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Expression and RNA binding properties of the chloroplast ribosomal protein S1 from *Chlamydomonas reinhardtii*

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Abstract

The gene encoding the chloroplast ribosomal protein S1 from *Chlamydomonas reinhardtii*, CreS1, was cloned and the RNA binding properties and the expression patterns were studied. Gel-shift analysis revealed that CreS1 binds AU-rich 5′-untranslated regions (5′-UTR) of chloroplast mRNAs with higher affinity than the corresponding sequence of a GC-rich nuclear transcript. The binding affinity of CreS1 for a mutant form of the *psbD* 5′-UTR with a deletion of a U-rich stretch that is required for translation decreases 4-fold as compared to the wild-type 5′-UTR. Our results suggest that CreS1 protein interacts with U-rich sequences. Most of CreS1 is bound to high-molecular-weight complexes which co-migrate with the 30S small ribosomal subunit, and only a small fraction of CreS1 exists in its free form. CreS1 is localized mainly to the chloroplast stroma albeit a significant fraction is associated with chloroplast membranes. The results suggest that most of CreS1 is associated with the 30S ribosomal subunit throughout the translation process. Upon a shift of cells from the dark to the light, the mRNA levels of CreS1 and Psrp-7, both components of the 30S ribosomal subunit, increase transiently and return to the dark levels after 8 h. However, during this dark-to-light transition the levels of CreS1 and of other components of the 30S subunit remain the same suggesting that either protein synthesis or degradation is regulated. The possible implications of these findings are discussed.

Introduction

Organisms are continuously subjected to a large number of environmental stimuli. In response to the constantly changing surroundings, they modify their gene expression patterns so as to optimize their metabolism. The effect of light on chloroplast development in land plants and green algae has been studied extensively. Light enhances synthesis of pigments and photosynthetic proteins and induces the formation of thylakoid membranes (von Wettstein *et al.*, 1995). Exposure to light promotes transcription of nuclear genes encoding chloroplast proteins involved in photosynthesis (Schindler and Cashmore, 1990) and also stimulates several post-transcriptional steps of chloroplast gene expression (Deng and Gruissem, 1987), in particular translation (Malnoe *et al.*, 1988; Danon and Mayfield, 1991). This increase in protein synthesis has been correlated with the light-promoted binding of ribosomes to thylakoid membranes (Chua *et al.*, 1976; Breidenbach *et al.*, 1988).

In Chlamydomonas, chloroplast protein synthesis continues at the same rate even when the chloroplast gene copy number and the levels of plastid mRNAs are reduced several-fold (Eberhard *et al.*, 2002). Thus, it is clear that in this alga the main regulatory steps of chloroplast gene expression occur at the post-transcriptional, translational and post-translational levels (Choquet and Wollman, 2002).

Because of their prokaryotic origin, it is not surprising that the plastid ribosomes and the basic translation factors resemble in many respects those of bacteria. However, the two systems differ in several ways from each other. Plastid-specific ribosomal proteins have been identified recently that are absent from bacterial ribosomes (Yamaguchi and Subramanian, 2000;

Yamaguchi *et al.*, 2000, 2002, 2003). Moreover, a surprising feature of the plastid system that has emerged mostly from genetic studies is the existence of a large number of nucleus-encoded ancillary factors that are required for the translation of specific plastid mRNAs. Inactivation of these factors leads to the loss of translation of specific chloroplast mRNAs. Little is known about how these factors interact with the plastid translational machinery and the basic regulatory mechanisms of translation in chloroplasts are still largely unknown.

