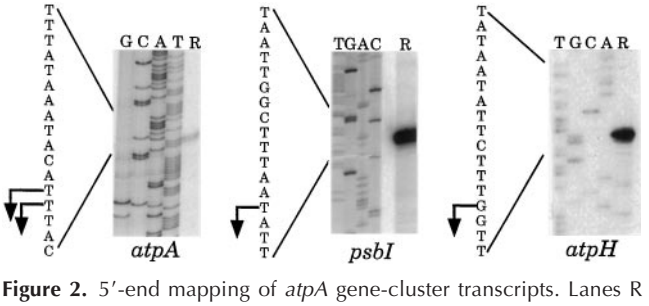

Figure 2. 5′-end mapping of *atpA* gene-cluster transcripts. Lanes R show primer-extension experiments with total RNA from wild-type cells, with the gene indicated under the panel. Relative to the translation-initiation codon, the primer for *atpA* annealed from +92 to +75, that for *psbI* annealed from +96 to +80, and that for *atpH* annealed from +27 to +9.

transformation. When DNA upstream of the *atpA*-coding region was fused to the bacterial *aadA* gene, spectinomycin-resistant transformants were recovered. These transformants accumulated chimeric *atpA-aadA* transcripts (Goldschmidt-Clermont, 1991), confirming that an active promoter lies upstream of *atpA*. To test for potential *psbI* and *atpH* promoters, upstream regions were fused to the *Escherichia coli uidA* gene, which encodes GUS, and the chimeric genes were introduced into *C. reinhardtii* chloroplasts. As shown in Figure 3A, the reporter genes were placed downstream of the *atpB* gene, in a vector previously used to express *uidA* fusion genes (Blowers et al., 1990, 1993; Sakamoto et al., 1993).

Figure 3B shows that when the *atpH-uidA* cassette was introduced either in tandem (+) or convergent (−) with the *atpB* gene, monocistronic *uidA* transcripts could be visualized by RNA-filter hybridizations; an additional co-transcript with *atpB* accumulated when the genes were in the tandem (+) orientation. Extension from a *uidA* primer revealed similar 5′ termini for monocistronic transcripts from both strains (data not shown); the sizes of the products were consistent with the mapping shown in Figure 2. These results strongly suggest that there is an *atpH*-specific promoter. Although we cannot completely rule out the possibility that *uidA* mRNA is produced exclusively by read through of *atpB* in the (+) orientation or from within the chloroplast genome's large, inverted repeat in the (−) orientation, followed by RNA processing directed by *atpH* 5′ sequences, we believe that this is very unlikely. The results of earlier studies (Stern and Kindle, 1993) suggested that in the (+) orientation, such read-through transcripts would either accumulate as dicistronic mRNAs or would be degraded by the *atpB* 3′ processing machinery, and that there is little transcription from within the chloroplast genome's large, inverted repeat.

Results with *psbI* were more equivocal. *psbI-uidA* transformants bearing the cassette convergent to the *atpB* gene did not accumulate a discrete *uidA* transcript, but did accumulate low levels of heterogeneous *uidA*-hybridizing transcripts (Fig. 3C). These transcripts could have arisen by a low level of read-through from the large, inverted repeat but, if so, they did not exhibit the discrete termini that might have been expected. To measure the transcription rate of the *uidA*-coding region in *psbI-uidA* transformants, cells were permeabilized by freeze-thaw cycles, and nascent RNAs were labeled with [^{32}P]UTP. Strains used were a wild-type strain lacking a *uidA* gene, the strain DG2, which contains a *petD-uidA* reporter gene known to be expressed in vivo (Sakamoto et al., 1993), and two independent *psbI-uidA* transformants. The labeled RNAs were hybridized with filter-bound plasmid DNAs comprising vector only, the *petD*-coding region as a control, or the *uidA* gene. The results in Figure 3D clearly show that the *uidA* gene is transcribed in *psbI-uidA* transformants at a rate similar to that in DG2, and at a much higher rate than a promoterless *uidA* gene inserted into the same site (see fig. 7C in Sturm et al., 1994). Slight background hybridization to vector sequences was also observed.

From these results we infer that although the *psbI* insertion in the chimeric gene does confer promoter activity, it lacks RNA processing and/or stability elements that are required to form stable *psbI* transcripts in wild-type cells. Although the test fragment extends 309 bp upstream of the mature *psbI* 5′ end, including the entire intergenic region and 22 bp of the *psbI* 5′ UTR, additional sequences in the 5′ UTR or coding region may be required for transcript processing or stabilization. One candidate sequence is an imperfect, inverted repeat (ATAGTTAtTAAN$_5$TAtTAACTAT) beginning at position −57 relative to the *psbI* initiation codon. Taken together, our data are most consistent with a

