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# Light regulated translational activators: identification of chloroplast gene specific mRNA binding proteins

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**Genetic analysis has revealed a set of nuclear-encoded factors that regulate chloroplast mRNA translation by interacting with the 5′ leaders of chloroplastic mRNAs. We have identified and isolated proteins that bind specifically to the 5′ leader of the chloroplastic *psbA* mRNA, encoding the photosystem II reaction center protein D1. Binding of these proteins protects a 36 base RNA fragment containing a stem-loop located upstream of the ribosome binding site. Binding of these proteins to the *psbA* mRNA correlates with the level of translation of *psbA* mRNA observed in light- and dark-grown wild type cells and in a mutant that lacks D1 synthesis in the dark. The accumulation of at least one of these *psbA* mRNA-binding proteins is dependent upon chloroplast development, while its mRNA-binding activity appears to be light modulated in developed chloroplasts. These nuclear encoded proteins are prime candidates for regulators of chloroplast protein synthesis and may play an important role in coordinating nuclear-chloroplast gene expression as well as provide a mechanism for regulating chloroplast gene expression during development in higher plants.**

*Key words*: chloroplast light regulation/mRNA-binding proteins/translational regulation

## Introduction

The chloroplast is the photosynthetic, chlorophyll-containing plastid found mainly in leaves, and is one of many developmental stages of the plastid organelle. During the light-induced biogenesis of the chloroplast there is a rapid accumulation of chlorophyll, photosynthetic membranes and the associated photosynthetic proteins, both nuclear and chloroplast encoded. Concomitant with light-induced chloroplast biogenesis there is an increase in the transcription and accumulation of cytoplasmic mRNAs encoding photosynthesis-related proteins (reviewed in Tobin and Silverthorne, 1985). Unlike photosynthetic proteins encoded by the nucleus, mRNAs for the chloroplast-encoded photosynthesis related proteins accumulate to relatively high levels in dark-grown plants although there is no accumulation of their protein products (reviewed by Mullet, 1988, Malnoë *et al.*, 1988). Exposure of dark-adapted plants to light results in a dramatic increase in the synthesis and accumulation of plastid proteins associated with photosynthetic membranes (Klein and Mullet, 1986; Klein *et al.*, 1988; Malnoë *et al.*, 1988). Direct measurement of plastid gene transcription during this transition shows no enhanced transcription of chloroplast genes (Krupinska and Apel, 1989) and blocking plastid transcription during the transition from dark to light has no effect on synthesis of photosynthetic proteins (Malnoë *et al.*, 1988). These observations clearly show that post-transcriptional processes are dominant mechanisms regulating plastid gene expression during chloroplast biogenesis and suggest that light-regulated translational control may be of primary importance in coordinating nuclear and chloroplast gene expression. Other regulatory mechanisms involved in chloroplast gene expression like those involving mRNA stability (Stern and Gruissem, 1987) and protein turnover (Mullet *et al.*, 1990) have been described at the biochemical level in higher plants.

In the green alga *Chlamydomonas reinhardtii* there is compelling genetic evidence for a class of nuclear genes that act as translational activators of specific chloroplast mRNAs (Jensen *et al.*, 1986; Kuchka *et al.*, 1988; Rochaix *et al.*, 1989). Each of these nuclear encoded genes affects the translation of only a single chloroplast mRNA. In some cases multiple nuclear genes appear to be required for the translation of a single chloroplast message. Genetic analysis of chloroplast mutants has implied that the 5′-untranslated regions (5′-UTRs) of the chloroplastic messages, probably stem-loop RNA structures, are the target for interaction with these nuclear encoded translational factors (Rochaix *et al.*, 1989).

To isolate these translational factors, we have identified transcript-specific RNA-binding activity and purified several of the mRNA-binding proteins associated with the 5′ leader of the chloroplastic *psbA* message encoding the D1 protein. Included in this set were two proteins that specifically associated with a stem-loop RNA structure located upstream of the ribosome binding site of the *psbA* mRNA. The *psbA* RNA-binding activity of these proteins correlated with the level of D1 protein translation *in vivo* in wild type (wt) cells grown in the light (high level of D1 synthesis), wt cells grown in the dark (low level of D1 synthesis) and *y-1* cells (a chloroplast deficient mutant that lacks D1 synthesis when grown in the dark). These RNA-binding proteins are candidates for the translational regulators that have been genetically and physiologically characterized in *C.reinhardtii*. Furthermore, regulation of the accumulation of these proteins could provide a mechanism for the regulated translation of plastid mRNAs that occurs in plant tissues lacking chloroplasts.

## Results

### *Identification and fractionation of RNA-binding activity of C.reinhardtii cell lysates*

To characterize the RNA-binding activity specific to the 5′ leader of chloroplast mRNAs, *in vitro* synthesized RNAs were incubated with crude cell extracts and subjected to RNase T1 protection-gel mobility shift (T1-GMS) assays (Leibold and Munro, 1988). A 245 base fragment containing

the 5′ leader of the *psbA* transcript (D1-HA; diagrammed in Figure 1) was utilized to identify the *psbA* RNA-binding activity. A 116 base portion (P6-SL) of the 5′ leader of the *psbC* mRNA encoding the P6 protein of PSII and containing a stem−loop implicated in translational control (Rochaix *et al.*, 1989) was used to identify the *psbC* mRNA specific RNA-binding activity. In the T1-GMS assay, RNA-binding proteins were detected according to their ability to protect radiolabeled RNA fragments from RNase T1 degradation, and by their ability to retard migration of the RNA fragment on native polyacrylamide gel electrophoresis (PAGE). The small size of the protected RNA fragment results in mobility that is largely determined by the mobility of the protein or proteins associated with the RNA. As shown in Figure 2,

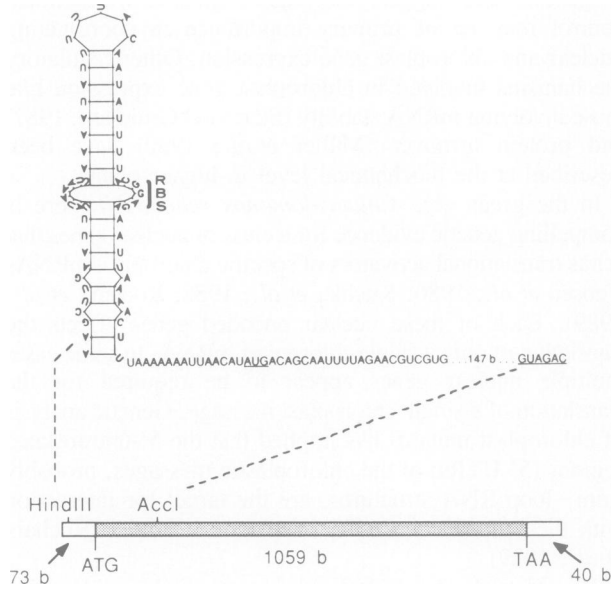


**Fig. 1.** Schematic representation of the *Hin*dIII−*Acc*I fragment of the *psbA* gene (D1-HA) which was used for *in vitro* synthesis of the 5′ end of the *psbA* mRNA. ATG and TAA mark the start and stop codons of the *psbA* mRNA. RBS denotes the potential ribosome binding site. Predicted RNA secondary structure was created using the Genetic Computer Group (Devereux *et al.*, 1984) Fold program of Zuker and Stiegler (1981).

cell lysates of *C.reinhardtii* contain three main proteins or protein complexes that bind to the *psbA* 5′ leader RNA (Figure 2A, lane 2) and two or three proteins or protein complexes that bind the *psbC* leader RNA (Figure 2B, lane 2). All assays were performed in the presence of excess tRNA to reduce non-specific RNA-binding activity.

