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Characterization of protein-binding to the spinach chloroplast *psbA* mRNA 5′ untranslated region

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ABSTRACT

RNA-binding proteins play a major role in regulating mRNA metabolism in chloroplasts. In this work we characterized two proteins, of 43 and 47 kDa, which bind to the spinach *psbA* mRNA 5′ untranslated region (*psbA* encoding the D1 protein of photosystem II). The 43 kDa protein, which is present in the stroma and in membranes, co-sediments with a complex of 68S. It was purified, and the N-terminal sequence was determined. Upon homology search it was identified as the chloroplast homologue of the *Escherichia coli* ribosomal protein S1. The 47 kDa protein, which, in contrast with the 43 kDa protein, sediments with a small sedimentation coefficient, is only detected in the stromal fraction. It is soluble in an uncomplexed form. By deletion analysis, an element within the *psbA* mRNA 5′ untranslated region was identified that is necessary but not sufficient for binding of stromal proteins. The 'central protein binding element' ranges from nucleotide –49 to –9 of the *psbA* mRNA 5′ untranslated region. It comprises the Shine–Dalgarno-like GGAG motif and, 7 nucleotides upstream, an endonucleolytic cleavage site involved in *psbA* mRNA degradation *in vitro*. The mechanistic impacts of this region in relation to RNA-binding proteins are discussed.

INTRODUCTION

In chloroplasts of higher plants, post-transcriptional mechanisms such as RNA processing, differential mRNA stability and translational control contribute to the regulation of gene expression during chloroplast development and light-dependent protein synthesis, respectively (1). Translational control as well as specific mRNA degradation is often mediated by RNA-binding proteins. Some of the chloroplast RNA-binding proteins from spinach, which are primarily involved in RNA processing, have already been cloned and identified.

A family of nuclear-encoded chloroplast RNA-binding proteins with a molecular weight in the range of 28 kDa has been described in several plant species, while the most detailed data have been obtained from spinach. Typical for these proteins is the presence of an RNA-binding recognition motif (2). In spinach, the respective protein is involved in the formation of mature 3′ ends because immunodepletion or addition of the recombinant protein to an *in vitro* processing system interferes with 3′ end formation (3,4). Similar proteins have also been identified in tobacco (5,6), *Arabidopsis* (7,8), bean (9) and maize (10). Furthermore, several chloroplast proteins of the mRNA degradation machinery have already been described (11). A protein with the molecular weight of 100 kDa was shown to have similar properties to the bacterial polynucleotide phosphorylase (PNPase), which is a key exonuclease involved in bacterial mRNA degradation (12). The amino acid sequence is 63% homologous between both enzymes (11).

Several other higher plant chloroplast RNA-binding proteins with enzymatic activity have been analyzed either in spinach or in mustard. These are mostly ribonucleases, such as the endonuclease of 54 kDa isolated from mustard which is required for the 3′ processing of the *trnK* (tRNA-lysine) and *rps16* (ribosomal protein 16 of the large ribosomal subunit) precursor transcript *in vitro* (13,14). A complex also involved in 3′ end formation is found at the *petD* pre-mRNA (encoding subunit IV of the cytochrome b_6/f complex). It consists of three protein species of 29, 41 and 55 kDa that recognize an AU-rich element downstream of the stem–loop structure that flanks most of the protein coding mRNAs in chloroplasts (15). Among these proteins, the 41 kDa proteins is a nuclease which cleaves RNA to small oligonucleotides (16).

RNA-binding proteins recognizing the 5′ end of chloroplast mRNAs are mostly described in *Chlamydomonas reinhardtii*, where they are involved in either in stabilizing mRNA transcripts (17) or in mediating translational regulation (18–21). A systematic study in which protein binding had been analyzed in *C.reinhardtii* under different light and nutritional conditions for several genes showed at least six species that could be detected by UV-cross-linking (22).

Earlier, we described several proteins binding to the 5′ untranslated region of the spinach *psbA* mRNA (encoding the D1 protein of photosystem II) (23). In stromal protein extracts we are most interested in two proteins with molecular weights of 43 and 47 kDa. The 43 kDa protein has been analyzed in great detail with regard to its binding properties. Binding activity of this protein can be detected only after plants have been illuminated. The protein has a high affinity for U-rich, single-stranded RNA; it can be cross-linked to the *psbA* mRNA 5′ untranslated region but not to the 3′ untranslated region of the *psbA* mRNA (23).

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In this work, we show that the proteins of 43 and 47 kDa are part of complexes which can be separated according to their sedimentation properties. We purified the 43 kDa protein, so we could obtain peptide sequence data that allowed us to identify the protein as the chloroplast homologue of the ribosomal protein S1, based on 14 amino acid identities. Furthermore, we determined an element within the *psbA* mRNA 5′ untranslated region which is necessary but not sufficient for protein complex formation by deletion analysis. This element comprises the Shine–Dalgarno-like GGAG motif and, 7 nucleotides upstream, an endonucleolytic cleavage site involved in *psbA* mRNA degradation *in vitro*.