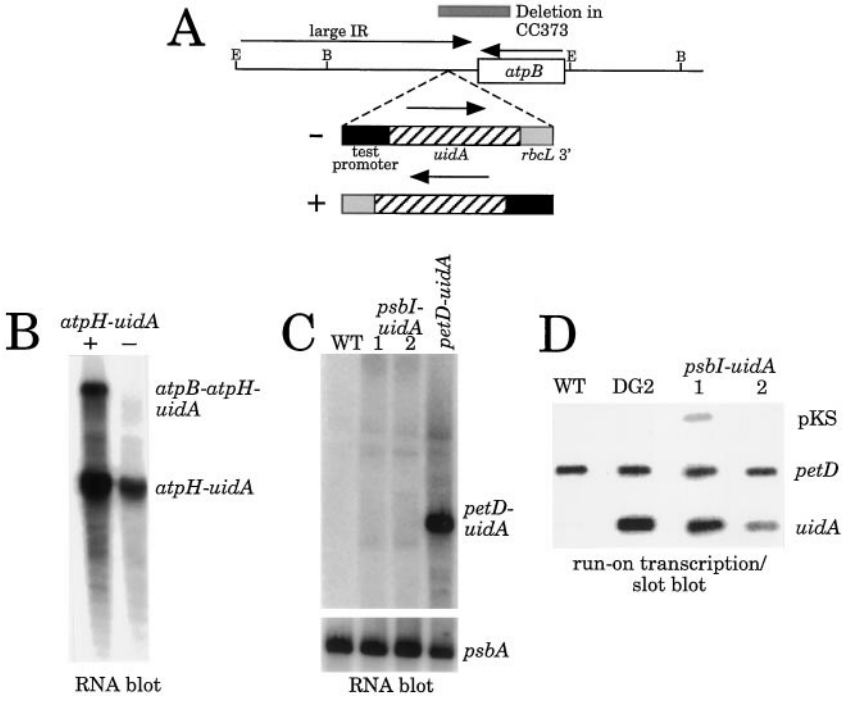

Figure 3. Analysis of chimeric *uidA* promoter fusions. A, The site of insertions of chimeric genes into the chloroplast genome. RNA-filter hybridizations are shown for *atpH* test constructs (B) and *psbI* test constructs (C). Both blots were hybridized with a *uidA*-coding-region probe; a *psbA*-coding-region probe was used for normalization of the *psbI* blot. For *atpH*, + and − indicate strains carrying the fusions in opposite orientations (A). For *psbI*, 1 and 2 are independent transformants in the (−) orientation, and the *petD-uidA* lane contains total RNA isolated from the strain DG2, which is known to accumulate *uidA* mRNA in vivo (Sakamoto et al., 1993). D, Run-on transcription from *psbI-uidA* transformants are shown. Two micrograms of each of the three plasmids shown at the right was fixed to a nylon filter using a slot-blot apparatus. pKS, pBluescript. Four separate filters were hybridized with ^{32}P-labeled transcripts from freeze-thaw-permeabilized cells of the strains shown across the top. Nonspecific hybridization can be seen for *psbI-uidA* lane1 (pKS) and for the wild type (*uidA*).

model in which each coding region in the *atpA* gene cluster, with the exception of *cemA*, is preceded by a promoter element. Nonetheless, the data do not distinguish whether the mature 5′ termini are formed by transcription initiation or RNA processing.

Transcript 3′ Termini

The RNA-filter-hybridization data suggested that there were four unique mRNA 3′ ends in the *atpA* gene cluster. It was of interest to map these ends to determine whether they coincided with obvious secondary structures, and also to determine whether any were coincident with 5′ ends of other transcripts within the cluster.

3′ ends were mapped to a resolution of ± 15 nt by RNase (*atpA*) or S1 nuclease (*cemA* and *atpH*) protection (Fig. 4). For *atpA*, two major protected products and several minor products were seen. The lower band corresponds to the 3′ end of the monocistronic *atpA* message (transcript 4), whereas the upper band represents full protection of the nonvector sequences in the probe, and thus corresponds to transcripts 1 through 3. The 5′ protection products from transcripts 5 through 7 would be 26 nt in length and thus not visible in this gel. The other minor bands are probably experimental artifacts, although we cannot rule out the possibility that they represent genuine in vivo 3′ termini. A single probe was used to map the 3′ ends of *cemA* and *atpH*, and two major protected bands were seen. The end of the smaller fragment, labeled *cemA*, maps downstream of *cemA* and presumably represents the 3′ ends of transcripts 2 and 6, whereas the larger band, labeled *atpH*, is consistent with a 3′ end downstream of *atpH*, and probably represents transcripts 1 and 5. (The DNA fragment fully protected by transcript 8 would not yield a labeled product.) The minor products just below the *atpH* band are probably artifacts, since they would map within the *atpH*-coding region. Taken together, our 3′ mapping data are consistent with the diagram in Figure 1.

Regulatory Elements in the *atpA-psbI-cemA* Intergenic Regions

To further examine the role of the region between *atpA* and *cemA* in the expression of this gene cluster, we made three deletions in the *psbI* region. To facilitate the introduction of these deletions into the chloroplast genome, we first created a nonphotosynthetic recipient strain, ΔatpA. As shown in Figure 5A, ΔatpA lacks most of the *atpA* gene and also *psbI*. This strain was created by co-transformation with a plasmid conferring spectinomycin resistance in 16S rDNA (see "Materials and Methods"). Figure 5B shows RNA accumulation, and Figure 5, C and D, respectively, show protein synthesis and accumulation in ΔatpA. A 5′ *atpA* probe revealed two transcripts corresponding to the deleted version of transcripts 1 and 2 of wild-type cells. When protein synthesis was examined by pulse labeling, the α-subunit was undetectable, as expected, but β-subunit synthesis occurred at roughly wild-type levels. However, steady-state accumulation of the β-subunit was strongly reduced, consistent with a posttranslational degradation mechanism (Lemaire and Wollman, 1989a).