In bacteria, the interaction between a purine rich sequence, called Shine-Dalgarno (SD), in the 5′-UTR of mRNAs, and a sequence, called anti-SD, at the 3′ end of the 16S ribosomal RNA, facilitates the binding of the 30S small ribosomal subunit near the initiation codon of the transcripts. This RNA-RNA association is promoted by the ribosomal protein S1 (Subramanian, 1983). A binary complex is first formed through protein-protein interactions between the amino terminal domain of S1 and the 30S subunit. This complex is then recruited to the 5′-UTR by the binding of the carboxyl-terminal domain of S1 to a pyrimidine-rich sequence adjacent to the SD sequence (Subramanian, 1983; Boni *et al.*, 1991). S1 has been shown to be essential for translation of any transcript in *Escherichia coli* (Sorensen *et al.*, 1998). In contrast, in chloroplasts, not all the transcripts contain a SD-like sequence and, if they do, this site is not always required for translation (Fargo *et al.*, 1998; Hauser *et al.*, 1998). However, the anti-SD sequence is highly conserved in the plastid 16S rRNAs (Harris *et al.*, 1994). The small ribosomal subunit could recognize other cis-acting sequences of the 5′-UTR in the absence of a SD sequence. A homologue of the bacterial S1 protein was found in spinach chloroplasts and cyanobacteria (Franzetti *et al.*, 1992a; Sugita *et al.*, 1995). Recently, the compositions of the chloroplast small and large ribosomal subunits of *C. reinhardtii* were determined by proteomic analysis (Yamaguchi *et al.*, 2002, 2003). A protein homologue of the bacterial S1 protein, CreS1, was detected in the 30S subunit (Yamaguchi *et al.*, 2002). However, the role of the chloroplast CreS1 protein in translation initiation is still unclear and its binding sites on the mRNAs are not known.

To investigate the mechanisms of the initial steps of chloroplast translation initiation and to characterize the factors involved, we have cloned the gene encoding the chloroplast homologue of the bacterial S1 protein of *C. reinhardtii*, CreS1, and studied its RNA-binding properties. We also analysed the localization of CreS1 in *Chlamydomonas* cells and its association to the 30S ribosomal subunit. In order to examine the effect of light on the synthesis of the chloroplast ribosomal machinery, we examined the expression of CreS1 and other 30S ribosomal proteins during a dark-to-light transition.

Materials and methods

Strains and growth conditions

Chlamydomonas wild-type or *cw15* mutant cells were grown in TAP medium (Harris, 1989) either under constant white light (70–100 μE m^{-2} s^{-1}) or in the dark for 3 days and then shifted to constant white light for 0, 1, 3, 5 and 8 h. Cells were collected at a concentration of 2×10^6 cells/ml.

Isolation of the CreS1 *cDNA*

The spinach CS1 sequence (Franzetti *et al.*, 1992a) was used to search for homologues in the *Chlamydomonas* EST/ORF database (www.biology.duke. edu/cgi-bin/blast.cgi). We found four EST sequences (accession numbers BI873502.1, BE024920.1, AV-396890.1 and BF862271.1) with significant sequence similarity to CS1. The two ESTs (BI873502.1 and BE024920.1) with the highest sequence identity to CS1 protein had overlapping sequences. However, one amino acid present in the common sequence was different in the two ESTs, probably due to a sequencing error. These two ESTs were characterized. Sequence alignment with the CS1 protein suggested that the amino-terminal region of the protein was missing. The 5′ RACE technique (Preitner *et al.*, 2002) was used to obtain the full-length cDNA. To synthesize the first-strand cDNA, total wild-type RNA was treated with RQ1 DNAse (Promega) and then incubated with reverse transcriptase (Gibco) and the backward-1 primer (5′-CTCCTGCAGCTGGCGGATG-3′) annealing close to the 5′ end of the BI873502.1 EST sequence. Because of the high GC content of *Chlamydomonas* nuclear genes, we performed the reverse transcription reaction at 50 °C. After removal of the mRNA template, the unincorporated dNTPs and primer, a poly(A) tail was added to the 3′ end of the cDNA by means of terminal transferase and dATP. After removal of the enzyme, a first round of PCR amplification was accomplished with the backward-1 primer and an oligo-dT containing an anchor at the 5′ end as forward primer (5′-

TACTCGAGGGTGAAATACGTG/TTTTTTTTTT-TTTTTTTTTT-3′). A second round of PCR was performed with a second nested backward primer (5′-AAGGTGGTGGCCTCCATG-3′) and the anchor as forward primer (5′-TACTCGAGGGTGAAATACGTG-3′). The sequence obtained predicted a protein sequence related to the amino-terminal part of the CS1 protein and contained in-frame stop codons upstream to the first ATG, suggesting that the obtained cDNA encodes the entire protein (Figure 1).