MATERIALS AND METHODS

Nucleic acids

Plasmid DNA. The *psbA* mRNA 5′ untranslated region had been cloned as a 142 bp *Hin*cII–*Mvn*I fragment, –119 to +23 (24), into the *Hin*cII–*Sma*I sites of pBluescript-SK+ (Stratagene, Heidelberg, Germany).

PCR products. PCR fragments for *in vitro* transcription covered the desired region of the *psbA* gene fused to a T7 promoter. Primers used for PCR were: psbA_-86-T7, 5′-GCGCCAGATCT-TAATACGACTCACTATAGGATAACAATCTTTCAATTTCTA-TTTCT-3′ (–87 to –60 of the *psbA* gene); psbA_-49-T7, 5′-GCGCCAGATCTTAATACGACTCACTATAGGTGTGCTT-GGGAGTCC-3′ (–49 to –35 of the *psbA* gene); psbA_3′_+8, 5′-GCAGTCATGGTAAAATCTTGGTT-3′ (complementary to –15 to +8 of the *psbA* gene); psbA_3′_-9, 5′-CTTGGTTTATTT-AATTTAATCATC-3′ (complementary to –32 to –9 of the *psbA* gene). The nucleotide positions are numbered according to Zurawski *et al*. (24). PCR reactions were performed using buffers and protocols supplied by the manufacturer (Promega, Madison, USA). As template DNA, 1 ng linearized plasmid containing a fragment covering the *psbA* mRNA 5′ region was used in the PCR reactions, in which 37 cycles (92°C, 90 s; 45°C, 60 s; 72°C, 90 s) were performed. PCR products were purified by spin columns (Qiaquick, Qiagen, Hilden, Germany) according to the manufacturer's instructions. For *in vitro* transcriptions, 1/20 of a PCR reaction was used.

Antiserum

Antiserum against the *E.coli* ribosomal protein S1 was kindly provided by Dr R.Brimacombe (Max-Planck Institut für Molekulare Genetik, Berlin, Germany). It was used in a dilution of 1/1000 in gel retardation assays.

In vitro RNA synthesis

Radiolabeled RNA transcripts were synthesized using the T7 *in vitro* transcription system. Briefly, for UV-crosslinking, conditions were as follows: 0.5 µg linearized DNA, 20 mM NaP_i, pH 7.7, 10 mM DTT, 8 mM $MgCl_2$, 20 mM spermidine, 1 mM each of ATP, CTP and GTP, 25 µM UTP, 35 µCi [α-^{32}P]UTP, 20 U RNasin, 30 U T7 polymerase; 1 h at 37°C. Transcripts were purified from unincorporated nucleotides by precipitation with 0.5 vol 7.5 M (NH_4)OAc, 2.5 vol EtOH. For gel retardation, 1/20 of a PCR reaction was used as template. The portion of radioactive [α-^{32}P]UTP was reduced to 10 µCi, otherwise the same conditions as described above were employed. End-labeled transcripts were synthesized under the same conditions, except that 1 mM UTP was used and 2 mM GpG was added. Transcripts were gel purified and 5′-end-labeled with 10 U T4 polynucleotide kinase in the presence of 50 µCi [γ-^{32}P]ATP for 1 h at 37°C using the buffer supplied by the manufacturer. End-labeled transcripts were again gel purified. To obtain 3′ end-labeled RNA, transcripts were synthesized under the same conditions, except that 1 mM UTP was used. Transcripts were end-labeled using T4 RNA ligase according to Bruce and Uhlenbeck (25). Briefly, 10 pmol RNA were labeled in 50 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 10 µg/ml BSA, 3.3 mg/ml DTE, 10% DMSO, 1 mM ATP, 25 µCi 5′ [^{32}P]pCp and 2 U T4 RNA ligase for 2 h at 37°C. All RNA transcripts were purified by gel electrophoresis.

Deletion mutants. To obtain 3′ and 5′ deletions, respectively, end-labeled transcripts were digested with 2 U RNase T1 (Boehringer, Mannheim, Germany) for 5 min at room temperature. After phenol/chloroform extraction, fragments were separated on 8% sequencing gels (7 M urea, 1× TBE) and eluted after exposure.

Protein extracts

Protein extracts representing the stroma and membrane fraction were obtained from intact chloroplasts, which had been isolated by percoll gradients as described (26). For plastid fractionation (27), intact chloroplasts were resuspended in 20 mM Tris–HCl, pH 8.8, 20 mM KCl, 10 mM $MgCl_2$, 0.5 mg/ml heparin, 5 mM DTT. After rigorous suspension, membranes were sedimented for 15 min at 15 000 r.p.m. (Beckman JA20). The supernatant, containing the soluble stroma fraction, was frozen at –70°C after adjustment to 15% glycerol. The sedimented membranes were resuspended in a small volume of the above-mentioned buffer. Protein concentrations were determined according to Bradford (28).