The three deletions mentioned above were designed to eliminate the *atpA-psbI* intergenic region (Δ3), the *psbI-cemA* intergenic region (Δ2), or both (Δ1). These deletions could remove intergenic 3′ and 5′ processing sites and/or transcription initiation sites. Figure 6A shows the extents of

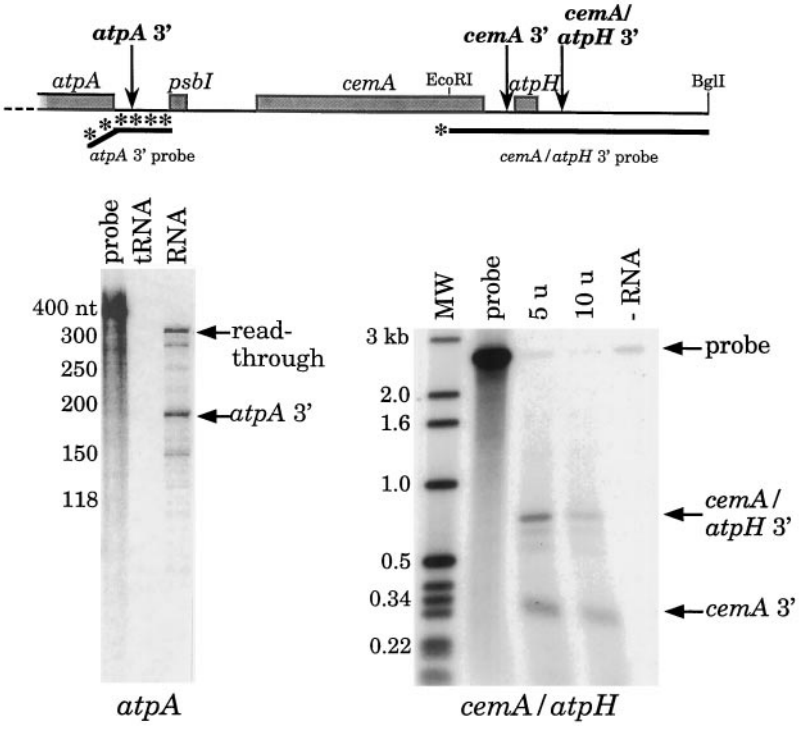

Figure 4. 3′-End mapping of *atpA* gene cluster transcripts. Mapping was carried out by RNase (*atpA*) or S1 nuclease protection with the indicated number of units per microgram of RNA (*cemA* and *atpH*). Locations of probes and deduced 3′ ends are shown at the top of the figure and are described in "Materials and Methods"; the *cemA* 3′ end is close to or coincident with the *atpH* 5′ end. For *atpA*, the tRNA lane contained 10 μg of yeast tRNA instead of *C. reinhardtii* total RNA. For *cemA* and *atpH*, total RNA from strain ΔatpA was used; this strain accumulates increased levels of transcripts ending at *cemA*. Note that the probe for *atpA* was a uniformly labeled RNA (the bent part indicates pBluescript vector sequences), whereas that for *cemA* and *atpH* was an end-labeled DNA fragment. Electrophoresis was in a denaturing polyacrylamide gel for *atpA*, and in an alkaline agarose gel for *cemA* and *atpH*. The *atpH* 3′ end is marked on the gel as *cemA/atpH* 3′ because with this probe only *cemA-atpH* co-transcripts will be visible as protected fragments. MW, Molecular weight.