Heparin−agarose chromatography was chosen as a first step for the fractionation of the RNA-binding activity based on its demonstrated affinity for nucleic acid-binding proteins. Fractions eluted from the heparin−agarose column were incubated with $^{32}$P-labeled *psbA* or *psbC* RNA leaders and then analyzed by the T1-GMS assay. The three RNA-binding activities observed in the crude lysate were resolved by heparin−agarose chromatography although there was some overlap between peaks of activity (Figure 2A and B, lanes 3−15). The major RNA-binding activity for both D1-HA and P6-SL RNAs was observed as several RNA−protein complexes that ran as a fast migrating set of bands (see arrow on Figure 2).

### *The psbA RNA-binding activity cannot be competed by total RNA or psbC RNAs*

To establish further the specificity of the *psbA* RNA-binding activity, labeled D1-HA RNA was incubated with heparin−agarose isolated protein lysates in the presence of several competitor RNAs. As shown in Figure 3, the binding to the labeled D1-HA RNA was competed by 50-, 100- and 200-fold molar excesses of unlabeled D1-HA RNA (lanes 1−4). The binding to the labeled D1-HA RNA was not competed by the inclusion of 50-, 100- and 200-fold mass excesses of total RNA (lanes 5, 6 and 7), revealing that the binding activity was not common RNA in general. Competition with 50-, 100- and 200-fold molar excesses of unlabeled P6-SL RNAs (lanes 8, 9 and 10) also did not compete the RNA-binding activity, suggesting that the binding activity was not common to all chloroplast encoded mRNAs. The *psbA* RNA-binding activity was also found to be resistant to the inclusion of general competitors for single stranded nucleic acids binding proteins such as heparin, dI−dC or poly(U) (data not shown). It should be noted that all binding reactions were carried out in the presence of 10,000 fold excess of tRNA. These data indicate

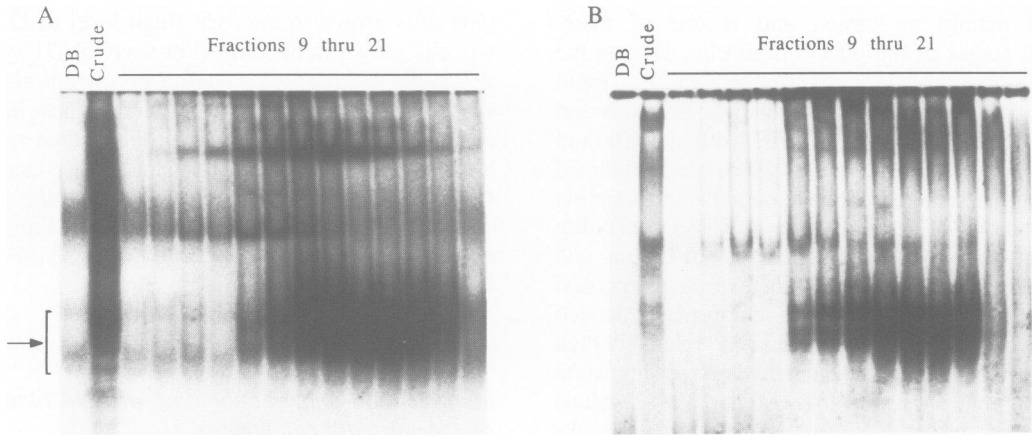


**Fig. 2.** Autoradiogram showing fractionation of transcript-specific RNA-binding activity by heparin−agarose chromatography. RNA-binding activity was detected by RNase T1 protection−gel mobility shift (T1-GMS) assays in the presence of (**A**) D1-HA or (**B**) P6-SL $^{32}$P-labeled transcripts. T1-GMS assays were performed with dialysis buffer (DB), crude cell lysate (Crude) or heparin−agarose fraction numbers 9−21. The arrow indicates the location of the fast migrating RNA-binding activity. The location of free unbound RNA (Free Probe) is indicated in Figure 3.

that the identified D1-HA RNA-binding activity was specific to the *psbA* mRNA and not general RNA-binding activity.

### *Light enhancement of RNA-binding activity*

The translation of *psbA* mRNA is minimal in dark-grown cells, while D1 is one of the most abundantly synthesized proteins in illuminated cells. A mutant of *C. reinhardtii* (y-1), which like higher plants lacks photosynthetic membranes in the dark, is completely deficient in D1 translation in the dark (Malnoë *et al.*, 1988). Cell lysates from each of these cell types were used to characterize the level of the *psbA* RNA-binding activity. Wild type (wt) and *y-1* cells were grown for 7 days in complete darkness or under continuous fluorescent light. Lysates of each were subjected to heparin-agarose chromatography. Equal amounts of total protein from each cell lysate were analyzed for binding of the D1-HA labeled RNA transcript by T1-GMS assay. As shown in Figure 4, differences in the resulting T1-GMS pattern were evident among the light- and dark-grown wild type cell lysates and dark-grown *y-1* cells at the fast migrating major RNA-binding region. Crude and heparin−agarose isolated lysates from dark-grown wild type cells contained a single fast-migrating RNA−protein band (lanes 3 and 6), while lysates from light-grown cells contained a RNA−protein triplet at this position (lanes 2 and 5). None of these fast-migrating RNA−protein bands were observed in the dark-grown *y-1* cell lysates (lanes 4 and 7). Thus, the level of *psbA* RNA-binding activity at the fast migrating RNA−protein complex correlated with the level of translation of D1 protein in each of these three cell types. Two less abundant slow migrating RNA−protein bands present in crude lysates from light-grown cells were also observed in the dark-grown wild type and in *y-1* cell lysate fractions at similar levels.

Reduced levels of free probe were observed in T1-GMS assays that included dark-grown crude cell lysates (Figure 4 lanes 3 and 4). However, the possibility that some nuclease activity intrinsic to the crude lysate affected the apparent RNA-binding activity was excluded since the heparin−agarose fractions, that lacked the nuclease activity showed the same differences in D1-HA RNA-binding activity between light- and dark-grown lysates.