UV-crosslinking

For analysis of protein binding, label transfer experiments were performed according to Klaff and Gruissem (23). Unless stated otherwise, 20 µg of protein extract was incubated with 10 fmol of synthetic radiolabeled RNA in 5 mM Tris–HCl, pH 9.0, 20 mM KCl, 3 mM $MgCl_2$, 5 mM EDTA, 0.1 mM DTT in microfuge tubes. After 10 min of incubation at 25°C, the opened tubes were irradiated with UV light of 254 nm and an energy of 1.8 J/cm^2 at 25°C using a stratalinker (Stratagene, Heidelberg, Germany). The RNA was digested with 10 U RNase T1 and 10 µg RNase A per sample at 37°C for 30 min. For SDS–gel electrophoresis, 1 vol 7% SDS, 15% β-mercaptoethanol, 30% glycerol, 0.19 M Tris–HCl, pH 6.8, and 0.001% bromphenol blue was added. After 5 min incubation at 85°C, the samples were loaded onto 10% polyacrylamide–SDS gels according to Laemmli (29). The gels were stained with silver nitrate (30), dried and exposed to Kodak XAR X-ray films.

Gel retardation analysis

For analysis of complex formation of RNA with proteins, gel retardation assays were performed according to Konarska and Sharp (31) with some modifications. Twenty micrograms of protein (or the amount of protein as noted in the figures) were incubated for 5 min at 22°C in 10 µl of 20 mM Tris–HCl, pH 8.5, 20 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 2 mg/ml heparin, 0.5 mg/ml tRNA. After addition of 5 fmol RNA (or the amount as

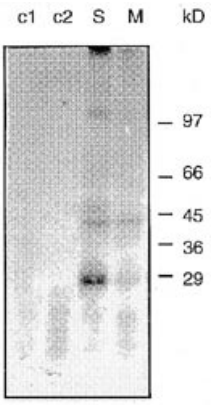

Figure 1. RNA-binding proteins to the *psbA* mRNA 5′ untranslated region in stroma and membrane fraction by UV-crosslinking. Thirty micrograms of protein were incubated with 20 fmol of radiolabeled RNA and treated for UV-crosslinking as described in the Materials and Methods. c1: RNA, UV-treated, RNase-treated; c2: RNA with stromal extract, not irradiated, RNase-treated. S, UV-crosslinking with stromal protein; M, UV-crosslinking with membrane protein.

noted in the Results), complex formation was allowed to take place during 15 min incubation at 22 °C. Afterwards, 5 µl 80% glycerol containing bromphenol blue and xylene cyanole were added for electrophoresis. Samples were separated on 8% polyacrylamide gels (acrylamide:bisacrylamide ratio 20:1) containing 50 mM Tris, 0.38 M glycine (2× Laemmli-buffer) at 200 V for 75 min at 22 °C.

Sucrose gradient centrifugation

For sucrose gradient centrifugation, intact chloroplasts were isolated and fractionated as described above. The stromal extract (2 ml) was layered onto 38 ml 10–40% continuous sucrose gradients containing 20 mM Tris–HCl, pH 8.8, 20 mM KCl, 10 mM $MgCl_2$, 0.5 mg/ml heparin, 5 mM DTT, 50 µg/ml chloramphenicol (32). After centrifugation for 19 h at 19 000 r.p.m. using a Beckman SW28 rotor, 20× 2 ml fractions were harvested and stored at –70 °C prior to use. Protein concentrations were determined according to Bradford (28). Sedimentation coefficients were determined according to Griffith (33) based on sucrose concentrations within the respective fractions and centrifugation conditions [$\omega^2 t = (2.72 \times 10^{11})/s$]. Sucrose concentrations were determined by refractometer.

Protein sequencing

To purify the 43 kDa protein for protein sequencing, a sucrose gradient as described above was loaded with 50–60 mg stromal protein. After centrifugation for 19 h at 19 000 r.p.m. using a Beckman SW28 rotor, 10× 4 ml fractions were harvested. Fractions containing the 43 kDa protein as analyzed by UV crosslinking were separated by SDS–gel electrophoresis (29) and transferred to polyvinylfluoride membrane (Immobilon, Millipore) in 10 mM CAPS, pH 11, 10% methanol (34). Electrophoretic separation of the fractions containing the 43 kDa protein allowed excision of the corresponding band without contaminating additional protein species. N-terminal sequencing of the protein after transfer was performed by Dipl.-Ing. Dagmar Müller (Institut für Enzymtechnologie, KFA Jülich, Germany).

RESULTS

Proteins binding to the *psbA* mRNA 5′ untranslated leader RNA are components of different complexes

To obtain information about the mRNA-binding 43 and 47 kDa proteins and their association with chloroplast fractions, the distribution of proteins binding to the *psbA* mRNA 5′ untranslated region between the soluble stroma and the membranes was analyzed. Figure 1 shows the comparison of the protein binding pattern in both fractions by UV-crosslinking analysis. The 43 kDa protein, which has a binding preference to the *psbA* mRNA 5′ untranslated region (23), is present in the membranes as well as in the stroma. The 47 kDa protein can only be detected in the stromal fraction. The 100 kDa RNA-binding protein, which is detectable in the stromal fraction, is the chloroplast homologue of the *E.coli* polynucleotide phosphorylase, an exonuclease involved in chloroplast RNA 3′ end processing and degradation (11). The 28 kDa RNA-binding protein detectable in both fractions has been described as an activity that is necessary for the formation of the mature mRNA 3′ ends (3).

