Translation of the *psbA* mRNA of *Chlamydomonas reinhardtii* Requires a Structured RNA Element Contained within the 5' Untranslated Region

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Abstract. Translational regulation is a key modulator of gene expression in chloroplasts of higher plants and algae. Genetic analysis has shown that translation of chloroplast mRNAs requires nuclear-encoded factors that interact with chloroplastic mRNAs in a message-specific manner. Using site-specific mutations of the chloroplastic *psbA* mRNA, we show that RNA elements contained within the 5' untranslated region of the mRNA are required for translation. One of these elements is a Shine-Dalgarno consensus sequence, which is necessary for ribosome association and *psbA* translation. A second element required for high levels of *psbA* translation is located adjacent to and upstream of the Shine-Dalgarno sequence, and maps to the location on the RNA previously identified as the site of message-specific protein binding. This second element appears to act as a translational attenuator that must be overcome to activate translation. Mutations that affect the secondary structure of these RNA elements greatly reduce the level of *psbA* translation, suggesting that secondary structure of these RNA elements plays a role in *psbA* translation. These data suggest a mechanism for translational activation of the chloroplast *psbA* mRNA in which an RNA element containing the ribosome-binding site is bound by message-specific RNA binding proteins allowing for increased ribosome association and translation initiation. These elements may be involved in the light-regulated translation of the *psbA* mRNA.

EXPRESSION of photosynthetic genes in plants and algae is keyed to both developmental and environmental signals. The accumulation of chloroplast-localized photosynthetic proteins is regulated such that component proteins, whether nuclear or chloroplast encoded, always accumulate in a coordinate manner. A key signal for this coordinate gene expression is light. For nuclear-encoded photosynthetic genes, light activates transcription, via the cytoplasmic photoreceptor phytochrome, by a molecular mechanism not yet fully understood (Benfey and Chua, 1989; Neuhaus et al., 1993). For chloroplast-encoded genes, translational regulation has been identified as a key component of light-activated gene expression (reviewed in Rochaix, 1992; Gillham et al., 1994). Additional regulatory signals, involving interactions of the chloroplast and nucleus, have also been identified as having regulatory roles in coordinate gene expression (Mayfield, 1990), but the nature of these signals remains unclear (Susek et al., 1993). In addition to translational regulation, several key steps in chloroplast gene expression have been identified as crucial to photosynthetic protein accumulation, including those affecting mRNA transcription (Deng and Gruissem, 1988; Mullet, 1993), mRNA stability (reviewed in Gruissem and Schuster, 1993; Salvador et al., 1993), and protein turnover (Mullet et al., 1990). To date, none of the regulators of these processes have been characterized.

Genetic analysis in the unicellular green algae *Chlamydomonas reinhardtii* has identified a number of nuclear genes that specifically affect chloroplast gene expression. These nuclear gene products are involved in mRNA processing (Goldschmidt-Clermont et al., 1990), mRNA accumulation (Kuchka et al., 1989), and translation (Jensen et al., 1986; Kuchka et al., 1988; Rochaix et al., 1989). The set of nuclear genes that control chloroplast mRNA translation appear to encode proteins that interact directly with chloroplast mRNAs, acting as translational activators of these messages (Rochaix et al., 1989). Chloroplast mutants have also been characterized in which the translation of a single mRNA is lost, although the affected mRNA accumulates to normal levels (Rochaix et al., 1989). Analysis of these chloroplast mutants has revealed that sequences within the 5'-untranslated region (UTR)[1] of these RNAs are essential for translation. In one case, a mutation in the 5'-UTR suppresses a nuclear mutation and restores translation (Rochaix et al., 1989). These data suggest that RNA elements contained within the 5'-UTR may be target sites for interaction with the nuclear encoded translational activator proteins.

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1. *Abbreviations used in this paper:* RBS, ribosome-binding site; rt, room temperature; UTR, untranslated region; wt, wild type.

To characterize the components of translational activation in the chloroplast, we previously identified and isolated a set of four proteins that bind with high specificity and affinity to the 5′-UTR of the chloroplast encoded *psbA* mRNA (encoding the D1 protein of the photosystem II reaction center). Binding of these proteins to the *psbA* mRNA correlates with the translation of this message under growth in bright light (maximum *psbA* translation), during dark growth (minimal *psbA* translation), and in a mutant that fails to translate any *psbA* in the dark (Danon and Mayfield, 1991). The binding of these proteins to the *psbA* mRNA can be regulated by at least two independent processes: ADP-dependent phosphorylation (Danon and Mayfield, 1994*a*), and redox potential (Danon and Mayfield, 1994*b*). From these previous studies, a model for translational activation of chloroplast mRNAs can be drawn in which nuclear-encoded translational activator proteins are transported into the chloroplast, where they interact in a message-specific manner with the 5′-UTRs of chloroplast mRNAs to initiate or enhance translation. Binding activity of these translational activators is light modulated, suggesting that they may be participating in the 100-fold increase in *psbA* translation observed during a dark to light shift (Fromm et al., 1985; Malnoë et al., 1988; Klein et al., 1988; Mullet, 1988; Krupinska and Apel, 1989).