Interestingly, AV396890.1 EST, also found in our database search for homologues of the CS1 protein, encodes the Psrp-7 protein. Recently, Psrp-7 was identified as a novel S1 domain-containing protein and is part of the chloroplast small ribosomal subunit of *Chlamydomonas* (Yamaguchi *et al.*, 2002, 2003).

Purification of recombinant CreS1 protein from E. coli

A DNA fragment containing the full-length coding sequence of *CreS1* was obtained by RT-PCR. The first-strand cDNA was synthesized with the backward oligo (5′-CGGCATAGCTAGCTTAGTAGACG-3′). PCR amplification was then performed with 5′-CGCCTCTCTGCTCGCAC-3′ and 5′-GTAGACGA-AGGGCTGGCCC-3′ as forward and backward oligo, respectively. The amplified DNA fragment was cloned in a pGEX expression vector. CreS1 protein was expressed in bacteria as GST fusion and purified with the GST Gene Fusion System kit (Pharmacia). After purification of GST-CreS1 fusion protein from a 500 ml bacterial culture, the GST tag was cleaved during over-night incubation with 12.5 units of thrombin protease at room temperature. The final yield was 2 mg of CreS1 protein. The mobility of CreS1 in a SDS-PAGE gel was slightly slower than expected from its predicted molecular weight for reasons which are not known.

Production of antibodies against CreS1 protein

In order to raise an antiserum against CreS1, 120 μg of gel-purified recombinant protein was injected in a rabbit five times at three week intervals. Bleeds were collected 10 days after each injection. In the immunoblot experiments reported in this manuscript we used the fourth bleed at a dilution 1:10 000.

Immunoblot analysis

Protein extract (2.5 μg) was fractionated on a 10% gel by SDS-PAGE and immunoblotted with specific polyclonal rabbit antisera against CreS1, cytosolic thioredoxin h, small subunit of Rubisco, D2 and the chloroplast 30S small ribosomal subunit of *Chlamydomonas*. The ECL western blotting system (Durrant *et al.*, 1990) was used to detect immunoreactive proteins with anti-rabbit secondary antibodies.

Preparation of whole-cell and chloroplast extracts

To obtain total cell extracts, a cell pellet (2×10^6 cells) was re-suspended in 100 μl of lysis buffer (50 mM Tris pH 8, 2% SDS, 10 mM EDTA, protease inhibitors; Sigma) and incubated at room temperature for 30 min. Extracts were cleared by centrifugation for 15 min at 12 000 $\times$ *g* at 4 °C.

To prepare total chloroplast extracts, a *cw15* cell pellet (10^9 cells) was re-suspended in 10 ml chloroplast breaking buffer (300 mM sorbitol, 50 mM Hepes-KOH pH 7.2, 2 mM EDTA pH 8, 1 mM $MgCl_2$). Cells were broken in a Bionebulizer (Gascol Apparatus, Terre Haute, IN) at 2.3 MPa and loaded onto a discontinuous 75%–45% Percoll gradient in chloroplast gradient buffer (300 mM sorbitol, 30 mM Tris pH 7.9, 10 mM $MgCl_2$). Chloroplasts were collected at the interphase of the two gradient steps and washed with gradient buffer. Chloroplasts were then re-suspended in lysis buffer and treated as the total cell extracts.

To isolate membrane and soluble fractions, cell or chloroplast pellets were re-suspended in lysis buffer without SDS and sonicated 5 times for 15 s. Unbroken cells or chloroplasts were removed by centrifugation for 5 s at 9000 $\times$ *g* in a microfuge. Total extracts were then centrifuged for 20 min at 100 000 $\times$ *g* at 4 °C. The resulting supernatant was the soluble fraction. The pellet (membrane fraction) was washed twice with washing buffer (50 mM Tris pH 8, 10 mM EDTA, 0.4 M sucrose), re-suspended in lysis buffer and treated as the total extracts. Protein concentration was determined using the bicinchoninic acid assay (Sigma).