The association of the stromal RNA-binding proteins with macromolecular complexes was analyzed by sucrose gradient centrifugation. Under native conditions, complexes are separated in their native form and their constituents, in this case the RNA-binding proteins, can be determined subsequently. Determination of sedimentation coefficients may indicate to which complex in the chloroplast the proteins belong. Chloroplasts were isolated from light-grown plants (26) and separated into stromal and membrane fraction (27). The stromal extract was fractionated by ultracentrifugation as described in the Materials and Methods. The sedimentation profile of the proteins is shown in Figure 2A. Fractions are numbered from the top to the bottom of the gradient. Most of the total protein sediments in the upper third of the gradient in a broad peak, while only a small peak can be detected with a higher sedimentation constant. The protein pattern of each fraction is shown in Figure 2B as an SDS–gel stained with silver. A volume of 1.5% of each fraction was analyzed for the contents of proteins binding to the *psbA* mRNA 5′ untranslated region using gel retardation, as shown in Figure 2C, and the UV-crosslinking assay depicted in Figure 2D. Gel retardation shows two peaks within the gradient containing proteins which bind to the *psbA* mRNA 5′ untranslated region. Heparin is added to a concentration of 2 mg/ml and tRNA to a concentration of 0.5 mg/ml to avoid unspecific complex formation. The peak of complex formation at the top of the gradient is shifted against the peak of protein concentration. While the highest activity of RNA-binding proteins is detected in fraction 2, the highest protein concentration can be detected in fraction 5. This results from the large amount of ribulose-1,5-bisphosphate carboxylase, which sediments with a peak in fraction 5 (Fig. 2B; the large subunit of ribulose-1,5-bisphosphate carboxylase is labeled as LSU). The second peak of RNA-binding activity sediments with a higher sedimentation coefficient and a maximum of complex formation in fraction 13. Here, binding activity correlates with the concentration profile of proteins. The proteins of the different fractions which are in direct contact with the RNA are analyzed by UV-crosslinking as shown in Figure 2D. At the top of the gradient, the 28 kDa (3) and a 33 kDa protein can be detected, which copurifies with the PNPase using a size exclusion chromatography (11). In addition, the 47 kDa protein is found.