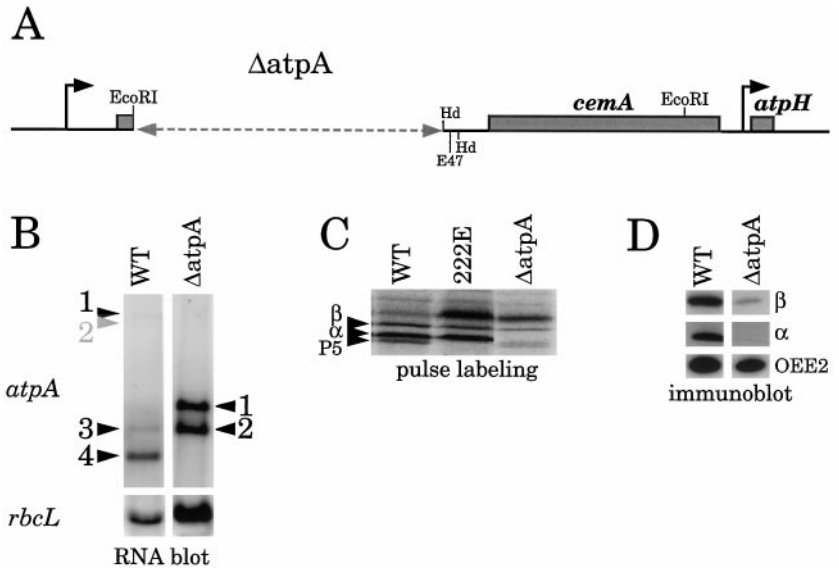

Figure 5. Characterization of the ΔatpA deletion strain. A, The horizontal gray dashed line shows the extent of the deletion. B, *atpA*-hybridizing transcripts, numbered as in Figure 1, after hybridization of an RNA blot with a 480-bp *Dra*II-*Eco*RI fragment of R15, the 5′ end of the *atpA* gene. For the wild type (WT), transcript 2 is not visible in this exposure and is therefore labeled in gray. C, Pulse-labeling with [^{14}C]acetate for 5 min, as described in "Materials and Methods"; β and α indicate subunits of the ATP synthase, and P5 is a PSII subunit. Strain 222E does not synthesize P5 because of a nuclear mutation causing instability of *psbB* mRNA (Monod et al., 1992), and was used as a negative control for this protein. In strains 222E and ΔatpA, there is increased labeling of the Rubisco large subunit, which migrates just above the β-subunit. This is typically seen for nonphotosynthetic mutants of *C. reinhardtii* under these experimental conditions (see fig. 3 in Drapier et al., 1992). D, Immunoblot using the antisera indicated at the right. The diminished accumulation of the β-subunit in Δ*atpA* reflects a posttranslational instability of the protein.

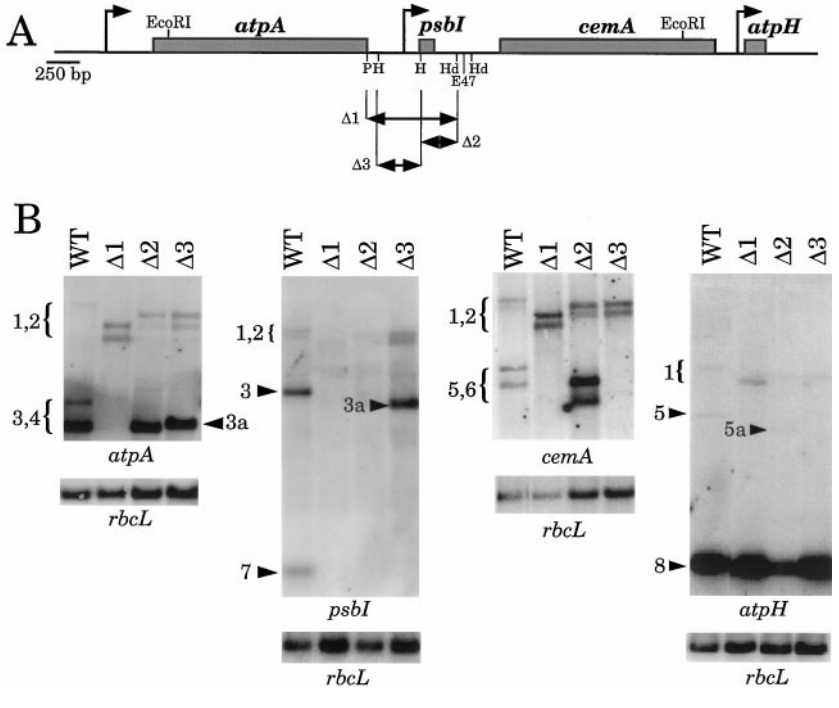

Figure 6. RNA accumulation in the deletion strains Δ1, Δ2, and Δ3. A, Map of the *atpA* gene cluster as shown in Figure 1. The extents of the deletions in Δ1, Δ2, and Δ3 are shown. B, RNA accumulation, with strains shown at the top of each panel and probes shown at the bottom. Transcripts 1 and 2 accumulate to relatively low levels in wild-type (WT) cells, and are more easily visualized with the *cemA* probe. An *rbcL* probe was used as a loading control.

deletions in each of these constructs. Although *atpA* is essential for photosynthesis, *psbI* is dispensable (Kunstner et al., 1995). Therefore, we were able to obtain phototrophic transformants carrying the deletions in Δ1, Δ2, and Δ3 by selection on minimal medium after transformation of Δ*atpA*.

Figure 6B shows RNA-filter hybridization results with a series of probes from the *atpA* gene cluster and RNAs isolated from a wild-type strain and the deletion mutants. Using a probe from the *atpA*-coding region, two transcripts were detected for Δ1, and three each for Δ2 and Δ3. The two Δ1 transcripts are equivalent to RNAs 1 and 2 from the wild-type strain, although they are shorter as a result of the 355-nt deletion. Therefore, the Δ1 mutant appears to lack the *cis* elements necessary to generate the mono- and dicistronic RNAs terminating in this region (transcripts 3 and 4). The Δ2 and Δ3 mutants also accumulated deleted versions of RNAs 1 and 2, which are intermediate in size between those of Δ1 and wild-type cells. These results indicate that the formation of RNAs 1 and 2 does not require any sequences in the deleted regions, for example, for correct RNA folding. Furthermore, the accumulation of these transcripts is somewhat higher than in wild-type cells, consistent with the idea that when processing signals are present in the *psbI* region, RNAs 1 and 2 can serve as precursors for RNAs 3, 4, and others. However, the increase in RNAs 1 and 2 in Δ1 transformants is still less than would be expected if the normal levels of RNAs 3 and 4 were now present as longer transcripts. This indicates either that the deletions destabilize the longer RNAs or that regulatory mechanisms limit the accumulation of RNAs 1 and 2.