Secondary structure analysis of the 5′-UTR of the *psbA* mRNA predicts a dominant stem-loop element located upstream and adjacent to a consensus Shine-Dalgarno sequence (putative ribosome-binding site). A 36-base fragment containing this stem-loop region was identified as the binding site for the *psbA*-specific RNA binding proteins using RNase protection assays (Danon and Mayfield, 1991). These data suggest that the binding of proteins to this region of the 5′-UTR might be involved in translational regulation of this mRNA. In this study, we examine the role of the 5′-UTR in translational regulation by introducing mutations into the *psbA* mRNA of *C. reinhardtii*. We specifically address how mutations in the consensus Shine-Dalgarno sequence and in the region containing the stem-loop affect mRNA accumulation and *psbA* translation in vivo. These data show that the Shine-Dalgarno sequence is required for *psbA* translation, suggesting that this region is a functional ribosome-binding site (RBS). We also show that mutations in the stem-loop region affect *psbA* translation, while mutations outside the stem-loop region have little effect on *psbA* translation. These results demonstrate that the region of the *psbA* UTR, which is bound by the message specific proteins, is in fact an RNA element functioning in *psbA* translation. Secondary structure of the RNA element is important for its function, and this element appears to be a translation attenuator that must be overcome for translation to proceed. Finally we present data that suggests that translation of *psbA* mRNA in the stem-loop mutants is affected to a greater degree in cells grown in the light than in the dark, indicating that the RNA element contained within the stem-loop region may play a role in light activated translation. These data suggest a mechanism for translational regulation of *psbA* mRNA in which specific RNA-binding proteins recognize a structured RNA element containing the RBS, bind to this structure, and alter it to allow for ribosome association. Under this model, the RNA element would act as a translation attenuator that is overcome during light growth to allow for *psbA* translation.

Materials and Methods

Construction of Site-directed Mutations in the 5′-UTR of the psbA Gene

Site specific mutations were introduced into the 5′-UTR of the *psbA* gene using standard PCR conditions (Gene Amp; Perkin Elmer Corp., Norwalk, CT) and synthetic oligo nucleotide primers containing the desired alterations (shown in Fig. 1). The oligos used to generate these mutations were as follows: 27-32 del (5′ GGAAGCTTGATAAATAAAA 3′), 32-36 del (5′ GGAAGCTTGAATTTATAAAATATTT 3′), 27-59 del (5′ GGAAGCTT-GACGGAGAAATTAAAAC 3′), Loop-del (5′ GGAAGCTTGAATTTATA-AATTAAAAATATTTTAC 3′), Alter (5′ GGAAGCTTGAATTTATAAATT-AAAATATTTTTACAATAAAAAACGGAGAAAT 3′), Insert (5′ ATTTTT-ACTATTTTTACACGGAGAAA 3′), RBS-del (5′ ATTTTTACAATATTTT-ACAAATTAAAACT 3′), RBS-paired (5′ CAAGCTTTAATTTCTCCGTAA AATATTTTT 3′). These oligos were used to amplify small fragments of the *psbA* gene. The PCR fragments were sequenced (Sequenase Version 2.0 Sequencing Kit; U.S. Biochemicals Corp., Cleveland, OH) to ensure that the desired mutation was present and that additional mutations had not been introduced during DNA amplification. Altered DNA fragments were ligated into the 5′-UTR of an intronless *psbA* gene contained within plasmid pBS (Stratagene, La Jolla, CA). The intronless *psbA* gene was used because it was easier to manipulate than its larger intron containing counterpart in the construction of the site-directed mutant *psbA* genes.

Introduction of Mutant Genes into C. reinhardtii Fud7 Cells

The *psbA*-deficient strain Fud7 (Bennoun et al., 1986) was grown in complete liquid media to a density of 5×10^6 cells/ml under continuous low intensity fluorescent light. Cells were pelleted, washed once with H_2O, and then spread on complete media plates containing 100 μg/ml spectinomycin at a concentration of 5×10^7 cells/plate. The cells were cotransformed with one of the mutant *psbA* genes and plasmid p228 (containing the ribosomal DNA 16S gene with spectinomycin resistance; Harris et al., 1989) using particle gun bombardment as described by Boynton et al. (1988). Transformants were replated onto new spectinomycin plates and then scored for *psbA* integration by Southern analysis (Sambrook et al., 1989). The *psbA* gene from each transformants was subcloned into pBS (Stratagene) and sequenced to ensure that the integrated genes contained the correct mutations.

RNA Isolation and Analysis

Strains were grown in complete media to a density of 5×10^6 cells/ml under constant light. RNA isolation, electrophoresis, and blotting were performed according to the procedures described by Rochaix et al. (1987). After fractionation of the RNA on formaldehyde denaturing agarose gels, the RNA was transferred by capillary action to Hybond-N (Amersham Corp., Arlington Heights, IL) using 25 mM sodium phosphate buffer (pH 6.5) as a transfer medium. The RNA was cross-linked to the membrane using UV light (Khandjian, 1986). Membranes were prehybridized for 30 min at 42°C in 50% formamide, 5X SSPE (pH 7.5), 0.1% SDS, 0.5% nonfat dry milk, and 10 μg/ml salmon sperm DNA. Hybridizations were carried out in the same solution for 16 h at 42°C with the addition of a random-primed, ^{32}P-labeled *psbA* cDNA. Membranes were washed three times at 60°C for 30 min in 1× SSPE/0.1% SDS. Hybridization was detected by exposure of the membranes to x-ray film (Kodak XAR) at −70°C.

Protein Isolation and Immunoblotting

Strains were grown in complete media to late log phase under constant light or dark. Cells were pelleted and resuspended in 700 mM Tris HCl (pH 8.0), 15% sucrose, and 100 mM 2-mercaptoethanol. The cells were disrupted by sonication with a microtip (Sonic 2000; B. Braun Biotech Intl., Allentown, PA) for 15 s at 80% maximum power. The lysed cells were pelleted at 10,000 *g* for 15 min at 4°C. The supernatant (soluble fraction) was removed and the pellet (membrane faction) was resuspended in the above mentioned buffer. To the resuspended membrane proteins, 2-mercaptoethanol and SDS were added to a final concentration of 2%. Proteins were separated by electrophoresis on 12% acrylamide gels containing 0.1% SDS. After electrophoresis, the gels were incubated in 25 mM Tris, 190 mM glycine, and 0.1% SDS for 20 min at room temperature (rt), and electroblotted to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) in the same

buffer with the addition of 10% MeOH. The nitrocellulose filters were blocked in TBST (50 mM Tris, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk for 1 h at rt. Rabbit polyclonal antisera specific for the D1 protein was added and the incubation continued at rt for 6 h. The filters were washed two times in TBST at rt, and were then incubated in TBST containing 5% nonfat dry milk and a goat anti–rabbit IgG alkaline phosphatase conjugate (Sigma Immunochemicals, St. Louis, MO) for 3 h at rt. The filters were washed two times in TBST for 10 min at rt followed by one 10-min wash in AP (100 mM Tris [pH 9.5], 100 mM NaCl, and 5 mM MgCl₂) buffer and developed by alkaline phosphatase activity staining (Mayfield and Kindle, 1990).