### *Selection of transcript specific RNA-binding activity by affinity chromatography*

To purify further the RNA-binding activity observed in the heparin column fractions, D1-HA and P6-SL RNAs were coupled to a column matrix and utilized in RNA affinity chromatography. RNA columns were synthesized by

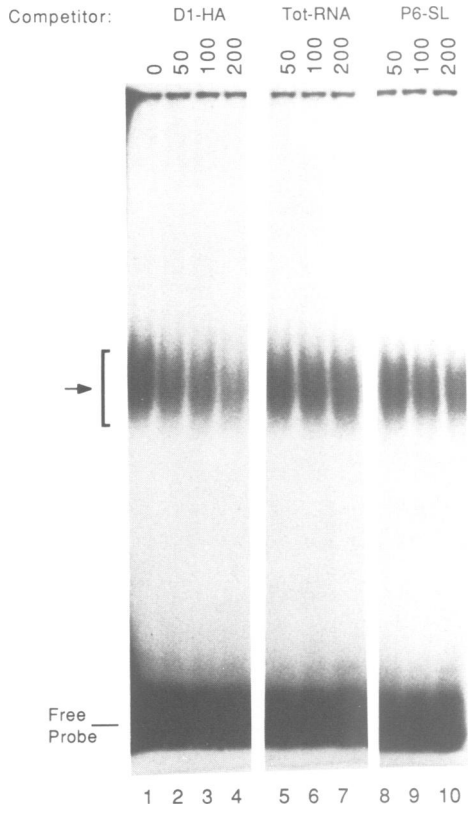


**Fig. 3.** The specificity of the *psbA* RNA-binding activity. Labeled D1-HA RNA was incubated with heparin−agarose fractions containing the *psbA* RNA-binding activity in the absence (0) or the presence of 50-, 100- and 200-fold molar excesses (50, 100, 200) of competitors: unlabeled D1-HA (D1-HA), P6-SL (P6-SL) RNA transcripts or total RNA (Tot-RNA) and subjected to T1-GMS assay. The arrow indicates the location of the fast-migrating RNA-binding activity. The location of free unbound RNA is indicated (Free Probe).

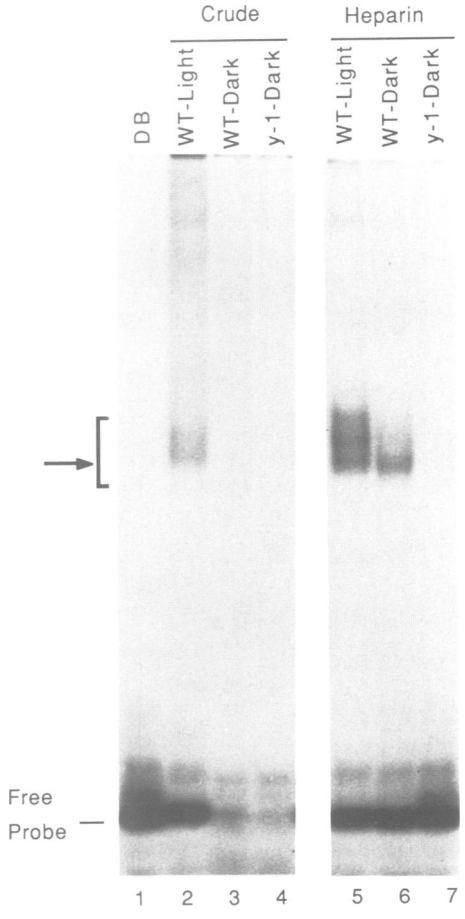


**Fig. 4.** Autoradiogram of RNA-binding activity from illuminated or dark-grown wild type cells and dark-grown *y-1* cells. Heparin−agarose fractions (Heparin) containing the fast-migrating major RNA-binding activity were pooled and subjected to T1-GMS assays in the presence of labeled D1-HA RNA. Additional assays containing either dialysis buffer (DB) or crude cell lysate (Crude) isolated from light or dark grown wild type cells (WT-Light, WT-Dark, respectively), or dark grown *y-1* cells (*y-1*-Dark) are also shown. The arrow indicates the location of the fast-migrating RNA-binding activity. The location of free unbound RNA is indicated (Free Probe).

coupling the 3′-terminal ribose moiety of D1-HA or P6-SL *in vitro* synthesized transcripts to an amino gel matrix allowing the whole RNA molecule to fold into its native structure. Heparin–agarose fractions that contained the *psbA* specific RNA-binding activity were pooled and applied sequentially to P6-SL and then to the D1-HA RNA columns. The columns were washed with low salt buffer containing excess tRNA to elute non-specific RNA-binding proteins. The specific RNA-binding proteins were then eluted from each column with buffer containing 0.55 M potassium acetate. Both light- and dark-grown wild type cell lysates were analyzed and the eluted RNA-binding proteins were subjected to T1-GMS assay as shown in Figure 5.

Proteins from light-grown cell lysate retained on the D1-HA RNA column contained the main fast-migrating major RNA-binding activity observed in the crude lysate and the heparin–agarose column fraction of light-grown cells (lane 2). Proteins from dark-grown cell lysate, retained on the D1-HA RNA column, contained the single RNA–protein band observed in the heparin fraction of dark-grown cells (lane 3). Equal amounts of proteins from light and dark fractions were used for the T1-GMS assays showing not only that there were differences in the pattern of RNA–protein bands, but also that the light-grown cell lysate (lane 2) contained a greater capacity for binding the *psbA* transcript than dark-grown cell lysate (lane 3). The RNA-affinity isolated binding activity to the D1-HA labeled transcript was competed with a 200-fold excess of unlabeled transcript (lanes 4 and 5) indicating that this activity, in both light and dark grown cell lysates, is specific to the *psbA* RNA.

Proteins from light-grown cell lysate, retained on the P6-SL RNA column, contained a slower migrating RNA–protein complex than the *psbA*-specific fast migrating complex (lanes 9 and 2 respectively). Also, proteins eluted from the P6-SL column contained only a small amount of *psbA* binding activity (lanes 6 and 7), again demonstrating the specificity of these proteins for their respective RNAs. The RNA-affinity isolated binding activity to the P6-SL labeled transcript was competed with a 200-fold molar excess of unlabeled transcript (lanes 11 and 12). The *psbC* specific RNA-binding activity was deficient in dark-grown cell lysate (lane 10) suggesting that *psbC* RNA-binding activity was under light modulation as is translation of the P6 protein *in vivo*.

