

# Processing of the *psbA* 5' Untranslated Region in *Chlamydomonas reinhardtii* Depends upon Factors Mediating Ribosome Association

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**Abstract.** The 5' untranslated region of the chloroplast *psbA* mRNA, encoding the D1 protein, is processed in *Chlamydomonas reinhardtii*. Processing occurs just upstream of a consensus Shine-Dalgarno sequence and results in the removal of 54 nucleotides from the 5' terminus, including a stem-loop element identified previously as an important structure for D1 expression. Examination of this processing event in *C. reinhardtii* strains containing mutations within the chloroplast or nuclear genomes that block *psbA* translation reveals a correlation between processing and ribosome association. Mutations within the 5' untranslated region of the *psbA* mRNA that disrupt the Shine-Dalgarno sequence, acting as a ribosome binding site, preclude

translation and prevent mRNA processing. Similarly, nuclear mutations that specifically affect synthesis of the D1 protein specifically affect processing of the *psbA* mRNA. In vitro, loss of the stem-loop element does not prohibit the binding of a message-specific protein complex required for translational activation of *psbA* upon illumination. These results are consistent with a hierarchical maturation pathway for chloroplast messages, mediated by nuclear-encoded factors, that integrates mRNA processing, message stability, ribosome association, and translation.

**Key words:** translation • mRNA processing • chloroplast • ribosome binding sequence • *psbA*

PHOTOSYSTEM II is comprised of proteins encoded within both the nuclear and chloroplast genomes. The expression of these proteins is regulated in response to light at the level of transcription, RNA processing, message stability, translation, and protein turnover (reviewed in Gillham et al., 1994; Mayfield et al., 1995; Rochaix, 1996). For many chloroplast-encoded proteins, translation appears to be the rate-limiting step of gene expression. Chloroplast mRNA levels remain relatively unchanged throughout dark/light transitions whereas protein synthesis rates increase dramatically upon illumination (Fromm et al., 1985; Deng and Gruissem, 1988; Malnoë et al., 1988). Genetic and biochemical evidence suggests that nuclear-encoded factors mediate this light-regulated translation of chloroplast-encoded proteins (Jensen et al., 1986; Kuchka et al., 1988; Rochaix et al., 1989; Girard-Bascou et al., 1992; Wu and Kuchka, 1995; Yohn et al., 1996; Zerges et al., 1997; Yohn et al., 1998b). These nuclear factors typically interact with specific elements within the 5'

untranslated regions (UTRs)<sup>1</sup> of the chloroplast mRNAs (Rochaix et al., 1989; Sakamoto et al., 1994a; Zerges and Rochaix, 1994; Stampacchia et al., 1997; Zerges et al., 1997).

The D1 protein, a core component of Photosystem II, is encoded by the chloroplast *psbA* gene. Throughout the light phase of photosynthesis, the D1 protein is subject to photodamage and is rapidly turned over (reviewed in Barber and Andersson, 1992). D1 inactivation is compensated by a 50–100-fold increase in the rate of D1 protein synthesis in response to light without a corresponding increase in *psbA* mRNA levels (Fromm et al., 1985; Klein et al., 1988; Malnoë et al., 1988; Krupinska and Apel, 1989). The use of reporter gene constructs in tobacco has demonstrated that the *psbA* 5' UTR is sufficient to confer light-dependent translational regulation in vivo (Staub and Maliga, 1994). An in vitro translation system derived from tobacco chloroplasts has identified critical regulatory elements for D1 synthesis in the *psbA* 5' UTR including potential ribosome

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1. *Abbreviations used in this paper:* cPABP, chloroplast poly(A)-binding protein; nt, nucleotide; RAC, RNA affinity chromatography; RB, RNA-binding; RBS, ribosome binding sequence; SD, Shine-Dalgarno; UTR, untranslated region.

binding sequences (RBS), an AU-box, and to a lesser extent, an upstream stem-loop element (Hirose and Sugiura, 1996). A stem-loop element has also been mapped within the 5' UTR of the spinach *psbA* mRNA (Klaff and Gruissem, 1995; Klaff et al., 1997). This element encompasses a putative RBS, an endonucleolytic cleavage site for mRNA decay (Klaff, 1995), and sequences recognized by stromal proteins (Klaff et al., 1997; Alexander et al., 1998).

In the unicellular green algae *Chlamydomonas reinhardtii*, similar elements within the 5' UTR have been identified as important for *psbA* expression. Among these elements is a stem-loop structure immediately upstream of a consensus Shine-Dalgarno (SD) sequence. Mutational analysis of the stem-loop region has shown that this element serves a role in *psbA* expression, although the exact nature of this role remains unresolved (Mayfield et al., 1994). Deletion of the SD sequence prevents ribosome association with the *psbA* mRNA and synthesis of the D1 protein, consistent with its proposed function as an RBS (Mayfield et al., 1994).

A complex of proteins, thought to serve as light-dependent translational activators, specifically recognizes the *C. reinhardtii psbA* 5' UTR (Danon and Mayfield, 1991). The binding activity of this complex is modulated in response to changes in photosynthetic activity via a redox switch (Danon and Mayfield, 1994; Kim and Mayfield, 1998). The principal RNA-binding (RB) protein in this complex is a chloroplast-localized poly(A)-binding protein (cPABP) homologue (Yohn et al., 1998a). Several nuclear mutants have been isolated in which the loss of the cPABP is accompanied by the absence of D1 protein synthesis due to a block in *psbA* mRNA association with polyribosomes (Yohn et al., 1996; Yohn et al., 1998b). A previous study suggested that this complex binds the stem-loop element upstream of the SD sequence (Danon and Mayfield, 1991). A model has been proposed (summarized in Mayfield et al., 1995) in which the stem-loop element acts as a translational attenuator in the dark, preventing ribosome association with the SD sequence. Upon illumination, and subsequent increase in photosynthetic activity, binding activity of the protein complex increases and stimulates translation initiation by disrupting the repressive stem-loop element or by providing a platform for ribosome association.