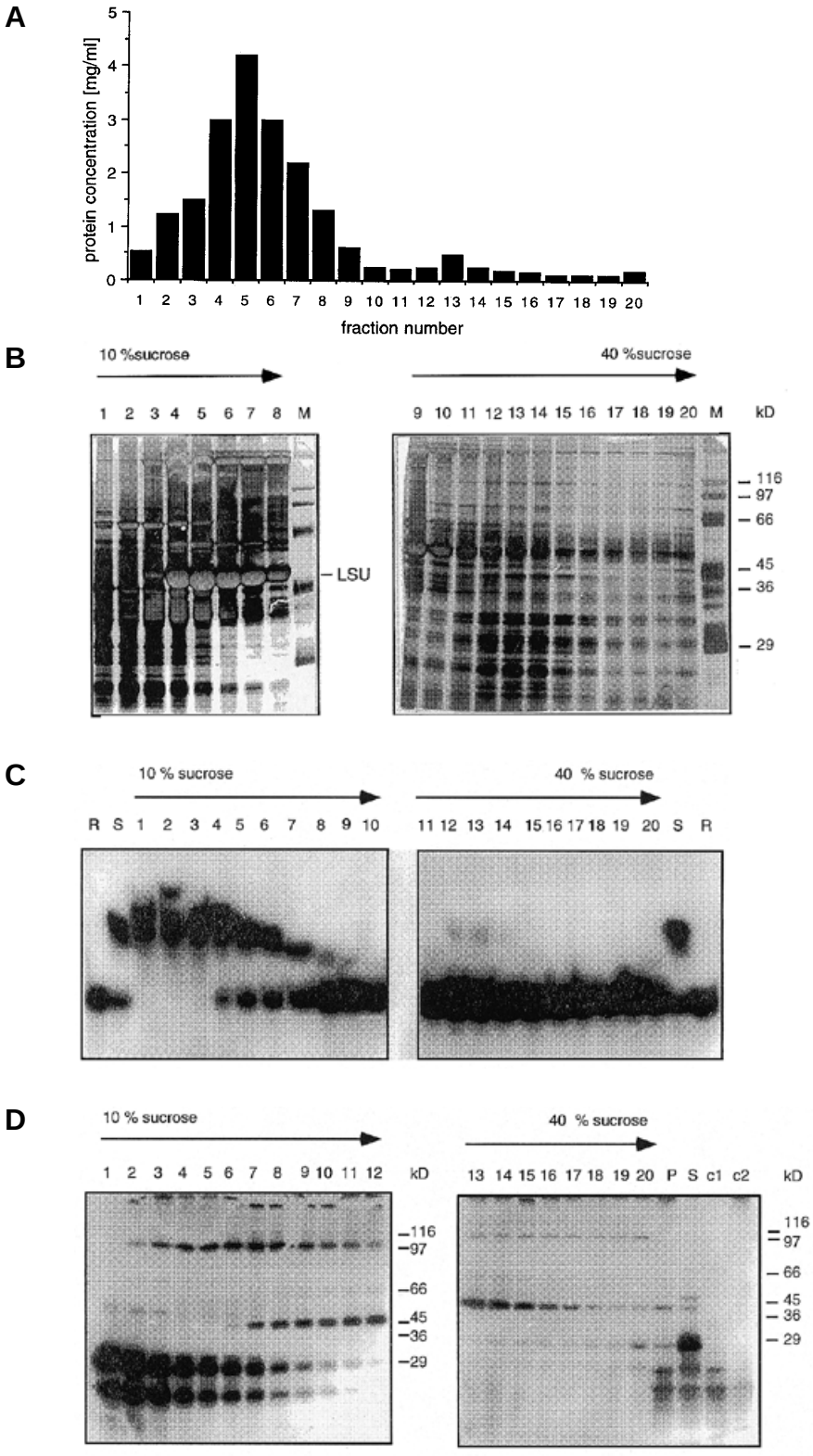

Figure 2. Sucrose gradient fractionation of stromal complexes. After separation, the stromal extract was fractionated by a 38 ml 10–40% sucrose gradient for 19 h at 19 000 r.p.m. (Beckman SW28). Twenty 2 ml fractions were harvested. (**A**) Profile of protein concentration along the gradient. (**B**) Profile of protein distribution along the gradient as shown by SDS–gel electrophoresis and silver staining; 1.5% of each fraction was used. The large subunit of ribulose-1,5-bisphosphate carboxylase is labeled as LSU. (**C**) Profile of RNA-binding proteins along the gradient, analyzed by gel retardation; 1.5% of each fraction was used. (**D**) Profile of RNA-binding proteins along the gradient, analyzed by UV-crosslinking; 1.5% of each fraction was used. R, free RNA; S, stromal protein; P, pellet of the gradient. c1: RNA, UV-treated, RNase-treated; c2: RNA with stromal extract, not irradiated, RNase-treated. The numbers given in the figure refer to the numbers of fractions, with fraction 1 at the top and fraction 20 at the bottom of the sucrose gradient.

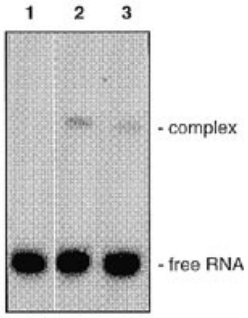

Figure 3. Presence of the chloroplast ribosomal protein S1 in RNA–protein complexes. Five fmol of radiolabeled RNA representing the 5′ untranslated region of *psbA* mRNA were incubated with 0.5% of fractions 12–14 of the sucrose gradient in the presence and in the absence of an antiserum raised against the *E.coli* S1 protein and separated on 8% polyacrylamide gels. 1, 5 fmol RNA; 2, 5 fmol RNA, 0.5% from fractions 12–14; 3, 5 fmol RNA, 0.5% of protein from fractions 12–14, polyclonal antiserum raised against the *E.coli* ribosomal protein S1. Binding of the antibody to the RNA–protein complex can be detected by the reduction of complex band in lane 3. RNA incubated with antiserum shows no effect (data not shown).

The chloroplast PNPase, with a molecular weight of 100 kDa, sediments in a broad distribution starting from fraction 2, with a peak in fractions 6/7 and is detectable along the whole gradient (Fig. 2D). The 43 kDa protein is only found sedimenting with higher sedimentation constants peaking in fractions 12/13. It also sediments in a broad distribution. The broadened peaks result from a slight overloading of the gradient. Comparison of the patterns of RNA-binding activities obtained by gel retardation analysis and UV-crosslinking shows the same peak fractions of proteins binding to the *psbA* 5′ untranslated region. The difference in binding patterns of fractions adjacent to the peaks as found by both methods results from their physical principles. While in gel retardation the equilibrium between the binding partners is observed, in UV-crosslinking, complexes are convalently linked fixing contacts with short half-lives. When working in a concentration range of the reciprocal binding constant, a higher concentration of RNA-binding proteins has to be present to form complexes detectable by gel retardation as by UV-crosslinking.

The 43 and 47 kDa proteins are separated by sucrose gradient centrifugation. The 47 kDa species sediments at the top of the gradient, indicating that under these conditions it is not associated with any larger complex. The 43 kDa species, which sediments into the gradient, has to be associated to additional components. The peak fraction of the 43 kDa protein (No. 13) contains a sucrose concentration of 27% as determined by refractometer. According to Griffith (33), a sedimentation constant of 68S can be calculated based on the sucrose concentration and the centrifugation conditions [$\omega^2 t = (2.72 \times 10^{11})/s$], indicating that the 43 kDa protein co-sediments with the ribosomes.

Using sucrose gradients loaded with a lower amount of stromal protein, we obtained fractions in which the 43 kDa protein could be separated from co-sedimenting proteins by SDS–gel electrophoresis. After transfer to polyvinylfluoride membrane the N-terminal sequence of the protein could be determined without background. Fourteen N-terminal amino acids were identified: Ala–Val–Ala–Val–Ser–Asn–Ala–Gln–Thr–Arg–Glu–Arg–Gln–Lys–Leu. The homology search resulted in one protein that has this sequence with 100% identity: the spinach chloroplast homologue of the ribosomal protein S1 (35). Based on the probability of $1/20^{14}$ for the sequence to occur a second time in combination with the sedimentation data, we are confident that the 43 kDa protein is the spinach chloroplast homologue of the ribosomal protein S1. This interpretation is further supported by the finding that a polyclonal antiserum raised against the *E.coli* ribosomal protein S1 crossreacts with the 43 kDa protein (data not shown).