Pulse Labeling of Proteins with [¹⁴C] Acetate

Strains were grown under continuous light or dark in complete media to late log phase. Cells were pelleted and resuspended in media lacking acetate for 1 h before labeling. Cycloheximide was added to a final concentration of 10 μg/ml 10 min before the addition of [¹⁴C]acetate to eliminate cytoplasmic protein synthesis. [¹⁴C]acetate (1 μCi/ml; Amersham) was added to the cell cultures and allowed to incorporate for 10 min. Sodium acetate (pH 5.3) was added to a final concentration of 50 mM, and the cells were harvested by centrifugation for 2 min at 10,000 g and immediately frozen in liquid nitrogen. Membrane proteins were isolated, separated by size on SDS-PAGE, and visualized by fluorography as described by Malnoë et al. (1988).

Results

Construction of Site-directed Mutations in the 5'-UTR of the psbA mRNA

Site-specific mutations were introduced into the 5'-UTR of the psbA gene using synthetic oligo nucleotide primers containing the desired alterations, and PCR to amplify small fragments of the psbA gene. The altered PCR-generated

DNA fragments were cloned into a psbA gene that had previously been modified by removal of the introns. This intronless psbA gene shows normal wild type (wt) production of psbA RNA (see Fig. 2 A, cDNA) and protein (see Fig. 3 B, lane I) in transformed strains of C. reinhardtii.

Four mutations, Loop-del, Alter, Insert, and RBS-del were initially constructed and are diagrammed on a linear map of the psbA 5'-UTR (Fig. 1 A), and as indicated on a two-dimensional computer-predicted structure of the UTR (Fig. 1 B). These mutations were centered around a Shine-Dalgarno consensus sequence (putative RBS) and an adjacent upstream stem-loop structure (Fig. 1 B). We had previously identified this region as being the binding site for the translational activator proteins (Danon and Mayfield, 1991). The mutations include deletion of the RBS (Fig. 1, RBS-del), deletion of bases that form the loop of the stem-loop structure adjacent to the RBS (Loop-del), alteration of bases to disrupt formation of the stem (Alter), and an insertion of 10 bases (Insert) between the RBS and the dominant stem-loop. These four mutations plus the intronless psbA gene (Wild type) were transformed into a psbA gene–deficient mutant of C. reinhardtii (Fud7; Bennoun et al., 1986) by particle gun bombardment as described by Boynton et al. (1988). The Fud7 cells were transformed with one of the mutated psbA gene plasmids and plasmid p228 (containing the ribosomal DNA 16S gene with spectinomycin resistance, Harris et al., 1989), selected for spectinomycin resistance, and scored for psbA gene integration by Southern analysis. All transformed psbA genes were isolated and sequenced to ensure that the desired mutation had been introduced.
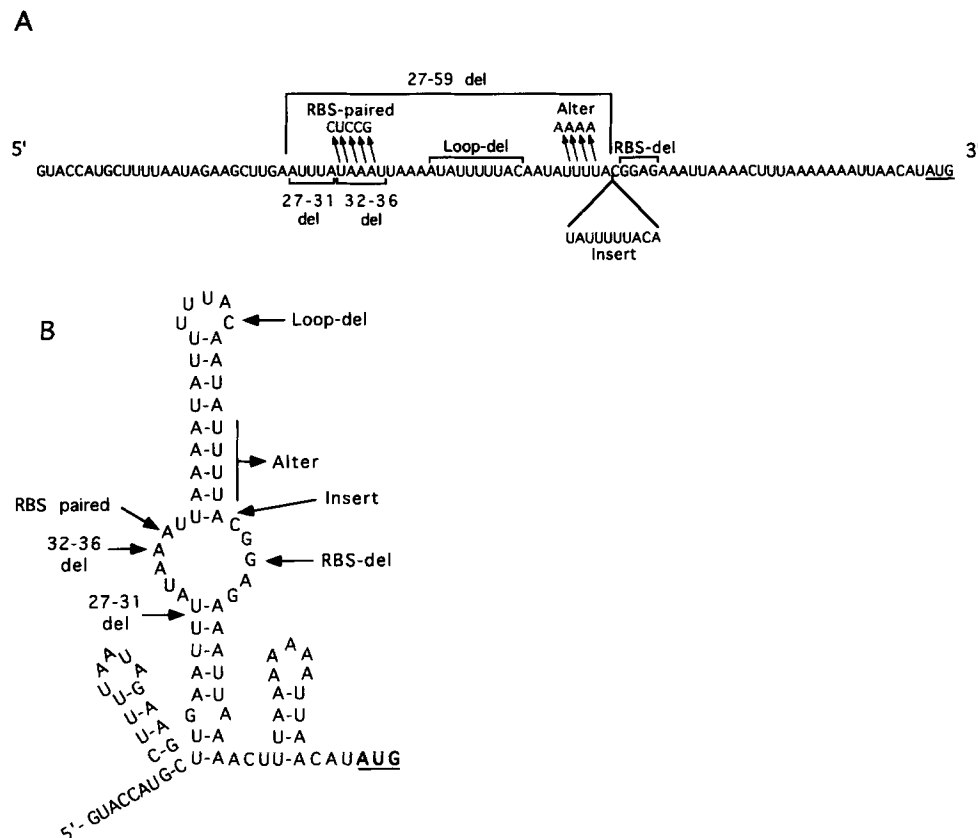
A