However, examination of *psbA* transcripts from *C. reinhardtii* indicated that in vivo the majority of this message lacks sequences upstream of the SD sequence including the stem-loop element (Erickson et al., 1984; Nickelsen et al., 1994; Shapira et al., 1997). In this study, we investigate the differential accumulation of *psbA* mRNAs containing different 5' termini. These different 5' UTRs likely result from the processing of the 90-nucleotide (nt) 5' UTR to generate a new 5' terminus 36 nt upstream of the initiation codon. Processing of the *psbA* 5' UTR is shown to be closely correlated with ribosome association. In the absence of a competent and accessible SD sequence, D1 protein is not synthesized and the *psbA* 5' UTR is not processed. Nuclear mutations that block D1 translation, in conjunction with reduced association of *psbA* mRNA with ribosomes, also reduce *psbA* processing. However, removal of the stem-loop element as a consequence of processing does not prohibit the binding of the nuclear-encoded protein complex to this *psbA* 5' UTR in vitro, nor does it

preclude dynamic light-dependent translational regulation mediated by the *psbA* RB complex. Based on these observations, we propose a model for *psbA* mRNA maturation in which 5' end formation does not serve as a prerequisite for initiation complex formation but rather processing of the 5' UTR occurs in conjunction with the early stages of ribosome assembly at the RBS.

## Materials and Methods

### Cell Growth Conditions

Unless otherwise noted, all *C. reinhardtii* strains were grown at 25°C under constant light in complete media (Tris-acetate-phosphate; Harris, 1989) to a density of  $\sim 10^6$  cells/ml. Cells were harvested by centrifugation at 4°C for 5 min at 4,000 g, washed with H<sub>2</sub>O, and pelleted again at 4,000 g for 5 min at 4°C. Cell pellets were frozen in liquid N<sub>2</sub> and stored at -70°C.

### RNA Isolation

Total and polyribosome-associated RNAs were prepared according to published protocols (Barkan, 1988; Cohen et al., 1998). To prepare membrane-associated RNA, the ground frozen cell pellet was twice extracted with extraction buffer lacking the detergents Triton X-100 and polyoxyethylene-10-tridecyl-ether to remove soluble RNA. The insoluble pellet was then resuspended in extraction buffer containing these detergents and used to prepare polyribosome-associated RNA.

### Primer Extension Analysis

Approximately 1 pmol of 5'-radiolabeled DNA oligonucleotide complementary to positions +47 to +32 of the *psbA* coding region (5'-CGAGC-CATAGGCTAG-3') or positions +66 to +49 of the *psbD* coding region (5'-AAGCCAGTCATCAGCGTC-3') was annealed to 5–25 µg RNA in a 7-µl mixture containing 50 mM Tris (pH 8.3), 60 mM NaCl, and 10 mM DTT by slow cooling from 80°C to room temperature. The reaction mixture was incubated at 42°C for 30 min after addition of 8 µl reaction mixture (50 mM Tris, pH 8.3, 60 mM NaCl, 10 mM DTT, 15 mM MgCl<sub>2</sub>, 1.25 mM each dNTP, and 0.2 U AMV reverse transcriptase; Life Sciences Inc., St. Petersburg, FL). The RNA was degraded by addition of 20 µl degradation buffer (50 mM Tris, pH 7.5, 0.1% SDS, and 7.5 mM EDTA, pH 8.0) and 3.5 µl 3 M KOH followed by incubation at 90°C for 3 min then 42°C for 25 min. The sample was neutralized upon addition of 6 µl acetic acid and 10 µl 3 M NaOAc (pH 5.3). EtOH precipitated, and resuspended in gel loading buffer. Products were separated by electrophoresis in a 7.5% polyacrylamide/8 M urea gel and quantified by PhosphorImager analysis. Samples were loaded to achieve approximately equal signals and do not reflect the relative amounts of *psbA* mRNA in each strain of *C. reinhardtii*.

### Analysis of *psbA* 5' UTR Length by Northern Analysis after RNase H Cleavage

100 pmol of a DNA oligonucleotide complementary to positions +171 to +156 of the *psbA* coding region (5'-TGGCGGAGCAGCGATG-3') was annealed to 5 µg wild-type total RNA or 15 µg hf261 total RNA in a 3.5-µl mixture containing 0.5 µl buffer (200 mM Tris, pH 7.5, 200 mM KCl, 1 mM EDTA, pH 8.0, and 1 mM DTT) and 0.1 U PRIME RNase inhibitor (5 Prime → 3 Prime, Inc., Boulder, CO) by slow cooling from 80°C to room temperature. RNA cleavage was initiated upon addition of 0.5 µl 100 mM MgCl<sub>2</sub>, 0.5 µl 0.2 U/µl PRIME RNase inhibitor, and 0.5 µl RNase H (2.2 U/µl; GIBCO BRL, Gaithersburg, MD). Reactions were incubated at 37°C for 30 min. Products were separated by electrophoresis in a 5% polyacrylamide/8 M urea gel. RNA blotting and hybridization with a random-primed, [<sup>32</sup>P]-labeled *psbA* 5' cDNA fragment (HindIII-ClaI) were performed as described previously (Mayfield et al., 1994).