UV-crosslinking data indicate that the complexes formed in fractions 12–14 of the sucrose gradient resulting in a complex band in gel retardation are composed of the S1 protein and/or the 100 kDa PNPase. The addition of the *E.coli* antiserum to the incubation mixture for gel retardation results in the reduction of the complexed band as shown in Figure 3. As the antiserum is heterologous, the antibody binding is too weak to induce a supershift. The RNA–protein–antibody complexes dissociate during gel electrophoresis. The ternary complex can be deduced indirectly from the reduction of complex band, indicating that the S1 protein is part of these complexes.

A distinct part of the *psbA* mRNA 5′ untranslated leader RNA is necessary but not sufficient for protein binding

To define a region that is necessary for protein binding to the *psbA* mRNA 5′ untranslated region, deletion studies were carried out. Deletions were derived by limited RNase T1 digestion of 3′ end- and 5′ end-labeled RNA molecules reflecting the *psbA* mRNA 5′ untranslated region including 8 nucleotides of the coding region. Fragments were gel purified and analyzed for their binding activity by gel retardation. Figure 4A shows a schematic representation of the fragments tested for binding; in Figure 4B, the respective gel retardation assays are depicted. Fragment 1 (full length) and fragment 2 (ranging from nucleotide –86 to +3) are completely retarded, i.e. bound by proteins under these experimental conditions. Fragment 3 (ranging from nucleotide –86 to –9), however, already shows a lower binding constant. Here, complexes still can be detected as a retarded band, but the intensity of the complex- and RNA-band does not add up to the amount of RNA that was originally within the binding assay. Thus, the half-life of the complex is probably long enough to allow separation of the complexed and uncomplexed RNA, but it is too short to show one distinct complex band. Instead, a broadened band can be observed. This effect is even more dramatic with fragment 4 (ranging from nucleotide –86 to –29), where complex formation can only be inferred because of the reduction of free RNA. Here, the half-life of the complex is too short to allow any complex to survive the time of electrophoresis. For fragment 5 (ranging from nucleotide –86 to –32) and the following shorter fragments, no complex formation can be observed at all. In deletions from the 5′-end of the RNA, fragments 11–16, retardation can be observed for fragments 11–14. In fragment 14 (ranging from nucleotide –49 to +8), the 5′ terminal 37 bases are deleted. Deletion of an additional two bases completely abolishes binding. These data define a region of the *psbA* mRNA 5′ untranslated region from nucleotide –49 to –9 which is necessary for protein binding.

A systematic analysis of the binding affinity of fragments 3 and 14 in comparison with the complete *psbA* mRNA 5′ untranslated region and the RNA-binding element determined by deletion analysis was performed in the experiment shown in Figure 5. RNA transcripts analyzed in these experiments are synthesized by *in vitro* transcription using PCR products as templates (see