The *atpA* probe also identified major, shorter transcripts in the Δ2 and Δ3 transformants. The major transcript in Δ2 corresponds to the monocistronic *atpA* transcript, RNA 4, in wild-type cells. The accumulation of this transcript indicates that the site(s) of transcription termination and/or processing for the monocistronic *atpA* transcript is unaltered by the deletion downstream of *psbI*. The major *atpA* transcript in Δ3 also hybridized with a *psbI* coding-region probe (RNA 3a) and thus contains *atpA* and *psbI* 3′ sequences; we infer that it is a deleted version of transcript 3. Because this transcript terminates at the *psbI* 3′ processing site and no shorter transcript accumulates, we conclude that the processing site for monocistronic *atpA* lies within the region deleted in Δ3.

Figure 6B also shows results with three other probes, which confirm the identities of the transcripts described above. For example, a *psbI* probe detected nothing in Δ1 and Δ2, since these sequences had been deleted, but identified RNAs 1 and 2 in Δ3. Monocistronic *psbI* mRNA was seen only in the wild-type strain; we infer that Δ3 lacks sequences required for 5′-end formation of *psbI* mRNA. A *cemA* probe also identified full-length and internally deleted versions of RNAs 1 and 2. In addition, it hybridized with RNAs 5 and 6 from wild-type cells and internally deleted versions in Δ2. RNAs 5 and 6 did not accumulate in Δ1 or Δ3; we conclude that sequences required for transcription or 5′-end formation were deleted in these transformants. Finally, an *atpH*-specific probe detected transcripts 1, 5, and 8. As seen with the *cemA* probe, RNA 5 accumulated only in wild-type and Δ2 (RNA 5a) cells, presumably because signals for 5′-end formation have been deleted in the other strains. Accumulation of *atpH* RNA (RNA 8) was not dramatically affected, which was not surprising because the deletions lie far upstream of *atpH*.

The total content of *atpA*-containing transcripts in Δ2 and Δ3 was significantly reduced relative to those of wild-type cells when normalized to the control transcript *rbcL*. Based on phosphor imager quantification relative to *rbcL* in multiple experiments, these strains accumulated approximately 50 and 40% of the wild-type content, respectively, mostly as versions of RNA 3 or 4. In contrast, the amount of the longer RNAs 1 and 2 increased approximately 3-fold in Δ2 and 1.5-fold in Δ3 relative to wild-type RNAs 1 and 2. Moreover, the amounts of RNAs 5 and 6 increased approximately 4-fold in Δ2 relative to wild-type cells. In Δ1, *atpA* transcript accumulation was more severely affected; RNAs 1 and 2 represented approximately 15% of the total *atpA* transcripts accumulating in wild-type cells. These alterations in transcript accumulation reflect complex relationships between RNA processing and stability that may be influenced by transcript structure in the deletion strains, as well as by other, as yet undefined mechanisms.

Translation of the ATPase α-Subunit

The *atpA*-containing mRNAs of Δ1, Δ2, and Δ3 varied from wild type both in terms of the polycistronic distribution of the *atpA*-coding region and in the total steady-state level of *atpA* transcripts. We wondered whether these variations might affect the synthesis and/or accumulation of the ATP synthase α-subunit. These were assessed by pulse labeling with [^{14}C]acetate, and by immunoblotting, respectively. Figure 7A shows the results of an experiment in which wild-type, Δ1, Δ2, and Δ3 cells were pulse labeled for 5 min in the presence of cycloheximide to inhibit cytosolic translation. The rates of synthesis in both the α- and β-subunits were similar to those in wild-type cells, displaying the characteristic higher rate of synthesis for the α-subunit than for the β-subunit (Drapier et al., 1992). No differences were seen in protein accumulation as determined by immunoblot analysis (Fig. 7B). It is remarkable that Δ1, which accumulates only about 15% of the wild-type level of *atpA*-containing mRNAs, and only in the form of polycistronic transcripts, nevertheless displayed essentially wild-type rates of synthesis for the α-subunit.

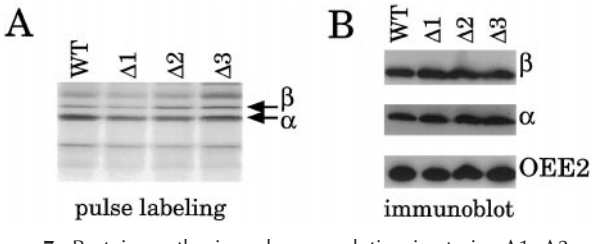

Figure 7. Protein synthesis and accumulation in strains Δ1, Δ2, and Δ3. The strains were pulse labeled for 5 min with [^{14}C]acetate (A) or analyzed by immunoblotting (B). Labeling is as for Figure 5.