### Gel Mobility Shift Assay

Approximately 1 µg total protein from *C. reinhardtii*, partially purified by heparin-agarose chromatography according to Cohen et al. (1998), was incubated for 10 min at room temperature with 0.5 U PRIME RNase inhibitor in a total volume of 8 µl dialysis buffer (20 mM Tris-HCl, pH 7.5, 100

mM KOAc, 0.2 mM EDTA, pH 8.0, 2 mM DTT, 20% glycerol) supplemented with 4 mM MgCl<sub>2</sub>. To each sample, 0.08 pmol of in vitro transcribed [<sup>32</sup>P]-labeled RNA (beginning with two G residues added 90 bases [-90] or 36 bases [-36] upstream of the start codon and ending 171 bases downstream of the start codon), 20 μg of wheat germ tRNA (Sigma), and 3 μg of FuD7 (a *C. reinhardtii* strain lacking the *psbA* gene) total RNA were added and the reaction was incubated at room temperature for 10 min. Where indicated, 8 pmol unlabeled in vitro transcribed RNA was also added as a competitor. RNA-protein complexes were separated in a 5% nondenaturing polyacrylamide gel.

### UV Cross-linking Assays

UV cross-linking assays were performed as gel mobility shift assays with the following alterations. Approximately 5 μg heparin-agarose purified protein was incubated with 1.0 pmol of internally labeled RNA (-90 or -36) generated by in vitro transcription using both [<sup>α-32</sup>P]ATP and [<sup>α-32</sup>P]UTP. After complex formation, the reactions were irradiated with short-wave UV light for 1 h at 4°C. The RNA was digested at 55°C for 45 min after addition of urea, EDTA (pH 8), and RNase A to final concentrations of 3 M, 3.75 mM, and 0.5 mg/ml, respectively. Labeled proteins were separated by SDS-PAGE and visualized using a PhosphorImager.

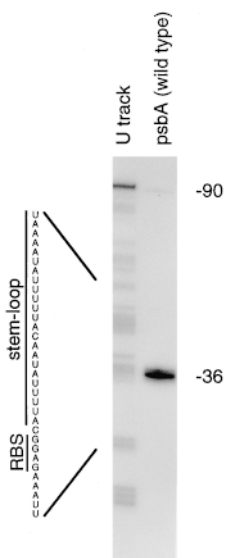
### RNA Affinity Chromatography (RAC) and Immunoblot Analysis

RAC was performed as described by Cohen et al. (1998). In brief, 125 μg of in vitro transcribed RNA (-90 or -36) was coupled to 100 μl amino gel 1702 (Sterogene Bioseparations Inc., Carlsbad, CA). 1 ml heparin-agarose purified protein was passed over the resulting columns, washed, and recovered by elution with a high salt buffer. Equal quantities of RAC proteins were mixed with 2× sample buffer (5% SDS, 5% β-mercaptoethanol, 400 mM Tris, pH 6.8, 10% sucrose), heated to 65°C for 5 min, and separated by SDS-PAGE. The gels were either stained with Coomassie blue or electroblotted to nitrocellulose (Schleicher and Schuell, Keene, NH) in 10 mM CAPS (pH 11)/10% methanol (Mayfield et al., 1994). Filters were treated with rabbit polyclonal antisera specific for RB38, RB47, or RB60.

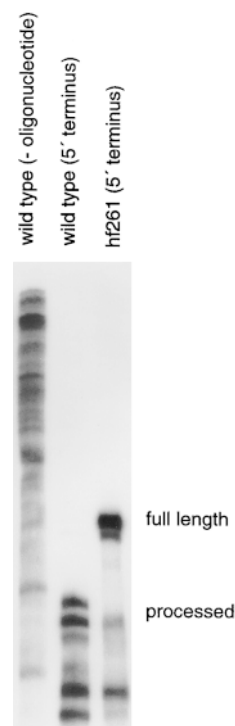
## Results

### Multiple *psbA* 5' UTR Exist In Vivo

S1 mapping of the *psbA* mRNA from *C. reinhardtii* previously identified two 5' termini, one 90 nt upstream of the start codon and a more pronounced terminus 36 nt from the start codon (Erickson et al., 1984). Multiple 5' termini



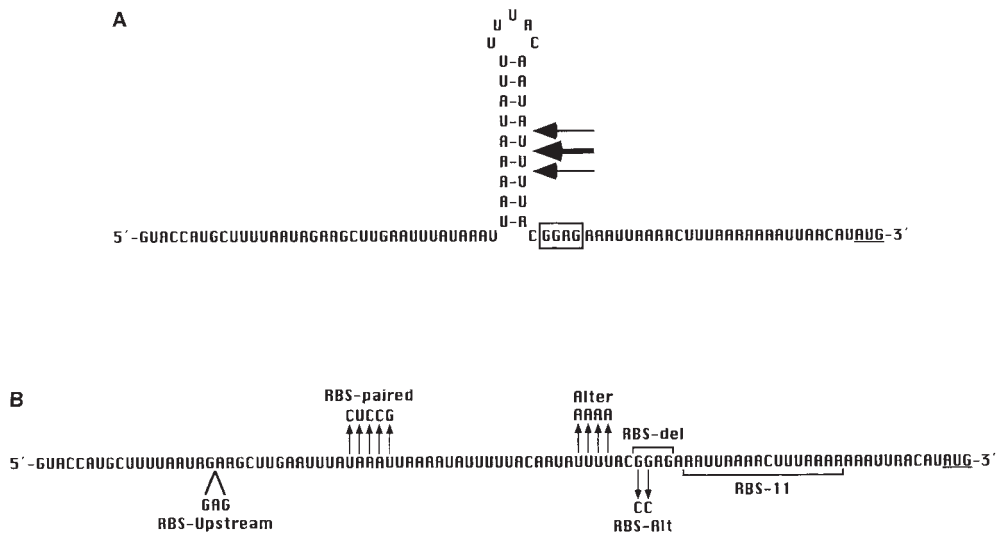
**Figure 1.** Primer extension analysis of the 5' UTR of *psbA* mRNA in *C. reinhardtii*. Two principal extension products are observed for the *psbA* 5' UTR, one terminating 36 nt upstream of the start codon (-36) and another terminating 90 nt upstream of the start codon (-90). Primer extension of *psbA* from hf261, a nuclear mutant in which only the 90-nt 5' UTR of the *psbA* mRNA is observed, was carried out in the presence of ddATP (*U track*) to identify the exact cleavage site.