Analysis of CreS1-containing complexes

Cell pellets (2×10^8 cells) were re-suspended in 500 μl of polysome breaking buffer (200 mM Tris-HCl pH 9, 200 mM KCl, 35 mM $MgCl_2$, 25 mM

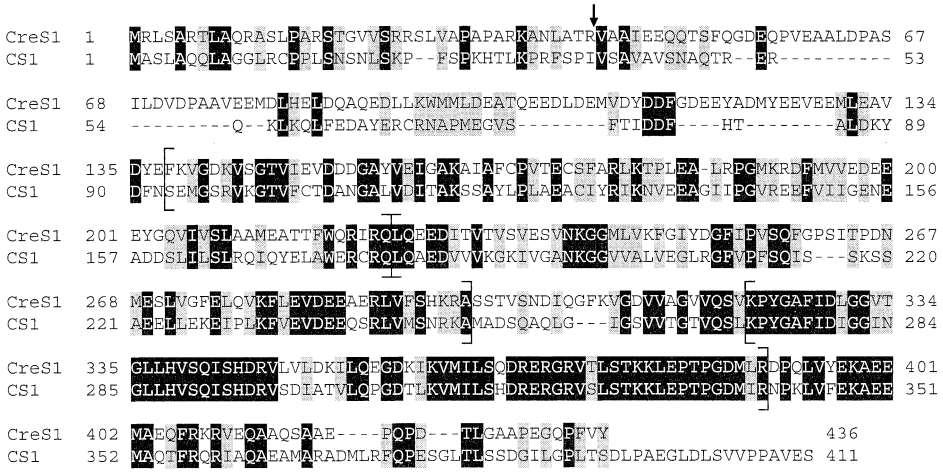

Figure 1. Sequence alignment of the chloroplast ribosomal protein S1 of spinach (CS1) and *Chlamydomonas* (CreS1). The three RNA-binding domains are between the square brackets. The CreS1 domain 1 (residues 138–223), domain 2 (residues 224–297) and domain 3 (residues 322–390) display significant sequence similarity to the corresponding domains of the spinach CS1 protein (Franzetti *et al.*, 1992a) (see Results). Identical residues and conservative replacements are depicted in dark and light grey, respectively. Gaps (−) were introduced to maximize alignment. The predicted cleavage site of the transit peptide of CreS1 is indicated with an arrow. The DDBJ/EMBL/GenBank accession number for CreS1 is AJ585191.

EGTA, 1% Triton X-100, 2% polyoxyethylen-10-tridecil ether, 0.1 mg/ml chloramphenicol, 0.5 mg/ml heparin), broken with a French press (medium ratio, 300 on gauge) and solubilized in 0.5% sodium deoxycholate for 5 min on ice. After centrifugation at 10 000 × *g* for 15 min, the supernatant (input) was loaded onto continuous 0.1 M–1.3 M sucrose gradients in polysome gradient buffer (40 mM Tris-HCl pH 8, 20 mM KCl, 10 mM $MgCl_2$, 0.1 mg/ml chloramphenicol and 0.5 mg/ml heparin). The sucrose gradients were centrifuged in SW40 rotors (Beckman) for 17 h at 30 000 rpm at 4 °C and 13 fractions of 1 ml each were collected from the top of the tube. For immunoblot analysis, 1 μl of input and 26 μl of each fraction were fractionated by SDS-PAGE.

RNA blot analysis

Upon re-suspension of frozen cell pellets (10^8 cells) in 750 μl of RNA lysis buffer (10 mM EDTA pH 8, 100 mM Tris-HCl pH 8, 600 mM NaCl, 4% SDS) and 750 μl of phenol/chloroform, RNA was isolated by extensive organic extraction and two successive precipitations (in 2 M LiCl overnight at 4 °C and in 70% ethanol for 30 min at −70 °C). Purified RNAs (10 μg) were loaded on formaldehyde 1% agarose gels, blotted onto nylon Hybond-N^+ membranes (Amersham Pharmacia) and hybridized overnight at 65 °C in modified Church and Gilbert buffer (Church and Gilbert, 1984; 7% SDS, 0.5 M phosphate buffer pH 7.2, 1 mM Na_2 EDTA, 1% BSA). Membranes were washed in washing buffer (40 mM phosphate buffer, pH 7.2, 1% SDS, 1 mM Na_2 EDTA) for 20 min at 65 °C. For detection of *CreS1*, *Psrp-7* and *Cbl* (Schloss, 1990) transcripts, DNA fragments of 700, 380 and 350 bp were amplified by PCR, respectively, and labelled by random priming with α-^{32}P-dATP. 5′-CCAGGTCATCGTGTCCC-3′ and 5′-CGGCATAGCTAGCTTAGTAGACG-3′, 5′-TTCGGTCCGTGTGTTCGCTGAG-3′ and 5′-CTC-GTCGTCATCGGTGCATCA-3′, 5′-GACGTCATC-CACTGCCTGTG-3′ and 5′-CGACGCATCCTCAA-CACACC-3′ were used as forward and backward primers, respectively, to obtain *CreS1*, *Psrp-7* and *Cbl* probes. Quantification of the band intensity was performed with a phosphorimager system (BioRad).