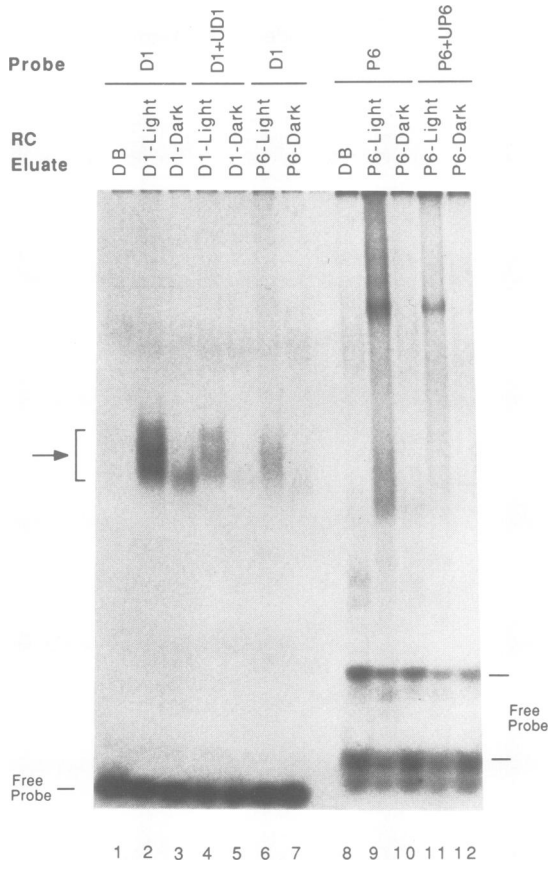


**Fig. 5.** RNA-binding activity of proteins enriched with either D1-HA or P6-SL RNA affinity columns. Proteins eluted from D1-HA RNA affinity columns (RC) incubated with dialysis buffer (DB) light- (D1-Light) or dark-grown (D1-Dark) cell lysate and proteins eluted from P6-SL RNA affinity column incubated with dialysis buffer (DB) light- (P6-Light) or dark-grown (P6-Dark) cell lysate were used for T1-GMS assays. T1-GMS assays included either D1-HA (D1) or P6-SL (P6) labeled RNAs. UD1 and UP6 designate reactions that included 200× unlabeled D1-HA or P6-SL RNAs as competitor, respectively. The arrow indicates the location of the fast-migrating RNA-binding activity. The location of free unbound RNA is indicated (Free Probe).

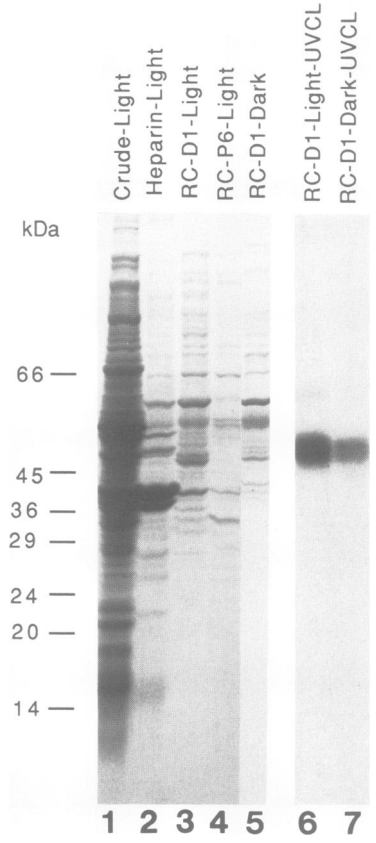


**Fig. 6.** Coomassie stained protein profiles following SDS–PAGE. Protein samples are from light-grown crude cell lysate (Crude-Light), heparin–agarose fractions (Heparin-Light), D1-HA RNA affinity-purified fractions of light-grown (RC-D1-Light) or dark-grown (RC-D1-Dark) cell lysates and P6-SL RNA affinity-purified fractions of light-grown cell lysate (RC-P6-Light). Autoradiogram of D1-HA RNA affinity-purified fractions of light-grown (RC-D1-Light-UVCL) and dark-grown (RC-D1-Dark-UVCL) cell lysates UV crosslinked to $^{32}$P-labeled D1-HA RNA and separated by SDS–PAGE along with the stained proteins. $M_r$s of reference proteins are shown on the left.

### *The psbA and psbC RNA-binding activities are associated with specific sets of proteins*

The progressive selection of a specific set of proteins by heparin–agarose and transcript specific RNA columns was apparent in stained protein gels, as shown in Figure 6 (lanes 1–5). A complex pattern of proteins was isolated by both the D1-HA or the P6-SL RNA columns (lanes 3 and 4). One-dimensional SDS–PAGE showed that although a few proteins may be common to both the *psbA* and *psbC* transcripts, the majority of the proteins appeared to be unique to the different RNAs, suggesting that these proteins were message specific. Washing the RNA columns with heparin or tRNA prior to protein elution did not change the complexity of the RNA-associated proteins (data not shown), again indicating that these proteins were not general RNA-binding proteins, but were specific to mRNAs in general and in some cases to the *psbA* or *psbC* mRNAs in particular. A complex pattern of proteins that was specific to the 3′ end of the *psbA* mRNA and distinct from the protein pattern associated with the 5′ end of the mRNA, as shown by this work, was also recently observed (Schuster and Gruissem, 1991). Comparison of proteins that were selected from light- and dark-grown cell lysates by the D1-HA RNA-affinity column (lanes 3 and 5, respectively), revealed differences in the abundance of several proteins, while others appeared to be present in approximately equal amounts in both light- and dark-grown cell lysates.

To determine which of the binding proteins were in close proximity to the *psbA* RNA, proteins eluted from the D1-HA RNA affinity column were incubated with labeled D1-HA RNA and then crosslinked by UV irradiation. RNA–protein complexes were digested with RNase A followed by SDS–PAGE fractionation. An autoradiogram of the labeled proteins displayed a doublet with molecular weight of ~ 47 kDa (Figure 6, lane 6). When equal amounts of proteins from light- and dark-grown cell lysates were incubated with labeled D1-HA RNA, more RNA-binding activity was detected in light- than in dark-grown cell lysates (Figure 6, lanes 6 and 7). The RNA-binding activity, as revealed by the intensity of the labeled UV crosslinked proteins, was in close agreement with the RNA-binding activity, as revealed by the T1-GMS assays (Figure 5, lanes 2 and 3).

### *The psbA RNA–protein complex identified by T1-GMS is composed of two proteins and a stem–loop RNA structure*

To identify the proteins and the protected RNA fragment which combined to form the *psbA* specific fast migrating RNA–protein complex, both RNA and proteins were isolated from T1-GMS gels. Gel slices containing the RNA–protein complex were excised and either extracted with phenol/chloroform to isolate the protected RNA fragment or placed onto a second dimension SDS–PAGE to visualize the associated proteins.

The protected RNA fragment was resolved on denaturing gel electrophoresis as a single 36 base fragment (Figure 7A, lane 1). The fragment could not be further digested with RNase T1, indicating that no G residues were present in the fragment. To determine the sequence of the protected RNA, unlabeled RNA fragment was isolated, end-labeled and sequenced under denaturing conditions with a C-specific and A- or U-specific RNases (RNases CL3 and Phy M respectively). As shown in Figure 7A, the sequence of the protected RNA fragment was located upstream to the ribosome binding site. This fragment could be folded into a stem–loop structure with 9 paired bases forming the stem and 5 bases in the loop (Figure 7B). To verify the predicted stem–loop structure, the labeled RNA fragment was sequenced under non-denaturing conditions with RNase Phy M. Under non-denaturing conditions Phy M preferentially cleaves unpaired bases. As shown in Figure 7A, lane 4, bases predicted to form the stem were more resistant to the RNase degradation than bases predicted to form the loop region, supporting the predicted secondary structure of this fragment.