**Figure 2.** Northern analysis of the 5' portion of *psbA* mRNA. Total RNA from the wild type or hf261 was incubated with an oligonucleotide complementary to the 5' coding region of *psbA*. RNase H cleavage of *psbA* mRNA at the RNA-DNA duplex allows for 5' termini of differing lengths to be resolved by PAGE and visualized using a radiolabeled 5' *psbA* probe. No oligonucleotide was added to total wild-type RNA in the first lane. The two predominant 5' termini are expected to correspond to the processed (36 nt) or full-length (90 nt) *psbA* 5' UTRs.

in this assay may reflect multiple transcription start sites, posttranscriptional cleavage events, or RNA modifications or structural elements which result in poor hybridization of the radiolabeled probe. We used primer extension analysis to examine the 5' termini of the *psbA* 5' UTR. A radiolabeled oligonucleotide complementary to the 5' coding region of *psbA* was annealed with total RNA prepared from wild-type *C. reinhardtii*. Reverse transcriptase was used to extend the probe to the 5' terminus of the message. Extension products were separated by PAGE and quantified by PhosphorImager analysis. As shown in Fig. 1, 95% of the *psbA* message contains a 5' terminus 36 nt upstream of the start codon whereas the remaining *psbA* mRNAs feature 5' UTRs of 90 nt in length. Similar results were observed previously by primer extension analysis (Shapira et al., 1997).

The two predominant extension products could reflect different 5' termini or may indicate partial blocks in primer extension resulting from strong structural elements within the 5' UTR. To resolve this issue we used RNase H to cleave the *psbA* mRNA at the site of hybridization to a complementary oligonucleotide to generate 5' *psbA* fragments of a size that could be resolved by PAGE and subsequently visualized by Northern analysis. The size of the wild-type *psbA* 5' UTR can be compared with the 5' UTR from the hf261 strain, a nuclear mutant in which only the 90-nt *psbA* 5' UTR was observed by primer extension analysis (see Fig. 6). As shown in Fig. 2, the 5' fragment of the *psbA* message from hf261 is longer than the predominant wild-type *psbA* 5' fragment, confirming that the primer extension products reflect 5' UTRs of differing size. Therefore, the vast majority of wild-type *psbA* transcripts from *C. reinhardtii* begin 36 nt upstream of the start codon, adjacent to the RBS (Fig. 3 A).



**Figure 3.** (A) Sequence of the *psbA* 5' UTR. The locations of the stem-loop element, start codon (*underlined*), SD-like RBS (*boxed*), and cleavage site (with arrows reflecting the relative intensity of primer extension products) are indicated. (B) Primary structure map of the *psbA* 5' UTR showing site-directed mutations. Alterations to primary sequence are indicated by arrows, deletions are delineated by brackets, and an insertion is indicated by a wedge. The start codon is underlined.

Neither primer extension analysis nor Northern analysis can distinguish between 5' termini generated by transcription initiation and termini generated by posttranscriptional mRNA processing. However, the data will show that the appearance of the 36-nt 5' UTR is most closely associated with posttranscriptional events, suggesting that this terminus most likely results from mRNA processing. This conclusion is further supported by the observation that partial deletion of a putative promoter element required for transcription initiation of the of the  $-36$  terminus (Erickson et al., 1984) had no affect on the appearance of the 36-nt 5' UTR (Loop-del [Mayfield et al., 1994]; data not shown).

***Processing Is Independent of the Primary mRNA Sequence at the Cleavage Site***

To identify RNA elements required for processing, site-specific mutations were engineered within the 5' UTR of the *psbA* gene which was then reintroduced into the *C. reinhardtii* chloroplast genome by particle bombardment (Mayfield et al., 1994). One of these constructs, Alter, replaced the four uridine nucleotides just upstream of the RBS with adenines (Fig. 3 B). In addition to disrupting the stem-loop element, this mutation changed the primary sequence at the processing site. This mutation results in a 95% reduction in D1 protein synthesis and an 80% reduction in D1 protein accumulation (Mayfield et al., 1994). However, primer extension analysis of total RNA isolated from this strain demonstrates that cleavage of the *psbA* 5' UTR is identical in extent and location to processing in wild-type *C. reinhardtii* (Fig. 4), indicating that processing is not a function of the primary RNA sequence at the cleavage site.

***Processing of the psbA 5' UTR Is Dependent upon the Presence of a Competent and Accessible RBS***

Many chloroplast-encoded mRNAs have a sequence resembling a prokaryotic-like SD that serves as an RBS. However, because these sequences tend to vary in size and location relative to their *Escherichia coli* counterparts, the

relevance of these RBSs in chloroplasts has been questioned (Fargo et al., 1998). Deletion of a putative SD sequence in the 5' UTR of the *C. reinhardtii psbA* mRNA (RBS-del) eliminated *psbA* mRNA polyribosome association (Yohn et al., 1996) and D1 synthesis in vivo (Mayfield et al., 1994), suggesting that this sequence is a functional RBS. Additional mutations designed to disrupt the RBS have been introduced into the *psbA* 5' UTR (Fig. 3 B). These mutations either eliminated the RBS (RBS-del, RBS-Alt), sequestered the RBS in an extended stem-loop structure (RBS-paired), or changed the location of the RBS by replacing the wild-type RBS with another RBS placed upstream of the stem-loop element (RBS-upstream). All of these changes block ribosome association and abolish D1 protein synthesis in vivo, providing further evidence that this SD sequence functions as an authentic RBS (Mayfield et al., 1994; Bruick, R.K., and S.P. Mayfield, unpublished results). Total RNA was prepared from



**Figure 4.** Primer extension analysis of Alter. Primer extension analysis of *psbA* mRNA was performed on total RNA from a *psbA*-deficient *C. reinhardtii* strain transformed with D1 cDNA or Alter, a D1 cDNA in which the uridine residues at the cleavage site have been replaced by adenine. The slight differences in the size of the extension products result from curving of the lanes during PAGE.

each of these mutants and primer extension analysis was used to characterize the processing of the *psbA* 5' UTR. As shown in Fig. 5, each of these mutants possess only one 5' terminus corresponding to the unprocessed *psbA* 5' UTR. These data show that a functional RBS, required for ribosome association and *psbA* translation, is also required for normal processing of the 5' UTR.