In vitro *synthesis of RNA probes for RNA mobility shift assays*

The sequences corresponding to the mature 5′-UTR of *Chlamydomonas psbD*, *psbA*, *psaD* transcripts were amplified by PCR. Backward primers (5′-CATTGCGTGTATCTCC-3′, 5′-CATATGTTAAT-TTTTTTAAAG-3′ and 5′-CATTTTGGCTTGTTGT-GAGTAG-3′) annealed up to the initiation codon of *psbD*, *psbA*, *psaD* transcripts, respectively. Forward primers (5′-TAATACGACTCACTATAGGG/CACA-ATGATTAAAATTAAAT-3′, 5′-TAATACGACTCAC-TATAGGG/TACCATGCTTTTAATAGAAG-3′ and 5′-

TAATACGACTCACTATAGGG/TCGTGCGAGGG-CTCCTGC-3′) annealed just downstream of the maturation site of *psbD* (−74), *psbA* (−90) and *psaD* (−165) transcripts, respectively, and contained at the 5′ end the sequence of the T7 promoter. The PCR fragments were used as DNA template for transcription reactions. The deletion of the stretch of Us introduced in the 5′-UTR of *psbD* to obtain ΔU *psbD* was as described (Nickelsen *et al.*, 1999).

In vitro synthesis of radioactively labelled RNA probes was performed in the presence of T7 RNA polymerase (Biolabs) and α-^{32}P-ATP (400 Ci/mmol, 10 mCi/ml, Amersham). After DNase treatment, RNA probes were gel-purified. The specific activity of the RNA probes was measured by counting 1 μl in a Beckman LS 6500 scintillation counter, using the Cherenkov channel.

RNA mobility shift assays

Purified recombinant CreS1 protein was incubated at different concentrations (3×10^{-6} to 3×10^{-5} M) with 40 fmol of radioactively labelled RNA probes (about 100 000 cpm). The RNA-protein complexes were allowed to form on ice for 30 min in 20 μl reactions containing 3 μg of purified *E. coli* tRNA and 80% binding buffer D (20 mM Hepes pH 8, 20% glycerol, 2 mM EDTA, 0.1 M KCl). When indicated, A, C, G or U ribohomopolymers (Sigma) or *in vitro* synthesized unlabelled RNA probes, corresponding to the 5′-UTR of the *Chlamydomonas psbA*, *psbD*, *psaD* transcripts, were added as competitors. Binding reactions were directly loaded onto native 4% polyacrylamide gels (60:1 acrylamide/bisacrylamide). The gels were run in 0.5× TBE buffer at 4 °C, at 50 V for the first 15 min (to allow the complexes to enter slowly into the gel) and then at 250 V. The gels were transferred onto 3 MM paper, dried and exposed to photographic film at room temperature. Quantification of the band intensity was performed with a phosphorimager system (BioRad).

Results

Characterization of the Chlamydomonas *CreS1 protein*

To study the function of the S1 protein in chloroplast translation, we isolated its cDNA from *C. reinhardtii* by screening the *Chlamydomonas* EST/ORF database (www.biology.duke.edu/chlamy_genome/) for homologues of the spinach CS1 sequence (Franzetti *et al.*, 1992a) (see Materials and methods). Three EST sequences with significant sequence similarity to CS1 were identified and the EST with the highest sequence identity to CS1 was further characterized by synthesizing the full-length cDNA. The corresponding ORF, named CreS1, codes for a 50 kDa polypeptide of 436 amino acids containing a putative 41 amino acid transit sequence at its N-terminal end (Figure 1, ExPASy Proteomics tools, www.cbs.dtu.dk/service/chloroP). The predicted mature protein consists of 395 amino acids with a molecular mass of 45 kDa. Sequence alignment, by means of the DNAssist (version 2.0) and Blast programs, indicates the presence of three RNA-binding domains with high sequence similarity (34%, 39% and 74% of identity, respectively) to the corresponding domains of CS1. The third RNA-binding domain is the most conserved between CreS1 and CS1. Shteiman-Kotler and Schuster (2000) have shown that deletion of the carboxyl-terminal part, containing the third and part of the second RNA-binding domains, impairs binding of recombinant CS1 protein to RNA targets. These results suggest that the third-RNA binding domain strongly contributes to RNA-binding activity of the protein. Furthermore, the *Chlamydomonas* CreS1 protein contains a specific amino-terminal extension.