To identify the associated proteins, the T1-GMS gel slices containing the *psbA* fast migrating RNA–protein complex were equilibrated in SDS sample buffer and separated by SDS–PAGE followed by silver staining. RNA–protein complexes isolated from both the heparin–agarose fraction and from the D1-HA RNA affinity-purified protein fraction were used. The 47 kDa protein which crosslinked under UV irradiation and an additional 60 kDa protein were detected in both the heparin–agarose and RNA affinity-purified samples (Figure 7C, lanes 1 and 2). Two additional proteins (30 and 33 kDa) were found in the heparin–agarose fractions but not in the RNA affinity-purified lane. These results indicated that the major *psbA* specific RNA-binding activity was comprised of the 47 kDa protein, and possibly an additional protein with a molecular weight of 60 kDa, which specifically associated with the stem–loop RNA structure of the *psbA* mRNA.

### *Accumulation of the 47 kDa protein depends on chloroplast development while its RNA-binding activity appears to be light modulated*

To characterize further and purify the 47 kDa protein, D1-HA RNA affinity chromatography selected proteins were

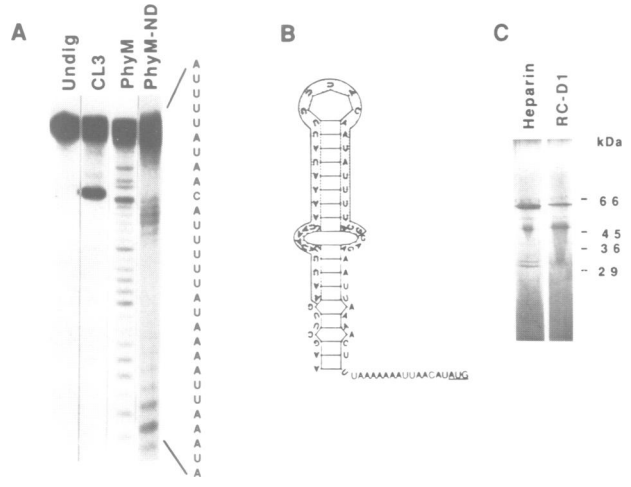


**Fig. 7.** The RNA fragment and proteins that comprise the fast migrating *psbA*-RNA–protein complex. **A.** Sequence of the protected RNA fragment excised from T1-GMS gel. The protected RNA (Undig) was extracted from the gel slices, end-labeled and sequenced under denaturing conditions with C-specific (CL3) and A- and U-specific (Phy M) RNases or non-denaturing conditions with RNase Phy M (PhyM-ND). **B.** Predicted secondary structure of the *psbA* 5′ leader with the RNase T1 protected fragment highlighted. **C.** Purified *psbA* stem–loop RNA-binding proteins excised from T1-GMS gels and separated by SDS–PAGE. Protein samples were from either heparin–agarose (Heparin) or D1-HA RNA affinity (RC-D1) associated proteins. Gels were stained with silver. $M_r$s of reference proteins are indicated.

fractionated by two-dimensional gel electrophoresis (2D-PAGE). The 47 kDa protein was labeled by UV crosslinking and identified as a cluster of four proteins migrating at the basic region of the gel. The four proteins varied in isoelectric point and apparent mass. The more basic proteins displayed decreased mobility in SDS−PAGE. Differences were observed in the abundance of these proteins between light- and dark-grown cell lysates followed by 2D-PAGE stained by Coomassie blue (Figure 8A). The more basic proteins accumulated to a higher level in the light- than in the dark-grown cell lysates, whereas the most acidic protein was present at equal amounts in both light- and dark-grown cell lysates. Mouse polyclonal antiserum was raised against each of the four proteins separately. Each of these sera was found to cross-react with all four proteins. Also, CNBr digestion of the protein eluted from each of the four different spots gave a very similar pattern when separated on reversed phase HPLC (data not shown), suggesting that the diffrent polypeptides were closely related.

To determine whether the observed differences in the RNA-binding activity of the 47 kDa protein were due to an increased abundance of this protein or to modulated activity, cell lysates containing equal amounts of protein for each group were fractionated on SDS−PAGE and analyzed by immunoblotting using one of the polyclonal 47 kDa protein antiserum. As shown in Figure 8B, the abundance of the 47 kDa protein in wt light-grown cell lysates was more than ten-fold that of *y-1* dark-grown cell lysates (lanes 1 and 3), whereas only a small difference (<2-fold) was detected between wt light- and dark-grown cell lysates (Figure 8B, lanes 1 and 2). Differences in the abundance of specific 47 kDa protein bands were observed between light- and dark-grown cell lysates (Figure 8B, lanes 1 and 2). These differences were in agreement with those detected in the 2D-PAGE (Figure 8A). The difference in abundance of the 47 kDa protein between light- and dark-grown cell lysates was more pronounced when the D1-HA RNA affinity-purified proteins were analyzed (Figure 8B, lanes 4 and 5). Also, differences in RNA-binding activity of the 47 kDa proteins isolated from light- or dark-grown cells were detected by both T1-GMS (Figure 4) and UV crosslinking (Figure 6, lanes 6 and 7) assays. Taken together, these data suggest that the 47 kDa proteins from light- and dark grown cell lysates have different RNA-binding activities.

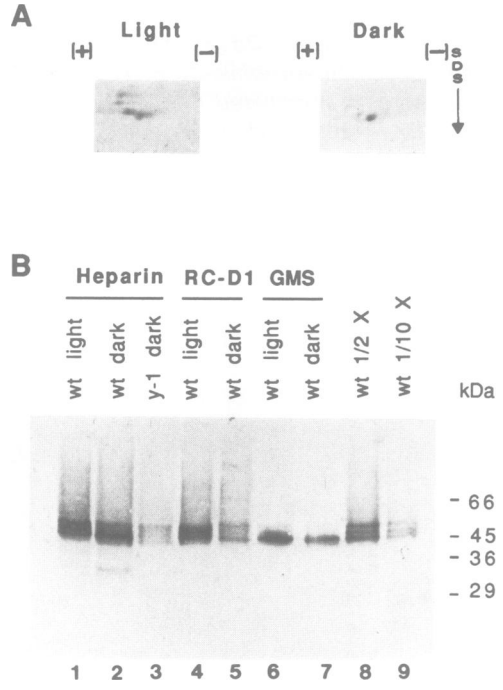


**Fig. 8.** Two-dimensional gel electrophoresis and immunoblotting analysis of the 47 kDa protein isolated from light- and dark-grown cell lysates. **A.** D1-HA RNA affinity-purified proteins from light- (Light) and dark-grown (Dark) cell lysates were subjected to two-dimensional gel electrophoresis (NEPHGE). Gels were stained with Coomassie blue and portions containing the 47 kDa proteins cluster are shown. The basic side of the gel is on the left. **B.** Heparin−agarose chromatography (Heparin), D1-HA RNA affinity chromatography (RC-D1) and Gel Mobility Shift (GMS) purified proteins of light- (light) and dark-grown (dark) cell lysates were fractionated by SDS−PAGE and analyzed by immunoblotting using a polyclonal 47 kDa antiserum. $M_r$s of reference proteins are indicated.