Though processing of the *psbA* 5' UTR requires a SD sequence, and presumably initial ribosome association, processing is not dependent upon D1 protein synthesis. For example, a deletion within the *psbA* 5' UTR that positions the RBS 11 nt upstream of the start codon (RBS-11, Fig. 3 B) reduces *psbA* association with large polyribosomes and eliminates D1 protein synthesis in *C. reinhardtii* while enhancing translation of the same message in *E. coli* (Bruick, R.K., and S.P. Mayfield, manuscript in preparation). As demonstrated by a primer extension assay, the *psbA* 5' UTR from this mutant is still cleaved to the same extent and in the same position relative to the RBS as in the wild type (Fig. 5). The exact location of the processing site was confirmed by RNA sequencing (data not shown). Thus, we imagine that the RBS in this construct remains accessible for recognition by factors involved in the early assembly of a translation initiation complex before processing but that this mutant RBS lacks the ability to specify the correct initiation codon.

### Processing of the *psbA* 5' UTR Is Reduced in Nuclear Variants Deficient in D1 Expression

Several nuclear mutations have been characterized in *C. reinhardtii* that specifically affect expression of the D1 protein (Yohn et al., 1996; Yohn et al., 1998b). In each of these mutants (F35, hf149, hf233, hf261, hf859, and hf1085), D1 protein synthesis is specifically lacking. While the primary defects of these mutations are not known, *psbA* association with polyribosomes is reduced or absent in the majority of these strains, suggesting that these mutations affect translation initiation (Yohn et al., 1996; Yohn et al., 1998b). Total RNA was prepared from each of these mu-

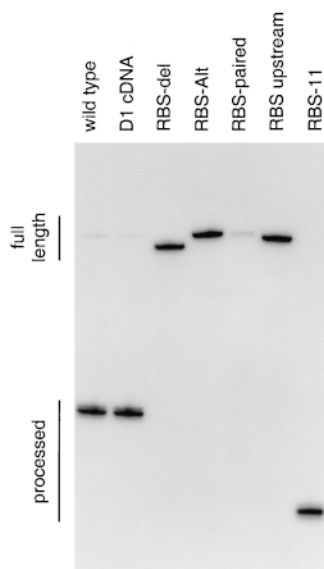


Figure 5. Primer extension analysis of total RNA isolated from *C. reinhardtii* strains containing mutations within the *psbA* 5' UTR. Differences in the size of the extension products are consistent with deletions or insertions shown in Fig. 4. Where observed, the processing site is always 4–6 nt upstream of the SD sequence.

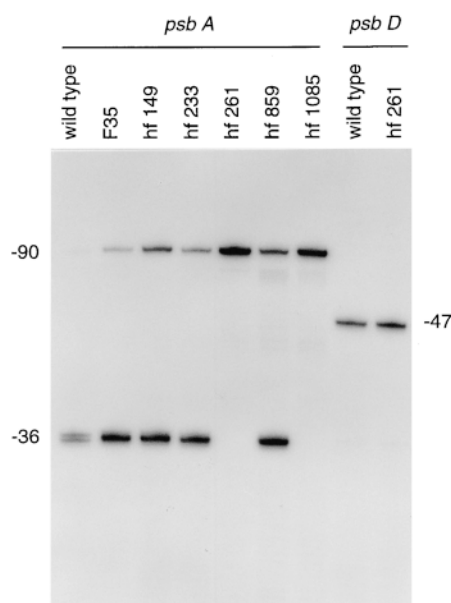


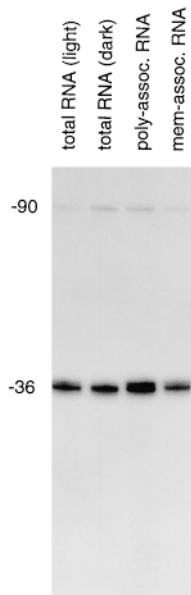
Figure 6. Primer extension analysis of total RNA isolated from *C. reinhardtii* strains containing mutations within the nuclear genome. Primer extension was performed with radiolabeled oligonucleotides specific for *psbA* or *psbD*. The principal extension product for *psbD* is consistent with a processed 5' terminus 47 nt upstream of the start codon.

tants and primer extension analysis of the *psbA* 5' UTR was performed (Fig. 6). Each of these nuclear mutations has a pronounced effect on the amount, but not the position, of processing of the *psbA* 5' UTR. In each case, the ratio of transcripts featuring a 90-nt 5' UTR (–90) to processed transcripts (–36) is increased compared with the wild type, with some mutants lacking any detectable *psbA* terminus 36 nt upstream of the initiation (hf261, hf1085).

Just as these nuclear mutants specifically affect translation of the *psbA* mRNA, the effect on mRNA processing also appears to be *psbA* specific. In addition to the *psbA* 5' UTR, the 5' UTR of the *psbD* mRNA (encoding the D2 protein) is also processed in *C. reinhardtii* (Rochaix et al., 1984). Primer extension analysis using a radiolabeled oligonucleotide complementary to the *psbD* 5' coding region was performed on total RNA prepared from the wild type and the hf261 strain (Fig. 6). Whereas processing of the *psbA* 5' UTR is completely absent in hf261, processing of the *psbD* 5' UTR remains unaffected in this mutant as does D2 protein synthesis (Yohn et al., 1998b).