The presence of a putative transit peptide and the sequence similarity to the chloroplast CS1 protein from spinach suggested that CreS1 is located in the chloroplast. To test this prediction, we raised an antiserum against the*Chlamydomonas* protein. Soluble and membrane fractions from total cells and from purified chloroplasts of *Chlamydomonas* were prepared and used for immunoblotting (Figure 2). CreS1 was found predominantly in the soluble fraction (lanes 3 and 6), but it was also present in lower levels in the membrane fraction (lanes 2 and 5). This distribution of CreS1 between soluble and membrane fractions was detected with both total cells and purified chloroplasts (lanes 1–3 and 4–6 respectively). The purity of the isolated chloroplasts was verified by the absence of the cytosolic thioredoxin h protein in this fraction (lanes 4–6). Incubation of the same protein blot with an antiserum raised against the chloroplast 30S ribosomal subunit from *Chlamydomonas* showed the same distribution of the proteins between membrane and soluble fractions (data not shown).

To verify the association of CreS1 protein to the chloroplast ribosome, total cell extracts were fractionated by sucrose density gradient centrifugation. The

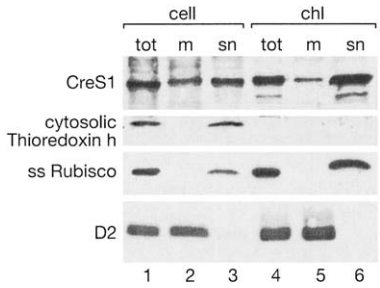

Figure 2. CreS1 protein localizes to the soluble and membrane chloroplast fractions. Total proteins (tot), membrane (m) and soluble (sn) fractions from whole cells (cell) and from purified chloroplasts (chl) of *Chlamydomonas* were prepared and used for immunoblotting using specific antisera against CreS1, cytosolic thioredoxin h, small subunit (ss) of Rubisco and D2 protein.

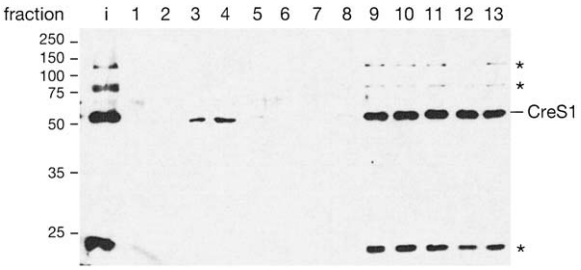

Figure 3. CreS1-containing complexes co-migrate with the small ribosomal subunit of the chloroplast ribosome. Proteins were extracted from whole cells (input) and fractionated by sucrose density gradient centrifugation. The presence of CreS1 protein in each fraction was monitored by immunoblotting with anti-CreS1 antibodies. The same membrane was then incubated with an antiserum raised against the 30S subunit of the chloroplast ribosome from Chlamydomonas. Bands detected by anti-30S antibodies are indicated with an asterisk. Fractions i, 1 and 13 correspond to the input, the top and the bottom of the gradient, respectively. The positions of the molecular weight markers are indicated on the left.

presence of CreS1 protein in each fraction was monitored by immunoblotting with anti-CreS1 antibodies (Figure 3). Only a small percentage of the total CreS1 population was present at the top of the gradient as free protein (fractions 3 and 4). Most of the CreS1 pool was associated with high-molecular-weight complexes, in the bottom part of the gradient (fractions 9–13). Incubation of the same protein blot with an antiserum raised against the chloroplast 30S ribosomal subunit from *Chlamydomonas* detected several bands (indicated with an asterisk) that co-migrated with the CreS1 complex (fractions 9–13). These data suggest that most of CreS1 is associated to the 30S ribosomal subunit.