B

Figure 1. Primary (A) and secondary (B) structure maps of the psbA 5' UTR showing site-directed mutations. Mutations include deletion of the putative RBS (RBS-del), deletion of the loop at the top of the stem (Loop-del), deletion of bases 27–31, deletion of bases 32–36, and deletion of the entire stem loop region (27-59-del). Other mutations include alteration of bases to disrupt formation of the stem (Alter), an insertion of 10 bases between the stem and the RBS (Insert) and a mutation in which bases 32–36 were altered to pair with the RBS (RBS-paired).

Accumulation of psbA mRNA in Stem-Loop and RBS Mutants

To assay for the accumulation of psbA mRNA in transgenic strains, total RNA was isolated from cells grown in complete media, separated on denaturing gels, and blotted to nylon membranes as described (Rochaix et al., 1987). The blots were hybridized with a radiolabeled psbA cDNA. As shown in Fig. 2 A, the wt and all four of the mutants accumulate psbA mRNA. psbA RNA accumulation is substantially less in the RBS deletion mutant than in wt or any of the other mutants, containing ∼20% of the wt psbA mRNA levels. To ensure that equal amounts of RNA were loaded in each sample lane, the filter was stripped and rehybridized with a radiolabeled psbD cDNA clone. As shown in Fig. 2 B, all of the lanes have a similar signal for the psbD mRNA.

Accumulation of the D1 Protein in Stem-Loop and RBS Mutants

To assay for the accumulation of D1 protein in the psbA mutants, proteins were isolated from wt and mutant strains grown in complete liquid media. Membrane proteins were separated by SDS-PAGE, and they were either stained with Coomassie blue (Fig. 3 A) or blotted to nitrocellulose membranes and decorated with anti–D1 antisera (Fig. 3 C). The blots were decorated with a secondary antisera and visualized by staining for alkaline phosphatase activity. As shown in Fig. 3 C, D1 protein accumulates in the wt transformant (lane 1), as expected. D1 protein fails to accumulate in the RBS deletion (Fig. 3 C, lane 3), showing that the Shine-Dalgarno sequence is required for psbA accumulation and suggesting that this sequence is in fact an authentic ribosome-binding site required for psbA mRNA translation. D1 protein accumulates to levels similar to or greater than wild-
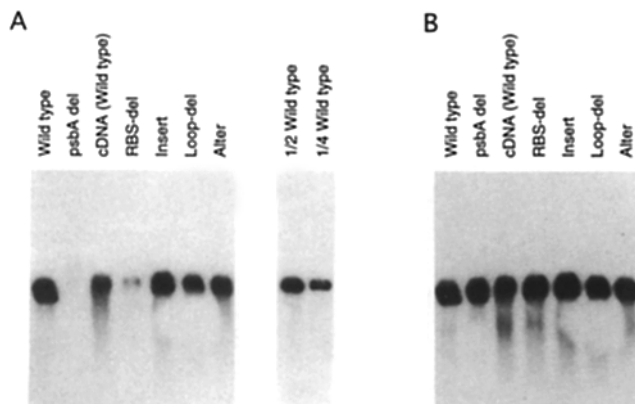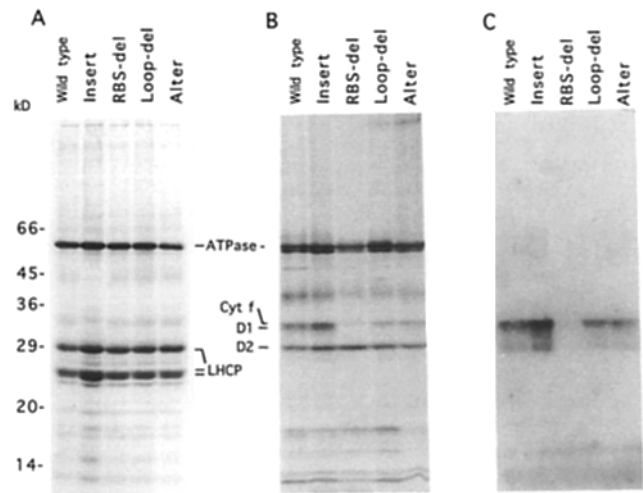

Figure 3. Synthesis and accumulation of D1 protein in stem-loop and RBS mutants. (A) Membrane proteins were separated by SDS-PAGE and stained with Coomassie blue. Proteins were isolated from Fud7 transformed with psbA cDNA (*Wild type*) and psbA cDNA containing the insertion (*Insert*), the RBS deletion (*RBS-del*), the loop deletion (*Loop-del*), and the stem alteration (*Alter*) mutations. (B) Synthesis of D1 protein in psbA mutant strains. Cells were labeled for 10 min with [14C]acetate in the presence of cycloheximide, and the membrane proteins were separated by SDS polyacrylamide gel electrophoresis, and visualized by fluorography. The positions of the chloroplast encoded proteins ATPase, cytochrome f (*cyt f*), D1, and D2 are indicated, as is the position of the nuclear encoded LHCP proteins. (C) Accumulation of D1 in wt and mutant strains. Membrane proteins were separated by size on polyacrylamide gels, transferred to nitrocellulose, and decorated with rabbit anti-D1 polyclonal antibody followed by alkaline phosphatase–conjugated goat anti-rabbit antibody. D1 protein was visualized by cleavage of NBT/BCIP substrate. Molecular mass of protein standards are given in kilodaltons at far left.