### Processing of the *psbA* 5' UTR Is Not Dependent upon Illumination

To determine if the *psbA* 5' UTR is differentially processed in response to environmental conditions, total RNA was isolated from *C. reinhardtii* grown under continuous light or continuous darkness. Primer extension analysis indicated no difference in the relative amounts of the 5' termini of the *psbA* mRNA in response to growth under continuous light or in constant darkness (Fig. 7). A previous study reported no change in *psbA* processing throughout the 18 h after a shift from low light to high light growth



**Figure 7.** Primer extension analysis of *psbA* mRNA. Total RNA was prepared from wild-type *C. reinhardtii* grown under continuous light or in constant darkness. Total RNA from light-grown *C. reinhardtii* could be further separated into RNA associated with polyribosomes and polyribosomal RNA associated with membranes.

conditions (Shapira et al., 1997). These results suggest that cleavage of the *psbA* 5' UTR is not directly related to light-dependent translational regulation.

#### Processing of the *psbA* 5' UTR Is Not Dependent upon mRNA Association with Large Polyribosomes

During growth under constant light, only 35% of *psbA* mRNA is associated with large polyribosomes (Yohn et al., 1996). Polyribosome-associated RNA was isolated and processing of the *psbA* 5' was analyzed by primer extension analysis. The extension products of *psbA* mRNA associated with polyribosomes were indistinguishable from those observed in total RNA (Fig. 7). Because the D1 protein is inserted into the membrane during, or soon after, synthesis, we examined the subset of polyribosome-associated *psbA* mRNA associated with thylakoid membranes. If this subset of mRNAs comprises the pool of functionally relevant *psbA* message, we might observe a difference in the amount of processed *psbA* 5' UTR in this fraction as compared with total mRNA. Again, primer extension analysis indicated no difference in the relative amount of processed *psbA* 5' UTR between membrane-associated mRNA and other RNA populations (Fig. 7).

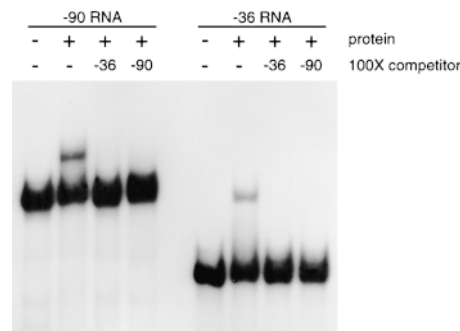
#### Removal of the Stem-Loop Element from the *psbA* 5' UTR Does Not Affect Binding of the Light-regulated Protein Complex In Vitro

The stem-loop element upstream of the RBS had been identified previously as a binding site for a protein complex involved in the dynamic regulation of *psbA* translation in response to light (Danon and Mayfield, 1991). However, as a consequence of processing, >95% of the steady-state levels of *psbA* message lacks this stem-loop element in vivo. To determine if the processed *psbA* 5' UTR was still capable of being recognized by the protein complex, a gel mobility shift experiment was used to compare complex binding to the two *psbA* 5' UTRs in vitro. Heparin-agarose purified proteins from wild-type *C. rein-*

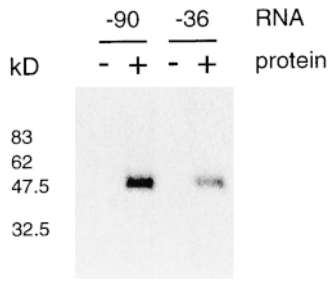
*hardtii* were incubated with in vitro transcribed RNAs corresponding to either the 90-nt *psbA* 5' UTR (–90) or the processed 5' UTR (–36). Complex binding is reflected by the appearance of an RNA–protein complex with reduced mobility in a nondenaturing polyacrylamide gel. As shown in Fig. 8, both RNAs are bound by protein. Furthermore, each RNA is able to compete the binding of the complex to the other RNA when added in excess as a cold competitor of the labeled RNA for the protein complex. The possibility of the –90 RNA being processed to the –36 RNA in vitro before complex formation is excluded by the observation that the migration of the –90 RNA–protein complex is retarded relative to the –36 RNA–protein complex.

Previous experiments have identified the primary *psbA* binding protein to be RB47, the cPABP (Danon and Mayfield, 1991; Yohn et al., 1998a). UV cross-linking of both radiolabeled transcripts (–90 or –36) complexed with heparin-agarose purified proteins resulted in the specific labeling of RB47 (Fig. 9). The lower intensity of RB47 labeling using the –36 transcript may be due to fewer radiolabeled residues in close proximity to the bound cPABP for the shorter RNA fragment or may reflect slightly lower binding affinity of the 36-nt 5' UTR compared with the 90-nt 5' UTR.

RAC was used to further characterize complex binding to the processed 5' UTR. RNA transcripts corresponding to the entire (–90) and processed (–36) *psbA* 5' UTRs were immobilized on solid supports. Heparin-agarose-purified *C. reinhardtii* lysate was passed over each support and bound proteins were eluted with high salt. An identical set of proteins was eluted from both columns as observed by Coomassie blue staining (Fig. 10 A). Western analysis with antibodies specific for the cPABP (RB47), the chloroplast-localized protein disulfide isomerase (RB60) (Kim and Mayfield, 1998), and a 38-kD component of the *psbA* binding complex (RB38) further demonstrated that both *psbA* 5' UTRs are recognized by an identical protein complex in vitro (Fig. 10 B). Taken together, these results predict that removal of the stem-loop element from the *psbA* 5' UTR does not prevent the binding of the *psbA*–



**Figure 8.** Gel mobility shift assaying the binding of the *psbA*-specific complex to the entire *psbA* 5' UTR (–90) and processed 5' UTR (–36). Radiolabeled in vitro transcribed RNA fragments corresponding to the two *psbA* 5' termini were incubated in the presence (+) or absence (–) of heparin-agarose purified protein and 100-fold unlabeled competitor RNA. RNA–protein complexes were separated by nondenaturing PAGE.