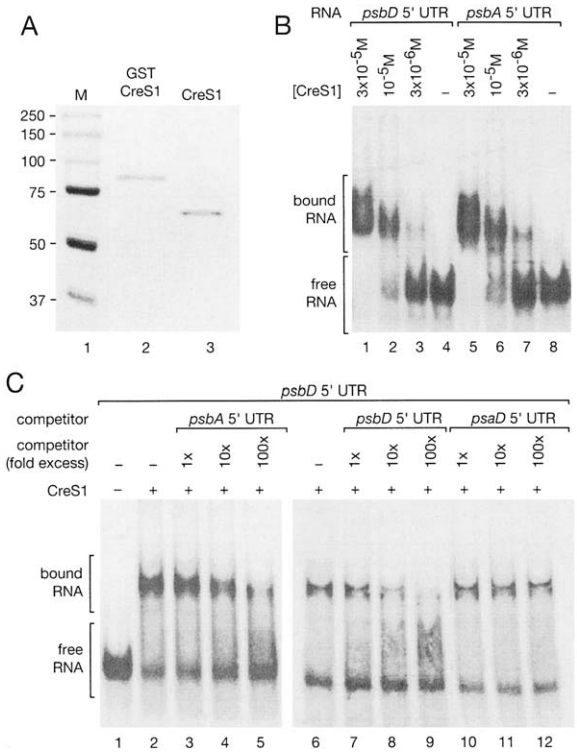

Figure 4. RNA binding properties of the CreS1 protein. A. Purification of recombinant GST-CreS1 protein from *E. coli*. To study the RNA binding activity of CreS1, the protein was expressed in bacteria as a GST fusion and purified (lane 2). Cleavage of the GST tag was obtained by thrombin digestion (lane 3). The positions of the molecular weight markers (M) are indicated (lane 1). B. CreS1 protein binds to the 5′-UTR of chloroplast transcripts. RNAs corresponding to the 5′-UTR of chloroplast *psbA* and *psbD* transcripts were transcribed *in vitro* and radiolabelled. The RNAs were incubated in the absence or presence of purified recombinant CreS1 protein at the indicated molar concentrations. Complexes were fractionated on native polyacrylamide gels. C. Binding of CreS1 protein to the *psbD* 5′-UTR is competed by the 5′-UTR of chloroplast, but not nuclear transcripts. Recombinant CreS1 protein was incubated at the fixed concentration of 5×10^{-6} M with an *in vitro* transcribed radiolabelled RNA corresponding to the 5′-UTR of the *psbD* transcript. Unlabelled *in vitro* transcribed RNAs corresponding to the 5′-UTR of the chloroplast *psbA* and *psbD*, and of the nuclear *PsaD* transcripts were added as competitors to the binding mixtures at the indicated molar excess. Complexes were fractionated on native polyacrylamide gels.

RNA-binding properties of CreS1

To study the RNA binding properties of CreS1, the protein was expressed in bacteria as a GST fusion. After purification of the recombinant protein (Figure 4A, lane 2) and cleavage of the GST tag (lane 3), the interaction of CreS1 with different RNA targets was tested in RNA mobility shift assays. *In vitro* transcribed radioactively labelled RNAs, corresponding to