Figure 2. Accumulation of psbA mRNA in stem-loop and RBS mutants. Total RNA was prepared from wild-type cells, Fud7 (*psbA del*), and Fud7 transformed with the wild-type psbA cDNA (*cDNA*) and psbA cDNA containing the insertion (*Insert*), the RBS deletion (*RBS-del*), the loop deletion (*Loop-del*), and the stem alteration (*Alter*) mutations. RNA was size fractionated by electrophoresis on a denaturing agarose gel, transferred to a nylon membrane, hybridized with a 32P-labeled psbA probe, and visualized by autoradiography. A 1/2 and 1/4 dilution of wild-type RNA are shown to compare RNA levels of mutants with wild type. (B) The filter was stripped of the psbA probe and rehybridized with a psbD-specific DNA fragment. The sample lanes are the same as in A.

type cells in the insertion mutant (Fig. 3 C, lane 2). The loop deletion and stem alteration mutants accumulate D1 protein, but only to ∼20% of wt levels (when quantitated with a serial dilution of wt proteins on the same Western blot, data not shown), demonstrating that the stem-loop region of the psbA UTR is essential for high levels of D1 accumulation. In each of these mutants, other chloroplast proteins accumulate to normal wt levels (Fig. 3 A).

Synthesis of D1 Protein Is Reduced in Stem-Loop and RBS Mutants

The steady-state accumulation of the D1 proteins gives only an indication of the translation rate from the psbA mRNA. Therefore, we directly measured psbA translation by pulse labeling proteins with [14C]acetate. Cycloheximide was added to eliminate protein synthesis from cytoplasmic ribosomes to allow for easier identification of the chloroplast synthesized proteins. [14C]acetate was added to the cells and allowed to incorporate for 10 min. Membrane proteins were isolated, separated by SDS-PAGE and visualized by fluorography as described (Malnoë et al., 1988). As shown in Fig. 3 B, psbA mRNA is translated in the wt transformant at normal levels. Translation of psbA in the insertion mutant (Fig. 3 B, lane 2) appears to be somewhat greater than in the wt

1540

strain, which is in good agreement with the Western analysis. As expected, no detectable D1 synthesis is observed in the RBS deletion mutant (Fig. 3 B, lane 3). Interestingly, the synthesis of D1 is so low in the loop deletion and alteration mutants that no D1 is visualized at this exposure (Fig. 3 B, lanes 4 and 5). Longer exposures show that D1 is synthesized in these mutants, but at levels <5% of the wt rate (data not shown). The accumulation of 20% of the wt levels of D1 protein in the loop deletion and alteration mutants, when synthesis is only 5% of the wt level, suggests that D1 protein is normally synthesized (in wt cells) at level greater then necessary for D1 accumulation. Thus, even a 5% synthesis rate (in the mutants) is sufficient to accumulate 20% of the wt level of the protein. It should be pointed out that D1 protein turnover should be at relatively low levels because of the growth conditions used for these studies (not saturating light), which may explain the high levels of D1 accumulation from relatively low levels of D1 synthesis in the mutants.

Deletion of Sequences Upstream of the Stem-Loop Region Does Not Affect psbA Translation

To determine if translation of psbA mRNA is sensitive to any deletion or alteration to the 5'-UTR, additional mutations were made into the region immediately 5' of the stem-loop and RBS region. These mutations include deletion of bases 27-31 and deletion of bases 32-36. These mutations are diagrammed in Fig. 1. Both of these mutations were constructed and transformed into the Fud7 strain and characterized for psbA integration and expression exactly as described for the previous set of mutations. The psbA gene was sequenced from each transformant to ensure that the mutations had been correctly incorporated into the C. reinhardtii chlo-

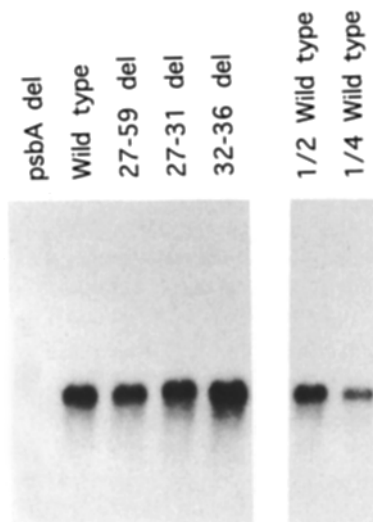Figure 4. Accumulation of psbA mRNA in mutants with deletions of the entire stem-loop region or bases upstream of the stem-loop region. Total RNA was prepared from Fud7 cells (psbA del) and Fud7 transformed with psbA cDNA (Wild type) and psbA cDNA containing a deletion of the entire stem-loop region (27-59 del), a deletion of bases 27-31 (27-31 del), and a deletion of basis 32-36 (32-36 del). RNA was analyzed as in Fig. 2. 1/2 and 1/4 dilution of wild-type RNA are shown to compare RNA levels of mutants with wild type.
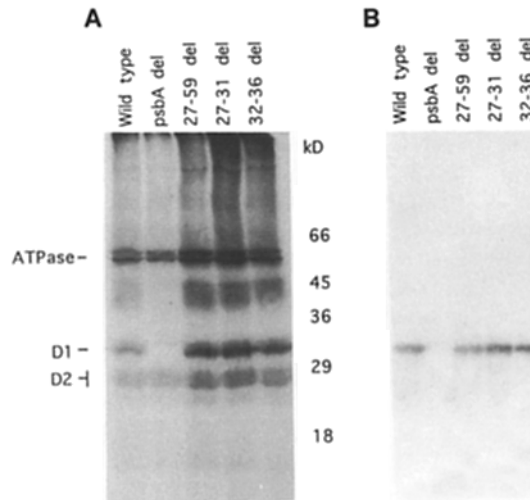