**Figure 9.** UV cross-linking of radiolabeled *psbA* RNA to cPABP. In vitro transcribed RNA fragments corresponding to the 90-nt *psbA* 5' UTR (-90) or cleaved *psbA* 5' UTR (-36) were incubated in the presence (+) or absence (-) of heparin-agarose purified protein followed by irradiation with short-wave

UV light and subsequent RNase A digestion. Radiolabeled proteins were separated by SDS-PAGE and visualized by Phosphor-Imager.

specific protein complex nor does it preclude the dynamic regulation of translation in response to light.

### Discussion

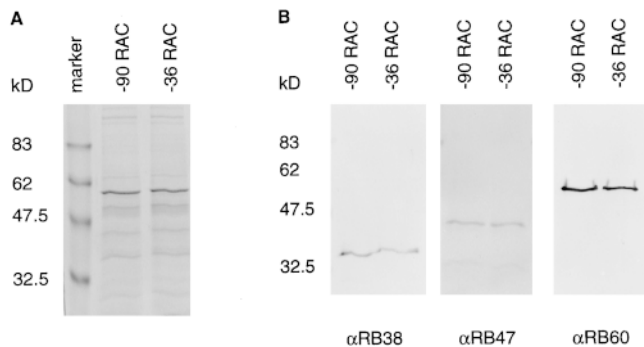
In both *C. reinhardtii* and higher plants, the expression of nuclear and chloroplast genes is tightly regulated to allow for a rapid response to fluctuations in environmental conditions. The regulation of chloroplast-encoded genes is dependent upon sequences within their 5' UTRs that influence message stability, translation, and possibly message localization (Gillham et al., 1994; Mayfield et al., 1995; Rochaix, 1996). Mutational analysis of the 5' UTR of the *psbA* mRNA has shown that RNA elements, including a consensus SD sequence and an upstream stem-loop element, mediate D1 expression (Mayfield et al., 1994). In this study, primer extension analysis of the *psbA* mRNA from a variety of *C. reinhardtii* mutants has revealed a correlation between processing of the 5' UTR and association of the mRNA with ribosomes. Alterations to the *psbA* 5' UTR that disrupt the RBS prevent RNA processing. Nuclear mutations that specifically block D1 protein synthesis, in part by reducing ribosome association, specifically reduce *psbA* mRNA processing. The proximity of the processing site to an accessible and competent RBS, and the influence on processing of nuclear-encoded factors that affect ribosome association, suggest a relationship between

ribosome association and processing of the 5' UTR of the *psbA* mRNA.

In contrast to *psbA* messages from *C. reinhardtii*, generally only one 5' terminus, corresponding to the unprocessed 5' UTR from *C. reinhardtii*, has been observed for the *psbA* mRNA isolated from higher plants (Zurawski et al., 1982; Link and Langridge, 1984; Sugita and Sugiura, 1984; Boyer and Mullet, 1986; Boyer and Mullet, 1988; Hanley-Bowdoin and Chua, 1988; Liere et al., 1995). Nevertheless, processing of mRNAs is a common phenomenon in higher plant chloroplasts, in which most messages are transcribed as polycistronic transcripts. For example, the *petD* mRNA, which is transcribed as a dicistronic message downstream of *petA*, contains elements within the 5' UTR that are required for accumulation of the monocistronic *petD* mRNA. Failure to process the *petD* 5' UTR results in the loss of translation of the *petD* message (Sakamoto et al., 1993; Sakamoto et al., 1994a,b).

Processing of the 5' UTR of the *rbcL* transcript, the chloroplast mRNA encoding for the large subunit of Rubisco, is also correlated with ribosome association and protein synthesis. In *C. reinhardtii*, for example, changes in large subunit synthesis in response to light levels coincide with changes in processing of the 5' UTR (Shapira et al., 1997). *rbcL* transcripts with longer 5' UTRs (beginning 168 nt upstream of the start codon rather than 93 nt) are not associated with polyribosomes and probably are not translationally competent. Recovery of large subunit synthesis is accompanied by an increase in the relative level of processed mRNAs. In mature barley leaves, inhibition of large subunit protein synthesis by methyl jasmonate is accompanied by a change in the predominant *rbcL* 5' UTR, resulting from alternative processing of the primary transcript. Once again, the longer 5' UTR is not associated with polyribosomes (Reinbothe et al., 1993). These observations further suggest that in both *C. reinhardtii* and higher plants, processing of 5' UTRs coincides with ribosome association of those mRNAs. Thus, although it is often assumed that 5' end formation occurs before, and is necessary for proper translation initiation, the available data also support the possibility that processing of mono- and polycistronic 5' UTRs may be a consequence of ribosome association, rather than a prerequisite for it.

Although processing of the *C. reinhardtii psbA* 5' UTR appears to be linked with translation initiation at the level of small ribosomal subunit recognition of the SD sequence association, no correlation was observed between processing and the dynamic regulation of translation in response to illumination. When *C. reinhardtii* is grown in the dark rather than the light, D1 protein synthesis is reduced in conjunction with reduced binding activity of the cPABP complex. Nevertheless, no difference in processing was observed between *psbA* mRNA isolated from *C. reinhardtii* grown in the light or in the dark, nor is there a direct linear relationship between the amounts of light-regulated translational activator proteins, such as the cPABP, and processing in the nuclear mutants. Therefore, processing is dependent upon at least the initial stages of ribosome assembly at the SD sequence, which occurs before, and independent of, dynamic light-dependent translation. However, some proteins may be common to both of these processes.