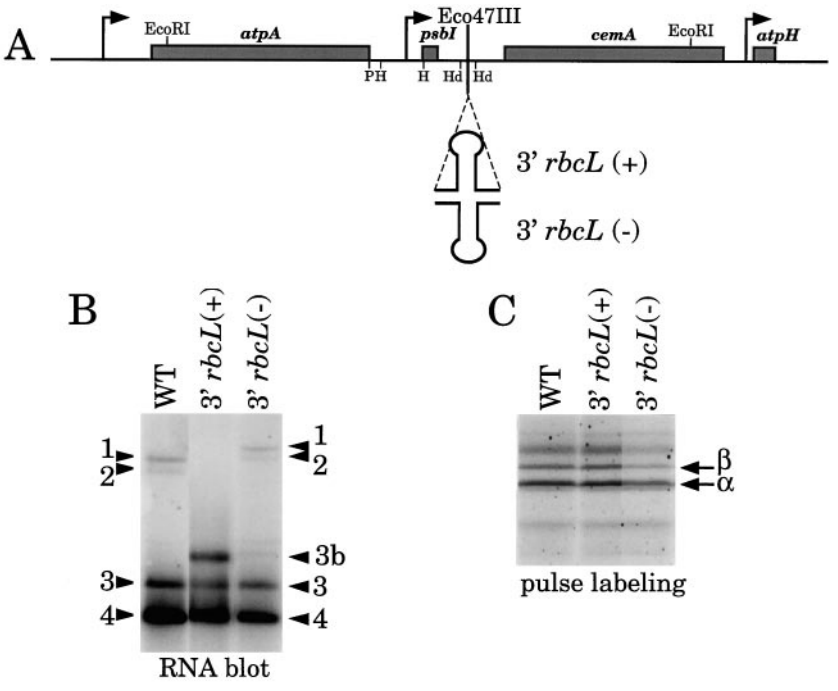

Figure 8. Analysis of transformants with an insertion of the *rbcL* 3′ UTR downstream of *psbI*. A, Map of the *atpA* gene cluster showing the site of the *rbcL* 3′ UTR insertion. B, RNA-filter hybridization analysis using an *atpA* coding-region probe. Transcripts are numbered as in Figure 1. C, Separation of proteins after 5 min of pulse labeling with [^{14}C]acetate. WT, Wild type.

Interruption of the Cluster by Insertion of the *rbcL* 3′ UTR

One possible interpretation of the results shown in Figure 7A, namely that α-subunit synthesis was unaffected by the deletions in Δ1, Δ2, and Δ3, is that it is translated only from the larger polycistronic mRNAs, transcripts 1 and 2, even in wild-type cells. To determine whether the α-subunit could be synthesized from mono- or dicistronic *atpA* mRNA, we constructed an *atpA* gene cluster that was modified to prevent accumulation of the tri- and tetracistronic *atpA* transcripts. To do this, the 3′ UTR of *rbcL* was inserted between the *psbI* and *cemA* genes, as shown in Figure 8A. In the sense (+) orientation, this sequence was suggested to act as a transcriptional attenuator, since RNA downstream of the stem loop did not accumulate in vivo (Blowers et al., 1993). However, because the *rbcL* 3′ UTR is not an efficient transcription terminator in vivo (Rott et al., 1996), we think it more likely that RNA processing and destabilization of the downstream sequences are responsible for the transcript accumulation patterns. The *rbcL* sequence was inserted in both the (+) and (−) orientations at an *Eco*47III site approximately 120 bp downstream of the mapped 3′ end of *psbI*.

The RNA-accumulation and protein-synthesis patterns of transformants homoplasmic for these modifications are shown in Figure 8, B and C, respectively. RNA-filter hybridization with an *atpA* probe revealed altered patterns of transcript accumulation relative to the wild-type strain for both 3′ *rbcL*(+) and 3′ *rbcL*(−). In the (+) strain, transcripts 1 and 2 were undetectable, and a new transcript (3b) accumulated. The size of transcript 3b is consistent with that expected for an RNA containing *atpA* and *psbI*, and terminating at the 3′ end of the *rbcL* insertion. For the (−) strain, the pattern was similar to that in the wild type. Transcripts 1 and 2 migrated more slowly because of the *rbcL* insertion, and two new transcripts with sizes consistent with 3′ ends near the antisense *rbcL* insertion accumulated to a low level. Other experiments have shown that the 3′ UTR of *rbcL* can act as an inefficient RNA 3′-end-formation element in this orientation (Rott et al., 1998).

To determine whether the lack of tri- and tetracistronic transcripts in the (+) transformants affected α-subunit synthesis, a pulse-labeling experiment was performed. As shown in Figure 8C, the α-subunit was synthesized at a rate equivalent to wild type, despite the fact that tri- and tetracistronic *atpA* transcripts did not accumulate to an appreciable level. We conclude that these longer RNAs are not obligate substrates for α-subunit synthesis.

DISCUSSION

Complex Gene Cluster in the *C. reinhardtii* Chloroplast Genome

We have described the organization and expression of a four-gene cluster encoding two components of the ATP synthase, a PSII polypeptide, and a putative envelope membrane protein involved in C uptake in chloroplasts. Although there are other closely spaced genes in *C. reinhardtii* chloroplasts, we define this set of four genes as a cluster based on the accumulation of abundant transcripts containing two, three, or four coding regions.

Although gene clusters are common in land-plant chloroplast genomes, there are only a handful of examples of genes that are known to be co-transcribed in *C. reinhardtii*. These include exon 2 of *psaA* with *psbD* (Choquet et al., 1988), *atpE* with the 3′ part of *rps7* (Robertson et al., 1990), *psbB* with *psbT* (Johnson and Schmidt, 1993; Summer et al., 1997), *psbF* with *psbL* (Mor et al., 1995), *rps9-ycf4-ycf3-rps18* (Boudreau et al., 1997), and *petA* with *petD*, as discussed in the introduc-

tion. Other possibly co-transcribed gene clusters in *C. reinhardtii* are *psbF-psbL-petG-ORF56* (Fong and Surzycki, 1992) and ribosomal protein genes related to the *E. coli* S10 and *spc* operons (Harris et al., 1994). More exhaustive analysis of transcript patterns in *C. reinhardtii* chloroplasts may reveal additional examples of co-transcribed gene clusters.