Figure 5. Synthesis and accumulation of D1 protein in mutants with deletions of the entire stem-loop region or bases upstream of the stem-loop region. (A) Synthesis of D1 in Fud7 cells (psbA del), and in Fud7 transformed with psbA cDNA (Wild type) and psbA cDNA containing a deletion of the entire stem-loop region (27-59 del), a deletion of bases 27-31 (27-31 del), and a deletion of bases 32-36 (32-36 del). Cells were treated and protein analyzed as in Fig. 3 A. (B) Accumulation of D1 in wt and mutant strains. Membrane proteins were analyzed as in Fig. 3 B.

roplast genome. Analysis of psbA mRNA accumulation in these mutations shows that each accumulates psbA RNA close to wt levels (Fig. 4). D1 protein also accumulates to near wt levels in these strains (Fig. 5 B). Pulse labeling of these deletion mutants with [14C]acetate shows that each synthesize D1 protein at levels comparable to wt (Fig. 5 A). That both the 27-31 and 32-36 deletions have no impact on D1 synthesis or accumulation shows that the 5' region of the psbA mRNA is, in general, tolerant of some mutations and suggests that the loop deletion and alteration mutations must be within some specific region of the psbA RNA, which is required for high levels of psbA translation.

A third deletion was made from bases 27-59. This last deletion includes the entire stem-loop structure but not the RBS (diagrammed in Fig. 1 A). Northern analysis shows that this deletion has psbA mRNA accumulation to near wt levels (Fig. 4, lane 3). Analysis of D1 protein accumulation and synthesis shows that this deletion also had little effect on psbA translation, with both D1 protein accumulation (Fig. 5 B, lane 3) and synthesis (Fig. 5 A, lane 3) appearing similar to wt. That a deletion of bases 27-59 had high levels of translation is especially interesting since this deletion encompasses the entire stem-loop structure including the loop deletion (bases 40-49) and alteration (bases 55-59), both of which show poor psbA translation.

Pairing of the RBS Abolishes psbA Translation and Reduces psbA RNA Accumulation

Earlier analysis had indicated that the stem-loop region located adjacent and upstream to the RBS might be a key component of translational regulation (Danon and Mayfield, 1991). Examination of the predicted secondary structure of

this region (Fig. 1 *B*), coupled with the above mutagenic analysis, suggests that the stem-loop region may contain an RNA element in which some secondary structure is recognized by protein factors and altered to allow access of ribosomes or other factors essential for translation initiation. With this in mind, a mutant was constructed in which bases 32-36 were altered so that they paired with the RBS (Fig. 1 *A, RBS-paired*). Bases 32-36 were chosen because deletion of these bases (32-36 deletion) had shown they were not essential for *psbA* translation. This mutation is predicted to increase the length of the stem and dramatically alter the secondary structure around the RBS without directly changing any of the bases in the stem-loop or RBS site. This mutation (RBS-paired) was constructed as described for the other mutants and transformed into the Fud7 strain and selected as described previously. Transformants were scored for *psbA* integration and sequenced to ensure the mutation had been correctly incorporated into the genome. Analysis of this mutant shows that no detectable D1 protein accumulates (Fig. 6 *B*), although other photosynthetic proteins such as LHCP (Fig. 6 *C*) accumulate in this strain to wt levels. Pulse labeling with [¹⁴C]acetate shows that no *psbA* translation is detected (Fig. 6 *A*).

Analysis of *psbA* RNA accumulation in the RBS-paired strain shows that *psbA* RNA accumulates to only a few percent of wt (Fig. 7). Although these levels of RNA are quite low, they should be sufficient for accumulation of measurable amounts of D1 protein if any *psbA* mRNA had been translated in this strain, as Western blot analysis should detect D1 protein accumulation, even if it were only 1% or less of wt. No D1 protein is detected, however, in the RBS-paired strain (Fig. 6 *B*). Thus, the RBS-paired mutant does not appear to translate any *psbA* mRNA. The low levels of *psbA* mRNA in this strain appears to be caused by instability of

the message, not to a reduction in transcription. Preliminary RNA pulse labeling experiments showed that transcription of *psbA* mRNA was similar in wt and RBS-paired mutant (data not shown).

psbA mRNA Translation in Dark-grown Cells May Be Similar in the Loop Deletion, Alteration, and Wild-type Strains

The loop deletion and alteration mutants translate *psbA* mRNA in the light at only a few percent of wt. The level of translation of *psbA* in these mutants is similar to wt cells grown in the dark (Malnoë et al., 1988). These data suggested to us that the RNA element defined by these mutations might have some function in light-activated translation. Conversely, these mutations may have identified RNA elements required for general translation that are not necessarily associated with light-activated translation. We reasoned that if these mutations caused a specific reduction in the light-activated translation, that translation in the dark might be similar in mutants and wt strains. If, however, these mutations were not involved in light-regulated translation, then translation in the dark should, as translation in the light, be 20-fold lower in the mutants as compared to wt. We measured D1 accumulation and *psbA* translation in dark grown wt, insertion, loop-deletion, and stem alteration strains. As judged by Coomassie staining, each of the transformants synthesize and accumulate approximately equal amounts of membrane-associated proteins (Fig. 8 *A*). As shown in Fig. 8 *C*, D1 protein accumulates in wt and the three mutants to similar levels, suggesting that D1 synthesis is similar in all strains. Pulse labeling with [¹⁴C]acetate reveals that translation in each of the strains, although greatly reduced compared with cells grown in the light, appears similar (Fig. 8 *C*). Although these data do not conclusively show that *psbA* translation is unaffected in the dark, they indicate that the loop deletion and stem alteration mutations affect translation of *psbA* mRNA in the light to a much greater degree than translation of *psbA* mRNA in the dark, and suggest that the stem-loop region may be a component of light activated translational regulation.