We have not detected *psbA* RNA-binding activity in dark-grown *y-1* cells (Figure 4, lane 7). We attribute the lack of binding of *psbA* RNA to the low abundance of the 47 kDa protein in these cells coupled with the apparent lower RNA-binding capacity of the 47 kDa protein in dark grown cells. Together, these factors may have reduced *psbA*-binding in dark grown *y-1* cells to a level lower than the sensitivity of the T1-GMS assay. Analysis of proteins isolated by T1-GMS gels verified that the T1-GMS purified protein is the 47 kDa protein identified by UV crosslinking (Figure 8B, lane 6). It is unclear why only a single band of the 47 kDa protein was observed in the T1-GMS lanes.

### *Antisera against the 47 kDa protein inhibits the RNA−protein complex formation*

To demonstrate further the participation of the 47 kDa protein in the *psbA* specific RNA−protein complex, we tested the capacity of the 47 kDa protein antisera to inhibit the formation of the *psbA* RNA−protein complex. As shown in Figure 9, formation of the *psbA* specific fast-migrating RNA−protein complex observed in T1-GMS assay (lane 2) was not inhibited by pre-incubation of heparin−agarose isolated proteins with increasing amounts of pre-immune sera (lanes 3−5). However, incubation of the 47 kDa antisera with heparin−agarose isolated proteins prior to incubation with labeled D1-HA RNA inhibited the formation of the RNA−protein complex by up to 77% (lanes 8). These data, combined with the UV crosslinking (Figure 5), SDS−PAGE (Figure 7C) and immunoblotting analyses of T1-GMS purified proteins (Figure 8B lane 6), clearly demonstrate that the 47 kDa protein is the protein which participates in the *psbA* specific RNA−protein complex to confer the mobility shift that is detected by the T1-GMS assays.

## Discussion

Translational regulation is a general mechanism for controlling gene expression in organisms as diverse as *Escherichia coli* and mammals (Portier *et al.*, 1990; Klausner and Harford, 1989; Springer *et al.* 1989; Tang and Draper, 1989). Usually, regulatory proteins interact with specific RNA sequences, stem−loop or pseudoknot structures, located in the 5′-untranslated region and act to repress translation. Activators of translation have also been identified (Altuvia *et al.*, 1987; Wulczyn *et al.*, 1989). Nuclear-encoded transactivating factors that affect translation

of specific mitochondrial proteins have been identified in *Saccharomyces cerevisiae* (Costanzo and Fox, 1988). At least three nuclear genes, *PET494*, *PET54* and *PET122*, and two-thirds of the 5′-UTR are required to activate the translation of the yeast mitochondrial mRNA encoding cytochrome *c* oxidase subunit III (coxIII). A class of nuclear gene products which act as translational activators of specific chloroplast mRNAs and interact with stem−loop structures located in the 5′-UTR of these RNAs has been genetically identified in *C.reinhardtii* (Jensen *et al.*, 1986; Kuchka *et al.*, 1988; Rochaix *et al.*, 1989). Taken together, these data identify the 5′-UTR of the mRNA, especially stem−loop structures, as the target site for protein−RNA interactions involved in translational regulation. Here we report the identification and isolation of chloroplast 5′-UTR transcript-specific RNA-binding proteins from *C.reinhardtii* cells.

The RNA-binding activity for one chloroplastic mRNA, that of the *psbA* gene, has been identified as a complex containing a 47 kDa protein that binds to a 36 base stem−loop RNA structure located adjacent to and upstream of the ribosome binding site of the *psbA* mRNA. The *psbA* mRNA−protein complex also appears to contain an additional 60 kDa protein. The 47 kDa protein has been shown by several criteria to be primarily responsible for binding to and protecting the 5′ stem−loop structure of the *psbA* mRNA. First, this protein has been identified as a component of the *psbA* fast migrating RNA−protein complex that contains the 5′ stem−loop RNA structure (Figure 7A and B), as shown by both the stained gel (Figure 7C) and immunoblotting analysis of T1-GMS isolated proteins (Figure 8B, lane 6). Second, the 47 kDa protein is crosslinked to the *psbA* RNA by UV irradiation (Figure 6, lane 6). Finally, formation of the *psbA* mRNA−protein complex is inhibited by incubation of cell lysates with 47 kDa antisera prior to the addition of labeled *psbA* RNA (Figure 9). Taken together, these results clearly demonstrate that the 47 kDa protein is part of the *psbA* fast migrating RNA−protein complex and that this protein specifically binds to the stem−loop RNA structure located in the 5′-UTR of the *psbA* RNA. The participation of an additional 60 kDa protein as part of the *psbA* RNA−protein complex is suggested by the stained gel (Figure 7 C). The 60 kDa protein was not detected after U.V. crosslinking. This suggests that the 47 kDa protein may be in closer association with the RNA molecule than the 60 kDa protein, which may associate with the complex by interacting with the 47 kDa protein.

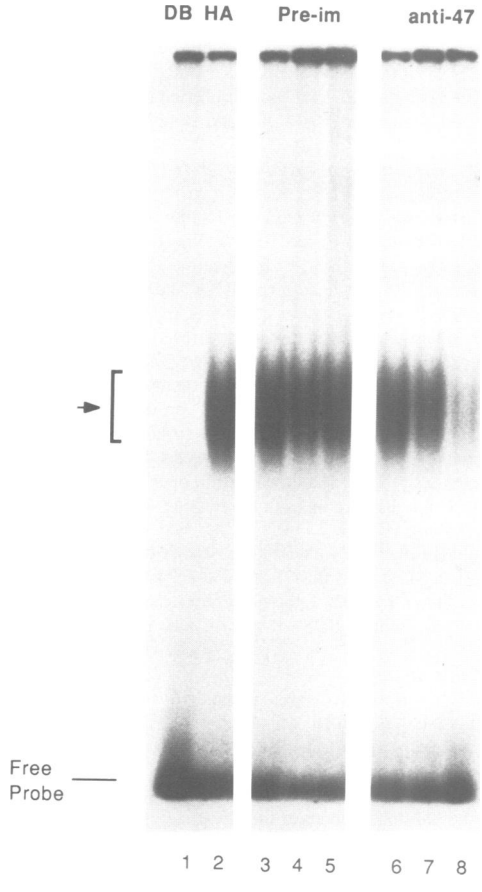


**Fig. 9.** Immuno-inhibition of the formation of the *psbA* specific fast migrating RNA−protein complex. Heparin−agarose isolated proteins containing the *psbA* specific RNA-binding activity were pre-incubated with either pre-immune (Pre-im) or 47 kDa antisera (anti-47) and subjected to T1-GMS assays containing labeled D1-HA RNA. Assays containing either dialysis buffer (DB) or heparin−agarose proteins (HA) that were not pre-treated with antisera were analyzed as control. The arrow indicates the location of the fast-migrating RNA-binding activity. The location of free unbound RNA is indicated (Free Probe).