Transcription Initiation and RNA-Processing Events in the *atpA* Gene Cluster

The *atpA* gene cluster has a partially redundant transcriptional organization, with independent promoters proximal to *atpA*, *psbI*, and *atpH*. Although tri- and tetracistronic *atpH*-containing transcripts accumulate in wild-type cells, the predominant *atpH* transcript is monocistronic. It is difficult to assess the contribution of the *atpH* promoter to *atpH* transcription because monocistronic *atpH* mRNA might be produced either primarily by transcription from the *atpH* promoter or by transcription from the *atpA*- or *psbI*-proximal promoter followed by RNA processing. In fact, the *atpA*-proximal promoter could suffice for transcription of the entire gene cluster. This situation is slightly more complex than that of the *petA-petD* region, where the downstream *petD* monocistronic transcript is presumably generated by the same processing event whether transcription is initiated at the *petA* or *petD* promoter (Sakamoto et al., 1994; Sturm et al., 1994). A similar situation may exist for *psbB* and the downstream *psbH* gene; *psbH* appears to contain a promoter able to provide wild-type levels of RNA when cut off from the *psbB* promoter (Summer et al., 1997). Rapid RNA processing may partially explain the relatively simple patterns of transcript accumulation in *C. reinhardtii* chloroplasts despite the fact that co-transcription of gene clusters is more common than originally thought. However, it is clear that redundant promoters exist in a number of gene clusters, and may afford a selective advantage by allowing more finely tuned responses to changing environmental conditions.

Our model for transcription of the *atpA* gene cluster invokes three sites of transcription initiation, up to three sites of transcript 5′ processing, and four sites of transcription termination or 3′ processing. The 5′ termini of all transcripts could be formed directly by transcription initiation or, alternatively, by 5′ processing. We favor processing as the mechanism for 5′-end formation because no primary transcripts have been detected in *C. reinhardtii* chloroplasts by capping with α^{32}P-GTP and vaccinia virus guanylyltransferase, a method that works readily for chloroplast mRNAs of land plants (Sugita and Sugiura, 1996).

Sites of 3′-end formation were analyzed by transcript 3′-end mapping and deletion analysis. From data shown in Figure 4, we place the *atpA* 3′ end approximately 150 nt downstream of the UAA stop codon. There is a small, inverted repeat immediately upstream of this terminus (GcAUUUA. . . [7 nt]. . . UAAAUaC) that could form a weak stem-loop structure. In contrast, the 3′ end of *psbI* mRNA was mapped 75 nt downstream of the UAA stop codon, approximately 25 nt downstream of a strong, potential stem-loop structure (UAAUUUAGCUAAGAGAUUGUUAccuUAACAAUCUCUUAGCUAAAUUA). Such structures are known to stabilize discrete chloroplast transcripts in *C. reinhardtii* (Stern et al., 1991; Blowers et al., 1993; Lee et al., 1996). In comparing the relative accumulation of transcripts 3 and 4, there is clearly no correlation with the theoretical stability of the stem-loop and transcript accumulation. This phenomenon has been previously noted for vascular-plant chloroplast mRNAs tested in vitro (Stern and Gruissem, 1987) and is also consistent with the orientation dependence of 3′ inverted repeats in stabilizing chimeric chloroplast mRNAs in *C. reinhardtii* (Blowers et al., 1993; Rott et al., 1998). These observations suggest that RNA-binding proteins or other RNA structures play a pivotal role in determining transcript abundance.

The 3′ end of *cemA* mRNA mapped approximately 180 nt downstream of the UAA stop codon, immediately downstream of an inverted repeat (AACCAAAGAAUAUaAUAUUCU-UUGGUU). The *atpH* 5′ end also maps in this region, to the second G in the inverted repeat sequence. This raises the possibility that the *cemA* 3′ end and the *atpH* 5′ end are formed by a common RNA-processing event, but the imprecision of the 3′ mapping must be taken into account and, thus, the ends may overlap slightly and be in competition for the processing machinery. Because the *atpH* 3′ end was mapped to a region for which we do not have the nucleotide sequence, it cannot be determined if there are obvious secondary structures close to it. Whether these 3′ termini are formed by transcription termination or RNA processing is unknown, but based on data for spinach (Stern and Gruissem, 1987) and *C. reinhardtii* (Stern and Kindle, 1993; Rott et al., 1996) chloroplasts, termination is unlikely to occur at a significant rate.

Therefore, many of the mRNAs from the *atpA* gene cluster may be produced by processing larger transcripts. Although a precursor-product relationship cannot be directly proven, we found that deleting the *atpA* 3′-processing site (strain Δ3) or the *psbI* 3′-processing site (strain Δ2) resulted in an increased accumulation of the larger *atpA* polycistronic transcripts (Fig. 6). The effect was even more pronounced when both processing sites were deleted (strain Δ1). This is consistent with the suggestion that RNA 1 is the primary transcript from which all of the smaller *atpA* transcripts are generated, analogous to the mechanism by which the many *psbB* operon transcripts are formed in land plants (Westhoff and Herrmann, 1988). However, we cannot rule out the possibility that a transcription-termination signal was deleted in the deletion strains, thereby increasing the frequency of transcriptional read through.