Discussion

The coordinate expression of nuclear and chloroplast encoded genes is a requirement for photosynthetic complex assembly and is an essential component of chloroplast and ultimately plant development. In plants and green algae, both
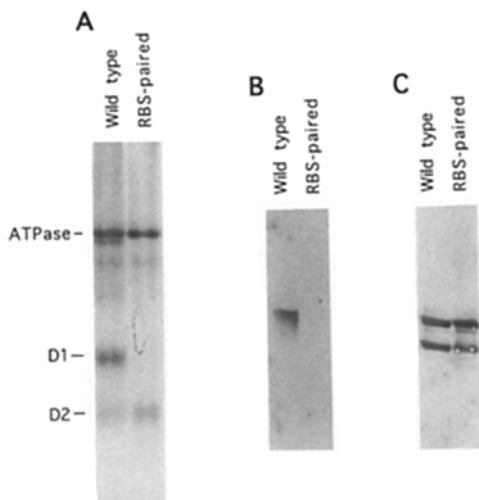
Figure 6. Synthesis and accumulation of D1 protein in mutant with base-paired RBS. (*A*) Synthesis of D1 in Fud7 transformed with *psbA* cDNA (*Wild type*) and *psbA* cDNA containing a mutation that basepairs the RBS (*RBS-paired*). Cells were treated and proteins analyzed as in Fig. 3 *A*. (*B*) Accumulation of D1 in wt and RBS-paired strains, and (*C*) accumulation of LHCP protein in wt and RBS-paired strains. Membrane proteins were analyzed as in Fig. 3 *B*.
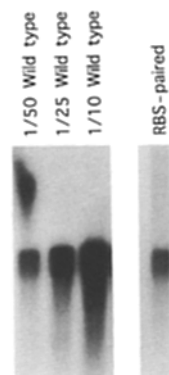

Figure 7. Accumulation of *psbA* mRNA in mutant with basepaired RBS. Total RNA was prepared from Fud7 transformed with *psbA* cDNA (*Wild type*) and *psbA* cDNA containing a mutation that basepairs the RBS (*RBS-paired*). RNA was analyzed as in Fig. 2. 1/50, 1/25, and 1/10 dilution of wild-type RNA are shown to compare RNA levels of RBS-paired with wild type.
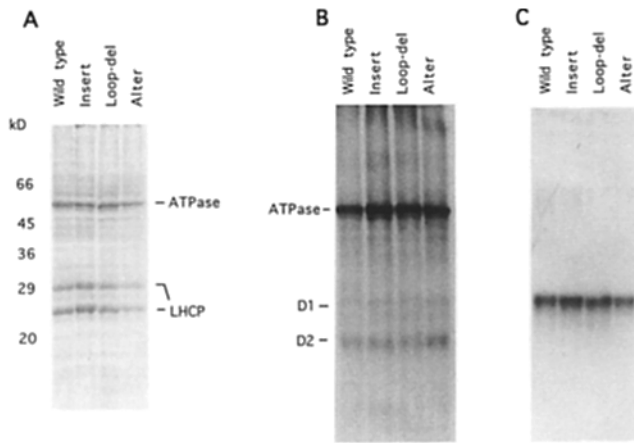
Figure 8. Synthesis and accumulation of D1 protein in stem-loop and RBS mutants grown in the dark. (*A*) Coomassie blue–stained gel of membrane associated proteins isolated from transformants of *psbA* cDNA (*Wild type*) and *psbA* cDNA containing the insertion mutation (*insert*), the loop deletion (*Loop-del*), and the stem alteration (*Alter*) separated by SDS-PAGE. (*B*) Synthesis of D1 in Fud7 transformants. Cells grown in the dark were otherwise treated and proteins analyzed as in Fig. 3 *B*. (*C*) Accumulation of D1 in cells grown in the dark. Membrane proteins were treated as in Fig. 3 *C*.

nuclear and chloroplast gene expression are activated in response to light, but in each case, a different mechanism is used to achieve this goal. Light-responsive transcriptional activation strongly influences expression of nuclear-encoded genes. For chloroplast gene expression, light-responsive translational activation appears to be the dominant mechanism regulating photosystem protein accumulation. Genetic analysis has revealed that nuclear-encoded factors are required for chloroplast mRNA translation (reviewed in Rochaix, 1992; Gillham et al., 1994). We have previously identified a set of proteins that bind with high affinity and specificity to the 5'-UTR of the chloroplast *psbA* mRNA. Binding of these proteins to the *psbA* RNA is light modulated and correlates with the translational activation of this mRNA (Danon and Mayfield, 1991). Binding of these proteins protects a region of the 5'-UTR located adjacent and upstream of a putative RBS (Danon and Mayfield, 1991). These data, coupled with the identification of the *cis*-acting RNA elements reported here, define a set of components necessary for translational activation of the *psbA* mRNA, and they suggest a mechanism by which translational activation may be achieved.

A Shine-Dalgarno Sequence Is Required for psbA Translation

Deletion of the putative Shine-Dalgarno sequence abolishes *psbA* translation and D1 protein accumulation, showing that this region is required for translation, and strongly suggesting that the GGAG sequence in this region is in fact an authentic ribosome-binding site. Although this result was expected, it is still interesting because although all *psbA* genes sequenced to date (>20) have Shine-Dalgarno consensus sequences, not all chloroplast genes contain this sequence. Why a Shine-Dalgarno sequence is required for *psbA* translation but is not found in other translated chloroplast mRNAs is not clear.