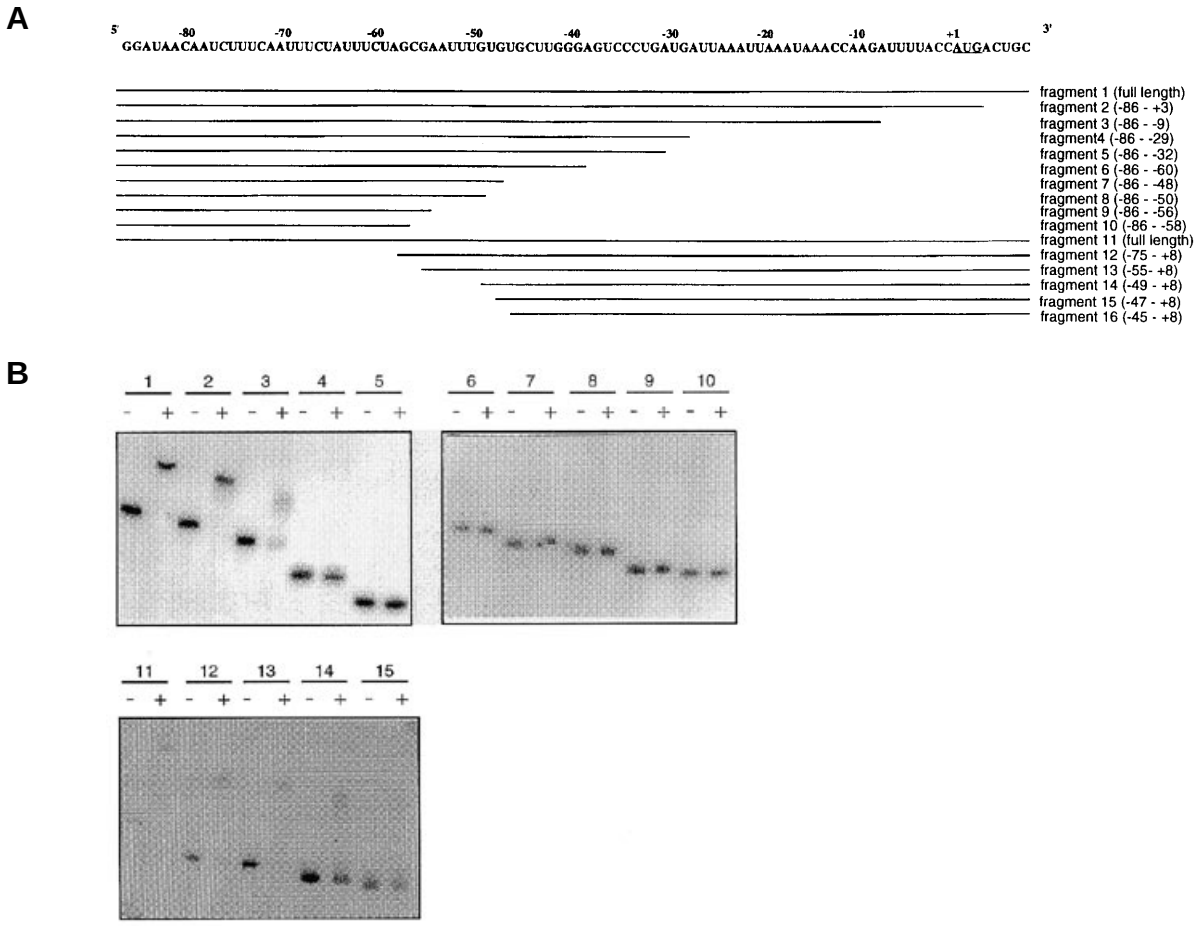

Figure 4. Determination of the sequence element of the *psbA* mRNA 5′ untranslated region necessary for binding of stromal proteins by deletion analysis. (**A**) Schematic representation of the deleted RNAs. They are prepared by limited digestion of 5′ or 3′ end-labeled transcripts with RNase T1 followed by gel purification. The numeration of the nucleotides is according to the translation start site with +1 (24). (**B**) Analysis of deletion mutants for protein binding using gel retardation assays. RNA was incubated with 2 mg/ml stromal protein and separated on 8% polyacrylamide gels. The numbers represent the fragments shown in Figure 3A; +, RNA–protein; –, RNA.

Materials and Methods). The titration of 4 fmol of each RNA with rising concentrations of protein extract shows that neither fragment 3 nor 14 has the same substrate activity for protein binding as the wild-type RNA. Higher protein concentrations are necessary to yield the same degree of complex formation as with the wild-type RNA; while with 1.0 mg/ml protein, the wild-type RNA is complexed to 50%, fragments 3 and 14 at the same concentration are complexed only to an extent of 10–20%, indicating a lower binding affinity for this RNA substrate. The analysis of the region from nucleotide –49 to –9 shows that this RNA molecule is hardly a substrate for protein complex formation. Only a slight reduction of the free RNA can be observed, and even at the highest protein concentration, no retarded complexed band can be detected. These data show that the region of protein binding determined by deletional analysis ranging from nucleotide –49 to –9 is necessary, but not sufficient, for efficient complex formation. Therefore, we termed this region the ‘central protein binding element’.

DISCUSSION

In this work, we identified a region of the *psbA* mRNA 5′ untranslated region which is necessary but not sufficient for complex formation with stromal proteins. This central protein binding element extends from nucleotide –49 to –9 of the *psbA* mRNA. As an isolated element, it is incapable of forming stable complexes with stromal proteins. The necessity for the adjacent regions may result from the relatively low content of uridine residues in this element, which is 26%. As shown earlier by competition experiments, the binding of proteins to the RNA is very sensitive to poly(U) (23). The region 5′ of the central protein binding element has a uridine content of 43% and the region 3′ of the element has a uridine content of 35%. In addition, U-stretches of three and four uridines can be found 3′ and 5′ of the central protein binding element; they may function as a nucleation site for specific complex formation. Furthermore, we showed earlier that the binding of stromal proteins depends on the structure of the *psbA* mRNA 5′ untranslated region (36). The central protein binding element may carry the sequence elements that are necessary for protein complex formation, but a certain structure that is also required for binding cannot be formed by the short stretch of nucleotides.

In Figure 6, the central protein binding element is drawn into the structural representation of the *psbA* mRNA 5′ untranslated region as it had been determined earlier by chemical modification (23,36). The element required for protein binding comprises two moieties functional in RNA metabolism; the only endonucleolytic

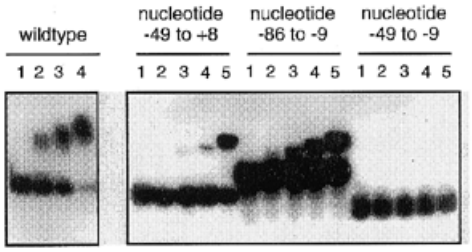

Figure 5. Protein binding to fragments of the *psbA* mRNA 5′ untranslated region. Four fmol of radiolabeled RNA were incubated in a volume of 10 µl with the following concentrations of protein. Wild-type: 1, free RNA; 2, 0.5 mg/ml protein; 3, 1 mg/ml protein; 4, 2 mg/ml protein. Deletion mutants: 1, free RNA; 2, 0.25 mg/ml protein; 3, 0.5 mg/ml protein; 4, 1 mg/ml protein; 5, 2.5 mg/ml protein. The numeration of the nucleotides is according to the translation start site with +1 (24).