Monocistronic mRNAs accumulate for all genes except *cemA*, which is present only as di- or polycistronic mRNAs 1, 2, 5, and 6. Although there is no direct evidence that *cemA* is expressed at the protein level in *C. reinhardtii*, it is a highly conserved gene, with 10 entries in the database, including crop plants, *Marchantia*, black pine, *Synechocystis* PCC 6803, and *Porphyra*. The putative protein in *C. reinhardtii* would necessarily be translated from a nonmonocistronic message, which would be somewhat unusual for this organism. However, it is possible that *cemA* is inefficiently translated, and the lack of 5′ processing serves as a regulatory mechanism. In *C. reinhardtii petD*, subunit IV translation from a dicistronic *petA-petD* message may be

inefficient or impossible (Sturm et al., 1994), and monocistronic *petD* seems to be the preferred form for translation in maize chloroplasts (Barkan et al., 1994). However, dicistronic mRNAs are the only mRNAs present for *psbF-psbL* (Mor et al., 1995), and monocistronic mRNAs could not be detected for *ycf3* or *ycf4*, which are known to be expressed at the protein level (Boudreau et al., 1997). Therefore, translation of downstream open reading frames in dicistronic transcripts is clearly possible in *C. reinhardtii* chloroplasts.

The *cemA* and *psbI* Genes Are Dispensable for Photosynthesis

The three deletions introduced downstream of *atpA* in strains Δ1, Δ2, and Δ3 inactivated *psbI* but did not abolish phototrophic growth of the transformants. This observation confirms a previous report (Kunstner et al., 1995) that *psbI* is dispensable for photosynthesis. This report also showed that *psbI* inactivation nevertheless caused a partial loss of PSII activity. In agreement with this result, we found that Δ1, Δ2, and Δ3 each had substantially altered fluorescence-induction patterns, and accumulated approximately 20% of the wild-type level of PSII proteins (data not shown).

The function of *cemA* is still under investigation. The *cemA* gene product was originally reported as a *c*hloroplast *e*nvelope *m*embrane protein of 34 kD (Sasaki et al., 1993). However, no protein of this size was detected after in vivo labeling of *C. reinhardtii* chloroplast envelope membranes with 35S (Clemetson et al., 1992). A later report suggested that the *cemA* protein could bind heme (Willey and Gray, 1990), whereas a *cemA* (*cotA*) mutant of *Synechocystis* PCC 6803 had defects in CO_2 transport (Katoh et al., 1996). C uptake was recently reported to be affected in *C. reinhardtii ycf10* (*cemA*) deletion mutants (Rolland et al., 1997), and the strains were found to be photosynthetically competent but high-light sensitive. We have constructed a *cemA*-deletion strain (ΔAH) that harbors a deletion from the *Hpa*I site immediately downstream of *atpA* to the *Eco*RI site in the carboxyl-terminal part of *cemA* (see Fig. 1). The ΔAH mutant was still capable of phototrophic growth (data not shown), in agreement with the results of Rolland et al. (1997) showing that *cemA* does not play an essential role in photosynthesis.

Changes in the Content of *atpA* Transcripts Do Not Affect α-Subunit Translation

In the strains described in the present study, the range of modifications in the pattern and number of *atpA* transcripts had no effect on the rate of α-subunit synthesis. The abundance of polycistronic messages beginning with *atpA* and having 3′ termini following *cemA* or *atpH* increased from 2- to 13-fold in Δ1, Δ2, and Δ3, whereas the deletion in Δ1 prevented monocistronic *atpA* mRNA accumulation altogether. In all cases, wild-type rates of α-subunit synthesis were observed. In contrast, the insertion in strain *rbcL*(+) (Fig. 8) caused a complete loss of tri- and tetracistronic *atpA* transcripts and, again, the rate of α-subunit synthesis was unaffected. Because the *atpA*-coding region lies the farthest upstream, these observations suggest that translation initiation at *atpA* is independent of the downstream coding regions.

The total amount of *atpA*-containing messages also varied among the wild-type strain and deletion mutants. The greatest effect was in Δ1, where only 15% of the wild-type amount remained, yet no effect on the rate of α-subunit synthesis was observed. The insensitivity of α-subunit synthesis rates to the number and type of *atpA* transcripts in *C. reinhardtii* has been noted previously. In the nuclear mutant *ncc1*, in which *atpA* transcript abundance declined by a factor of 10, there was only a moderate effect on the rate of α-subunit synthesis (Drapier et al., 1992). In addition, when *C. reinhardtii* cells were grown for 48 h in the presence of 5-fluorodeoxyuridine, an inhibitor of ctDNA replication, both the *atpA* gene-copy number and transcript level decreased by a factor of approximately 10, but the rate of α-subunit synthesis was unaffected (Hosler et al., 1989). Therefore, translation of the α-subunit is limited by some factor other than the availability of *atpA* transcripts. It follows that there is a large excess of *atpA* transcripts in wild-type cells, which are stable in spite of their translational inactivity. This is consistent with the 3-fold increase in the steady-state level in *atpA* transcripts reported for the F54 mutant, which is blocked at the level of α-subunit synthesis (Drapier et al., 1992). Together, these results would argue that in the case of *atpA*, ribosome association does not protect the transcript from degradation, but, instead, that their degradation may be a co-translational process. However, whether the translation-initiation complex has any role in RNA stability remains to be determined.

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