The level of *psbA* RNA-binding activity in cell lysates (Figure 4) correlates with the level of translation of the D1 protein *in vivo* under light and dark growth conditions (Malnoë *et al.*, 1988). Illuminated cells, which rapidly synthesize the D1 protein, contained high levels of *psbA* mRNA-binding activity, while dark-grown cells, which contain photosynthetic membranes but do not exhibit high levels of D1 translation, showed only a fraction of the *psbA* mRNA-binding activity observed in illuminated cells. Dark-grown cells of the *y-1* mutant, that lack photosynthetic membranes and do not translate *psbA* message, were completely deficient in *psbA* mRNA-binding activity. This three point correlation is consistent with the participation of the RNA−protein complex in the translational regulation of *psbA* mRNA in response to dark to light shifts and to chloroplast development (*y-1* cells lack chloroplasts in the dark).

The *psbA* specific RNA−protein complex constituted by the 47 kDa and perhaps the 60 kDa proteins is identified as a participant in the translational activation of *psbA* mRNA by the following criteria. Genetic analysis of both nuclear and chloroplast mutations in *C.reinhardtii* has predicted the existence of translational activators and identified the site of interaction as the 5′ UTR of chloroplast mRNAs. Biochemical analysis has shown that translation of *psbA* mRNA is light regulated. Here we show that the binding of the 47 kDa protein to the *psbA* RNA is light responsive as described by the T1-GMS assay (Figure 4), UV crosslinking assays (Figure 6), 1D-PAGE (Figure 6) and 2D-PAGE (Figure 8A) and immunoblotting analysis utilizing 47 kDa antisera (Figure 8B). Further, the 47 kDa and perhaps the 60 kDa proteins bind to and protect only the stem−loop RNA structure located upstream of the ribosome binding site. Thus, the *psbA* RNA−protein complex identified in this study satisfies both the genetic and biochemical predictions for participation in activation of *psbA* mRNA translation.

The protected RNA fragment associated with the *psbA* RNA−protein complex is a stem−loop RNA structure that is located adjacent and upstream to the ribosome binding

site (Figure 7A and B). The proximity of the stem–loop structure and the ribosome binding site suggests that regulation of D1 translation may be achieved by protein/RNA complex-induced conformational change in the *psbA* mRNA. In bacteriophage Mu, the Com protein has been shown to enhance translation of the *mom* mRNA by binding to and inducing a conformational change in the translational initiation region of the *mom* mRNA (Wulczyn and Kahmann, 1991). An alternative way of enhancing translation may involve protein–protein interactions between the 47 and 60 kDa protein complex bound to the *psbA* stem–loop and the ribosomal subunits at the adjacent ribosome binding site.

Large differences in the abundance of the 47 kDa protein are apparent between wt light-grown and *y-1* dark-grown cell lysates (fig. 8B, lanes 1 and 3), indicating that the level of accumulation of the 47 kDa protein is regulated during plastid biogenesis. *psbA* mRNA is transcribed and accumulates in non-photosynthetic plastids (tomato roots), but is apparently not translated (Deng and Gruissem, 1988). Blocking the accumulation of the nuclear-encoded *psbA* translational activators would allow for repression of D1 protein synthesis in root plastids independent of *psbA* mRNA accumulation within these tissues. We have not detected large differences in the abundance of the 47 kDa protein between light- and dark-grown wt cell lysates (fig. 8B, lanes 1 and 2), although differences in specific 47 kDa polypeptides were apparent (Figure 8A and B, lanes 1 and 2). Large differences in RNA-binding activity were observed when light- and dark-grown cell lysates were analyzed by T1-GMS (Figure 4, lanes 5 and 6) and UV crosslinking (Figure 6, lanes 6 and 7). These data support the model of light modulated RNA-binding activity of existing 47 kDa protein which would allow for quick changes in translational level in response to altered light intensity. Rapid response of D1 translation level is observed in *C.reinhardtii* (Malnoë *et al.*, 1988).

A combination of regulation of translational factor accumulation coupled with light responsive RNA-binding activity of the translational factor would provide a precise mechanism for coordinating nuclear and chloroplast gene expression in response to variation in light intensity and to changes in the developmental stage of the plastid. This model can be tested by determining the plastid specificity, accumulation and activity of these translational activators in different cell types during development in higher plants.

## Materials and methods

### *Recombinant plasmid construction*

All DNA templates were cloned under control of the promoter of the T7 DNA-dependent RNA polymerase of Bluescript, M13–, SK (Stratagene). The cloned *psbA* *Hin*dIII–*Acc*I (D1-HA) fragment includes 72 bases of the 5′-untranslated region (5′ UTR) and 173 bases of coding sequences, as shown in Figure 1. The stem–loop (P6-SL), located between bases −336 to −214 of the 5′-UTR of *psbC* (Malnoë *et al.*, 1988), was cloned out of the *psbC* *Ssp*I–*Hin*dIII fragment in a standard PCR reaction which included the following oligonucleotides: P6–5′, 5′-CCGAATTCGTTTTAAATC-AACT-3′ and P6–3′, 5′-CCGAATTCGAATAGAATTTTATAAC-3′. All the transformants were sequenced to verify the authenticity of the cloned sequence.

### *T7 DNA-dependent RNA polymerase isolation and in vitro transcription reactions*

T7 DNA-dependent RNA polymerase was isolated (Butler and Chamberlin) from a cloned T7 RNA polymerase that was kindly provided by F.W.Studier (Davanloo *et al.*, 1984). Radiolabeled RNA transcripts were synthesized (Davanloo *et al.*, 1984) from 0.5 μg recombinant plasmid DNA in the presence of 20 μCi [α-$^{32}$P]UTP (800 Ci/mmol)(Amersham), 1 mM ATP, CTP and GTP (Pharmacia), 40 mM Tris–Cl (pH 7.5), 50 mM NaCl, 20 mM DTT, 8 mM $MgCl_2$ and 10 U T7 RNA polymerase in a 10 μl reaction volume. Samples were incubated for 1 h at 37°C. The specific activity under these conditions is $2.5\times10^{13}$ d.p.m./μg RNA. For large scale synthesis of unlabeled RNA, transcription reactions were performed with 200 μg plasmid DNA in the presence of 3 mM ATP, CTP, GTP and UTP, 40 mM Tris–HCl (pH 7.5), 50 mM NaCl, 20 mM DTT, 24 mM $MgCl_2$ and 20 000 U of T7 RNA polymerase in a 2 ml reaction volume. Samples were incubated for 2 h at 37°C.