**Figure 10.** RAC purification of RB proteins specific for the 90-nt *psbA* 5' UTR (-90) and the processed *psbA* 5' UTR (-36). Proteins were separated by SDS-PAGE and stained with Coomassie blue (A) or transferred to an immunoblot and probed with anti-serum against RB38, RB47, or RB60 (B).

Previous studies have demonstrated that mutations introduced within the *psbA* 5' UTR upstream of the RBS can disrupt D1 expression. Typically, these mutants accumulate normal amounts of *psbA* mRNA but synthesize D1 protein at <5% of the wild-type level (Mayfield et al., 1994). Nevertheless, the *psbA* 5' UTR in each of these mutants is processed normally. Processing within these transcripts removes the mutated RNA upstream of the RBS and results in a steady-state pool of *psbA* transcripts that is virtually indistinguishable from the wild type. Therefore, sequences upstream of the RBS must influence D1 expression before processing and ribosome association, possibly by acting on steps such as formation of a preinitiation complex or perhaps even in localization of the *psbA* mRNA to specific regions of the chloroplast (Rochaix, 1996). The D1 protein must be synthesized in close proximity to the thylakoid membrane, and translational activators, including the cPABP, have been shown to be associated with a specific membrane fraction within the chloroplast (Zerges and Rochaix, 1998). Perhaps these upstream sequences act in concert with translational activator proteins to target the *psbA* mRNA to membrane-associated ribosomes.

Sequences upstream of the processing site could also influence chloroplast gene expression at the level of message stability, acting before the early stages of ribosome association. For example, the 5' UTR of the chloroplast *psbD* mRNA from *C. reinhardtii* is processed in a manner similar to that of the *psbA* mRNA (Rochaix et al., 1984; Erickson et al., 1986; Nickelsen et al., 1994). Analysis of nuclear mutants that destabilize the *psbD* mRNA has indicated that the sequences upstream of the processing site in the *psbD* 5' UTR may mediate this mRNA stability by interacting with nuclear encoded factors (Nickelsen et al., 1994). Furthermore, a deletion engineered within the *psbD* 5' UTR designed to initiate *psbD* transcription at the processing site resulted in a lack of *psbD* mRNA accumulation despite a wild-type rate of transcription of the mRNA (Rochaix, 1996). An analogous chloroplast deletion engineered to initiate *psbA* transcription at the processing site also resulted in failure to accumulate any *psbA* mRNA (data not shown). These data show that sequences upstream of the RBS can influence message stability, likely acting during the early stages of mRNA maturation. Interestingly, an endonucleolytic cleavage site associated with mRNA degradation has been mapped within the spinach *psbA* 5' UTR in vitro. Similarities exist between this endonucleolytic cleavage site and the processing site within the *C. reinhardtii psbA* 5' UTR. Both cleavage sites are located immediately upstream of a consensus RBS. In spinach, this site also defines the upstream boundary of the core RNA element recognized by an RB complex that includes the ribosomal protein S1 (Klaff, 1995; Alexander et al., 1998). The close proximity of this cleavage site in spinach *psbA* mRNA to the putative RBS and the site of ribosome assembly may reflect an intimate relationship between translation initiation, mRNA processing, and message stability. This relationship is also observed for *psbA* mutants in *C. reinhardtii* (RBS-del, RBS-Alt, RBS-paired) in which the loss of ribosome binding due to the absence of a SD sequence results in the absence of processing and a reduction in *psbA* mRNA accumulation (Mayfield et al., 1994).

Originally, mutations to the upstream stem-loop element of the *psbA* mRNA were thought to influence D1 translation by altering the binding site of the light-regulated cPABP complex as the stem-loop element was found to be protected by protein binding in a T1-gel mobility shift assay (Danon and Mayfield, 1991; Mayfield et al., 1994). However, gel shift assays, UV cross-linking, and RAC assays presented here all indicate that both the 90-nt and processed *psbA* 5' UTRs are recognized by an identical set of *psbA*-specific RB proteins. Thus, dynamic light-regulated translation via binding of these activator proteins should still be possible with the truncated *psbA* mRNA.

Based on these and other results, we propose a model for *psbA* maturation and translation in the *C. reinhardtii* chloroplast. The *psbA* mRNA is transcribed with a 5' UTR extending at least 90 nt upstream of the start codon. The 5' UTR is rapidly processed to the -36 transcript but not before the upstream RNA elements influence D1 expression. These upstream elements could affect expression at a number of key steps including RNA stability, formation of a preinitiation complex, or mRNA localization. Processing is mediated by proteins, including ribosomal subunits and possibly other initiation factors, that recognize the RBS during the early stages of ribosome association. The nuclease responsible for processing has not yet been identified but appears to be closely associated with a ribosomal subunit or with a protein complex required for ribosome association. Interaction of the ribosomal subunits and associated proteins with the processed 5' UTR may act to stabilize the message. Processing may also initiate an mRNA degradation pathway affecting the half-life of the transcripts. Whereas removal of the upstream sequences may be necessary to make the message competent for high levels of translation, processing alone is not sufficient for translation of the message as evidenced by mutations, both chloroplast and nuclear, that affect *psbA* translation without eliminating processing. Upon illumination, the binding activity of a set of *psbA*-specific RB proteins, including the cPABP, is enhanced in response to increased photosynthetic activity. Binding of this protein complex stimulates translational activity, probably acting via recognition of the *psbA* 5' UTR through a stretch of adenine residues between the RBS and the start codon (Bruick, R.K., and S.P. Mayfield, manuscript in preparation). While the molecular basis underlying each of these events remains unknown, we have begun to define distinct RNA elements within the *psbA* 5' UTR that are recognized by *trans*-acting factors to orchestrate message stability, mRNA processing, general translational competency, and dynamic translational activation.

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