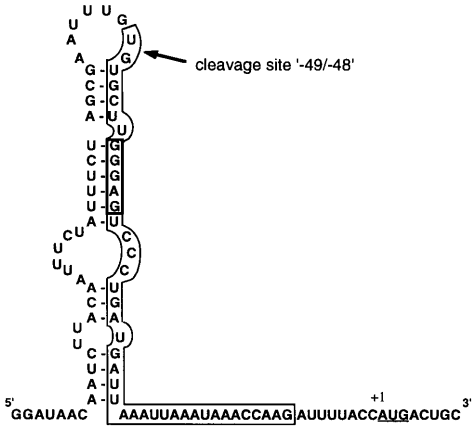

Figure 6. Position of the central protein binding element within the *psbA* mRNA 5′ untranslated region. The thin frame labels the region which had been identified by deletion analysis. The Shine–Dalgarno-like element is labeled by the bold frame and the endonucleolytic cleavage site in this region is labeled by an arrow. The numeration of the nucleotides is according to the translation start site with +1 (24).

cleavage site that could be identified on the *psbA* mRNA 5′ untranslated region *in vitro* (37) is localized there as well as the Shine–Dalgarno-like GGAG motif, which is located 7 nucleotides adjacent to the cleavage site. It has already been shown *in vitro* that in tobacco chloroplasts, the Shine–Dalgarno-like element is involved in translational regulation of *psbA* expression (38). It is postulated that the GGAG motif interacts with the ribosomal RNA during translational initiation. The close proximity of the Shine–Dalgarno-like element to an endonucleolytic cleavage site within a region which is central for protein binding can be of regulatory significance. After cleavage, the protein complex to activate translation cannot be formed any longer, resulting in a biologically inactive mRNA; on the other hand, if a translational complex is formed, the mRNA is protected against inactivation.

Furthermore, the localization of the endonucleolytic cleavage site within the central protein binding element leads to the speculation that accessibility of this cleavage site is regulated by RNA-binding proteins. It could be shown *in vitro* that the direction of *psbA* mRNA degradation is 5′ to 3′ (37). The cleavage site in the 5′ untranslated region may be one of the initiating endonucleolytic cleavages; therefore, the regulation of its accessibility would also regulate *psbA* mRNA degradation. A 43 kDa protein correlates in concentration to the steady state levels of *psbA* mRNA; no active protein can be detected in dark grown seedlings, where no *psbA* mRNA is observed by northern analysis. After illumination, the concentration of the actively binding protein rises in parallel with the amount of *psbA* mRNA (23,39).

We identified the 43 kDa protein by N-terminal sequencing, and homology search. It is the chloroplast homologue of the ribosomal protein S1. At the moment, we do not understand why the ribosomal protein S1 from etiolated seedlings cannot be cross-linked to the *psbA* mRNA 5′ untranslated region (23). Specific modifications occurring only after illumination may be necessary to allow binding of the S1 protein to the *psbA* mRNA. The spinach ribosomal protein S1 shares some homology with the *E.coli* ribosomal protein S1 which has two different RNA-binding domains, the S1-domain and an RNP motif (35,40). The S1 motif has also been found in several other proteins of the RNA metabolism: the polynucleotide phosphorylase, the bacterial and chloroplast initiation factors as well as in the eukaryotic initiation factor eIFα (40 and citations therein).The *E.coli* S1 protein is very well characterized. During translational initiation, it binds to a U-rich region 5′ of the Shine–Dalgarno sequence (41). The RNA-binding site of the *E.coli* S1 protein can also be a defined tertiary structure within an RNA molecule as it could be shown by *in vitro* evolution studies. Those experiments revealed a pseudoknot as the substrate with the highest affinity (42). Furthermore, the S1 protein has additional functions beyond translation which have been detected in the metabolism of phages. Here, the protein mediates contacts between the phage RNA and additional proteins: the RNA-dependent RNA polymerase of the phage Qβ can be completed (43); a ribonuclease of the phage T4 is stimulated by binding to the protein S1 (44), and in phage λ, the recombination protein β is bound (45). With regard to this multitude of functions, it still has to be shown whether the ribosomal protein S1, besides its function in translation, may also be part of the *psbA* mRNA degradation mechanism in chloroplasts. Several observations support this model: the endonucleolytic cleavage site detected in the *psbA* mRNA 5′ untranslated region is localized 7 nucleotides upstream of the Shine–Dalgarno-like element several uridines adjacent to it. If the chloroplast ribosomal protein S1 binds to RNA, as the protein S1 from *E.coli*, the binding site is upstream of the Shine–Dalgarno-like element. In this case, the 43 kDa protein would possibly interact with the sequence of the cleavage site and may also be involved in regulating mRNA degradation.

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