### *Growth conditions and preparation of cell lysates*

*Chlamydomonas reinhardtii* cells were grown in Tris-acetate-phosphate medium (pH 7.0) (Gorman and Levine, 1965) at 25°C to a cell density of $\sim1\times10^7$ cells/ml. Cells were harvested as previously described (Malnoë *et al.*, 1988) and immediately frozen in liquid nitrogen and stored at −70°C until use. About 50 g of frozen cells were thawed in 100 ml low salt extraction buffer [10 mM Tris–Cl (pH 7.5), 10 mM NaCl, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol] and passed once through a cell disruption bomb (Parr Instrument Co., Moline, IL) The cell extract was centrifuged for 1 h at 200 000 *g*, at 4°C and immediately applied to a heparin–agarose column. Unfractionated cell lysate (crude extract) was dialyzed against 500 vol of dialysis buffer [20 mM Tris–Cl (pH 7.5), 100 mM KOAc, 0.2 mM EDTA, 5 mM 2-mercaptoethanol, 20% glycerol] overnight at 4°C and stored at −70°C.

### *Heparin–agarose column chromatography*

Heparin actigel (Sterogene, Bioseparation) was washed with buffer A [20 mM Tris–Cl (pH 7.5), 3 mM $MgCl_2$, 0.1 mM EDTA, 5 mM 2-mercaptoethanol] with 2 M KOAc and equilibrated in buffer A before lysate fractionation. Cell extract was applied at 0.3 cm/min to 50 ml heparin actigel and the column was washed at the same flow rate with 4 column bed volumes of buffer A. Proteins were eluted with a 0–1.6 M KOAc gradient over 3 column bed volumes and collected in 5 ml fractions. Fractions were dialyzed against dialysis buffer and stored at −70°C.

### *RNase T1 protection gel–mobility shift (T1-GMS) assay and UV crosslinking*

Dialyzed lysate was pre-incubated for 15 min at room temperature with 0.25 U of Inhibit-Ace (5-Prime 3-Prime, Inc.) in the presence of 3 mM $MgCl_2$ and added to 66% (v/v) of the final binding reaction volume (15 μl) which contained 20 μg wheat germ tRNA and 0.065 pmol radiolabeled transcript ($6\times10^5$ c.p.m.). After 15 min at room temperature, 0.5 U of RNase T1 (Pharmacia) were added, the reaction incubated for another 10 min, loaded immediately onto a 1×TBE, 5% native PAGE and run at 130 V constant voltage for 3.5 h. RNA–protein complexes were detected by autoradiography. Equal amounts of proteins were estimated by fractionating the protein samples with SDS–PAGE and visual determination of the amount of Coomassie Blue-stained proteins. In UV crosslinking assays, binding reactions (15 μl) were irradiated in open 0.6 ml Eppendorf tubes, for 1 h on ice at a distance of 1 cm from top of the tube, with a hand-held Mineralight lamp (UVGL-25, UVP, Inc. San Gabriel, CA). The RNA transcripts were digested with 50 μg RNase A (Sigma) for 30 min in the presence of 3 M urea, 3 mM EDTA at 55°C. After adding SDS and 2-mercaptoethanol to 2.5% final concentrations, proteins were separated on a 7.5–15% gradient SDS–PAGE (Chua, 1980).

### *RNA affinity column*

Unlabeled RNA transcripts were oxidized with 10 mM $NaIO_4$ for 30 min on ice and in the dark to form a diketone on the 3′ terminal ribose residue. Glycerol was then added to a final concentration of 0.2% to quench the reaction. The diketone was coupled to the terminal amine of aminogel-B (Sterogene Bioseparation) in a ratio of 17 nmol RNA to 0.7 ml gel, by reducing the aldimine adduct in the presence of 10 mM $NaBH_4$ for 2 h at room temperature. The column was washed with 10 ml binding buffer + 1 M KOAc, followed by 10 ml binding buffer + 0.1 M KOAc. Protein samples in dialysis buffer were incubated with Inhibit-Ace (5-Prime 3-Prime Inc.) (5 U/ml) and 3 mM $MgCl_2$ for 20 min at room temperature. Protein samples were circulated twice over the column followed by a sequential wash with 5 ml binding buffer + 0.1 M KOAc including 1.3 mg/ml wheat germ tRNA, and 5 ml binding buffer + 0.1 M KOAc. Bound proteins were eluted with binding buffer + 0.55 M KOAc.

### *GMS purification of the psbA RNA-binding complex*

RNA–protein complexes were fractionated with T1-GMS as the first dimension. Following exposure of the non-fixed wet gels, the gels slices

containing the fast migrating *psbA* RNA–protein complex were cut out and equilibrated with SDS-sample buffer for 30 min at room temperature and proteins were separated with SDS–PAGE (Chua, 1980). Silver staining was performed according to Sammons *et al.* (1981). For RNA isolation, gel slices were cut and soaked over night in a buffer containing 20 mM Tris–Cl (pH 7.5), 1 mM EDTA, 300 mM NaCl, 0.1% SDS followed by phenol/chloroform extraction. The RNA was precipitated with ethanol and purified on a Sephadex G-50 fine (Pharmacia) 2 ml column. The purified RNA fragment was treated with alkaline phosphatase in a reaction containing 25 mM Tris–Cl (pH 8.0), 0.2 mM EDTA and 1 U calf intestinal alkaline phosphatase (Pharmacia) for 30 min at 37°C. The reaction was extracted with phenol/chloroform and the RNA was 5′ end-labeled for 30 min at 37°C in a reaction containing 25 mM Tris–Cl (pH 9.0), 5 mM $MgCl_2$, 3 mM DTT, 200 μCi 5′ [$\gamma$-$^{32}$P]ATP (5000 Ci/mmol, Amersham) and 10 U T4 Kinase (Pharmacia). RNases CL3 and PhyM (Pharmacia) were used to sequence the RNA fragment under denaturing or non-denaturing conditions according to the manufacturer's protocol. Sequencing reactions were fractionated on 8 M urea, 1×TBE, 15% PAGE.

***Isolation of the 47 kDa protein, production of specific antisera in mice, immunoblotting analysis and immuno-inhibition assay***

D1-HA RNA affinity-purified protein lysates were fractionated by two-dimensional gel electrophoresis (O'Farrell *et al.*, 1977), followed by electroblotting to nitrocellulose membrane. The cluster of 47 kDa proteins was identified by staining with amido black, and each spot was cut out and used to immunize AJ mice according to Knudsen (1985). For immuno-inhibition, heparin–agarose isolated proteins containing the *psbA* specific RNA-binding activity were pre-incubated for 20 min with an equal amount of protein G-purified IgG from either pre-immune or 47 kDa antisera and 0.25 U Inhibit-Ace and than subjected to T1-GMS assays containing labeled D1-HA RNA. The protein G purification step was needed to remove the nuclease activity inherent to the crude bleed.

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