An RNA Element Adjacent to the RBS Is Required for Normal psbA Translation

Two mutations introduced into the *psbA* 5'-UTR (loop deletion and alteration) demonstrate that the region adjacent to and upstream of the Shine-Dalgarno sequence is a functional component of *psbA* translation. The two small deletions (27-31 deletion and 32-36 deletion) immediately upstream of the stem-loop both synthesize and accumulate normal levels of D1, showing that deletions in general can be tolerated within the noncoding region of the *psbA* mRNA. These deletions also suggest an upstream boundary for the element, suggesting that the element functioning in *psbA* translation may be a relatively small region of the UTR. The insertion mutation, which is positioned between the stem-loop region and the RBS and has normal *psbA* translation, again suggests that the element identified by the stem-loop mutations may be localized to that region alone. Each of these data suggests that the stem-loop region adjacent to the RBS is a functional component of *psbA* translation.

Examination of the stem-loop region shows that the sequence (AUAUUUU$^\text{U}$AC) is repeated two times. Two mutants that have reduced translation (loop deletion and alteration) affect one or the other of these repeats. The insertion mutation, which translates *psbA* at wt levels, has this same sequence added downstream of the two repeats to form three direct repeats of the sequence. From these data one could argue that this repeat sequence is the functional element contained within the stem-loop region that is required for *psbA* translation. However, this simple view does not account for all of the observed phenotypes. The large deletion (27-59), in which both repeats have been removed, still translates *psbA* at high rates, suggesting that this repeat is not likely to be a positive element required for *psbA* translation. A second mutation which suggests that primary sequence within the stem-loop region can not be the only criteria for translational activation is the RBS-paired mutation. In this mutant, the repeat sequence is unaltered yet no translation is detected in the mutant. This mutation (RBS-paired) strongly suggests that secondary structure around the RBS may play an important role in translational activation. A simple explanation for these data might be that the stem-loop region contains a translational attenuator that must be overcome to allow translation.

Is the Stem-Loop Region a Translational Attenuator?

If the stem-loop region contains a translation attenuating element, we might expect small alterations within the element, (i.e., loop deletion or alteration mutations) to result in an altered element that still retains its translation attenuating nature but could no longer be overcome by binding of translational activator proteins. The large deletion (27-59 del) that removes the entire stem-loop region would remove the attenuating element completely thereby allowing unattenuated translation. If the attenuating element functions by sequestering the RBS then mutation such as the RBS-paired might be expected to increase the attenuating effect of the element by decreasing the availability of the RNA to ribosomes even further. Finally, mutations like the insertion mutation might have normal translation since this mutation could result in a more exposed RBS.

The exact nature of the attenuating element can not be de-

termined from the present data. However, this element does not seem to be dependent upon simple base pairing within the stem-loop region. If simple base pairing within the stem-loop were a requirement for attenuation, then we would expect the alteration mutation, which disrupts pairing in this region, to have higher levels of psbA translation, which it does not. It appears that any of the mutations that perturb RNA structure within the stem-loop region have reduced translation. Whether these mutations also reduce protein binding or protein function is now under investigation. Whatever the structural nature of the RNA element the simple explanation of the data is that the stem-loop region contains an RNA element that acts as a translation attenuator that is normally overcome in wt cells to induce translation.

Mutations That Affect Ribosome Binding also Affect mRNA Accumulation

An interesting effect of the RBS deletion and RBS-paired mutations is a decrease in psbA mRNA accumulation. Preliminary evidence suggests that the decrease in psbA mRNA accumulation is likely to be caused by more rapid turnover of the mRNA than by a reduction in transcription. It is interesting to note that the only mutants in which we observe this reduction in mRNA accumulation are those in which the RBS is unavailable, either by deletion of pairing of the bases. Other mutations in which psbA translation is reduced (alteration and loop deletion) still accumulate wt levels of the psbA mRNA. Several nuclear mutations of C. reinhardtii have been characterized in which translation of a specific chloroplast mRNA is absent. Each of these mutants accumulates wt, or in some cases more than wt, levels of the mRNAs. These data show that a reduction in translation, per se, does not necessarily affect chloroplast mRNA accumulation. Our data suggest that ribosome association rather than translation may be a factor influencing mRNA stability. The RBS-paired mutation, which increases the length and presumably the stability of a stem-loop structure, results in decreased psbA mRNA stability. This is contrary to reports for stem-loop structures located in the 3' end of chloroplast mRNAs, which are reported to increase mRNA stability (Stern and Gruissem, 1988; Stern et al., 1991). However, there is not data to suggest that 5' and 3' untranslated regions of chloroplast mRNAs have similar functions in this regard.

The Stem-Loop Structure May Be Used in Light-regulated Translation

The stem-loop region has previously been identified as the site of interaction for light modulated translational activator proteins (Danon and Mayfield, 1991). This, combined with the current identification of the stem-loop region as a functional component of psbA translation, led us to examine the role of this RNA element in light-activated translation. Although the experiments described here do not conclusively define the stem-loop region as a functional component of light-modulated translational activation, they do define this RNA region as required for maximum translation in the light. In the case of the loop deletion and alteration mutants, translation of psbA is <5% of wt when cells are grown in the light. If the stem-loop region is a specific component of light-dependent translational activation, then translation of psbA in the mutants should be similar to wt during dark growth.

Measurement of translation from psbA during dark growth shows that all strains accumulate D1 to approximately the same level and appear to synthesize D1 at similar rates. These data suggest that the stem-loop region may not only be a component of translational activation but may also be a component of light activated translation.

A Model for Translational Activation of the psbA mRNA

From these and other data a model for translational activation can be drawn in which the psbA 5'-UTR contains a structured RNA element that acts as a translational attenuator. This element is recognized by a set of message-specific RNA-binding proteins that bind to the RNA and allow for ribosome association or translation initiation. The precise nature of the RNA element remains unknown, but clearly involves a small region of the UTR located upstream and adjacent to the RBS. Although the molecular mechanism of translational activation involving this set of components remains unresolved, it is clear that nuclear factors (RNA-binding proteins) interact with chloroplast RNA elements to regulate chloroplast gene expression in a light-dependent manner.

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