the 5′-UTR of the chloroplast *psbA* and *psbD* mRNAs, were incubated in the absence or in the presence of increasing amounts of recombinant CreS1 protein and fractionated on native gels (Figure 4B). RNA-protein complexes were detected as bands with reduced mobility. Dissociation constants (K_d) were estimated as the protein concentration at which 50% of the RNA probe is shifted. The similar K_d values (5×10^{-6} M) of CreS1 complexes containing either *psbA* or *psbD* RNAs indicated that, as expected for a general translation factor, CreS1 protein binds the 5′-UTR of the two chloroplast transcripts with the same affinity, at least *in vitro*. However, the high K_d value suggested that CreS1 binds the target RNAs with low affinity, in agreement with the studies already reported for S1 from both spinach (Franzetti *et al.*, 1992a) and *E. coli* (Subramanian, 1983; Kalapos *et al.*, 1997). To investigate the binding specificity of CreS1, competition experiments were performed. A radiolabelled RNA corresponding to the *psbD* 5′-UTR was incubated with a fixed amount of CreS1 protein (at a concentration equal to its K_d (5×10^{-6}M)) in the absence or presence of increasing amounts of unlabelled competitor RNAs, corresponding to the 5′-UTR of the chloroplast *psbA* (Figure 4C, lanes 3–5), *psbD* (lanes 7–9), and of the nuclear *PsaD* (lanes 10–12) transcripts. Interestingly, binding of CreS1 to labelled *psbD* 5′-UTR was competed by the chloroplast 5′-UTRs (compare lanes 3–5 with lane 2 and lanes 7–9 with lane 6) but not by the nuclear 5′-UTR (compare lanes 10–12 with lane 6). These results indicate that CreS1 binds specifically the 5′-UTR of chloroplast transcripts, albeit with low affinity.

The identity of the target sites recognized by S1 and CS1 in bacterial and spinach chloroplast transcripts, respectively, is controversial (see Discussion). To study the sequence preference for the interaction of CreS1 with RNA, binding experiments were performed in the presence of increasing amounts of unlabelled A, C, G and U ribohomopolymers (Figure 5A). Binding of CreS1 to *psbD* 5′-UTR was preferentially inhibited by the addition of poly(U) (compare lanes 3–8 and 9–10 with lane 2). The quantification of these competition experiments is also shown (Figure 5A, right panel). The data suggest that CreS1 preferentially interacts with U-rich sequences.

In *E. coli*, the ribosomal protein S1 binds to a U-rich region upstream of the Shine-Dalgarno sequence during translation initiation (Boni *et al.*, 1991). Previous experiments showed that deletion of a highly U-rich sequence (−25 to −14) adjacent to the putative Shine-Dalgarno sequence in the Chlamydomonas *psbD* 5′-UTR affects translation and, only partially, stability of the RNA (Nickelsen *et al.*, 1999; Figure 5B, upper panel). We tested if this stretch of uridines is important for the interaction of CreS1 with the *psbD* 5′-UTR. A mutant form of the *psbD* 5′-UTR lacking the U-rich sequence (ΔU *psbD*) was incubated with CreS1 (Figure 5B). This deletion decreased the affinity of CreS1 for the *psbD* 5′-UTR almost fourfold (compare lanes 1–4 with lanes 5–8), as indicated by quantification of the band intensity performed with a phosphorimager system (right panel), thus suggesting that the stretch of Us close to the SD sequence of *psbD* is recognized by the CreS1 protein. However, because of the limited effect of the deletion on the binding affinity, we cannot exclude that CreS1 also interacts with other U-rich sequences in the 5′-UTR.

Expression of CreS1 is transiently induced by light

It is well established that chloroplast translation is strongly stimulated by light both in land plants and *Chlamydomonas* (Deng and Gruissem, 1987; Malnoe *et al.*, 1988). To test if this effect is partly also caused by the light-dependent synthesis of the chloroplast ribosomal machinery, we examined the expression of the *CreS1* gene under different light conditions. *Chlamydomonas* wild-type cells were dark-adapted for 3 days and then shifted to light for 0, 1, 3, 5 and 8 h (Figure 6A). Total RNA was then isolated and the levels of the *CreS1* transcript were determined by RNA blot analysis. *CreS1* transcript levels were low in dark-adapted cells and increased in response to light. *CreS1* mRNA abundance reached its highest level after 1 h of light treatment and diminished gradually until it returned to the initial dark level after 8 h in the light. *CreS1* gene expression was specifically induced by light because no increase of the *Cbl* transcript was observed under these conditions. Interestingly, a similar transient light induction was observed for the *Psrp-7* gene which encodes another component of the 30S ribosomal subunit containing S1 domains. Two bands were detected by the *Psrp-7* probe, probably due to processing or alternative splicing of the *Psrp-7* transcript. The levels of *CreS1*, *Psrp-7* and *Cbl* transcripts were quantified by the phosphorimager system. Light induction of transcripts levels was estimated from the ratio between the values at different times of light exposure and in the dark. Normalization was performed with *Cbl* transcript levels as loading control. The estimated values are indicated below each lane of the

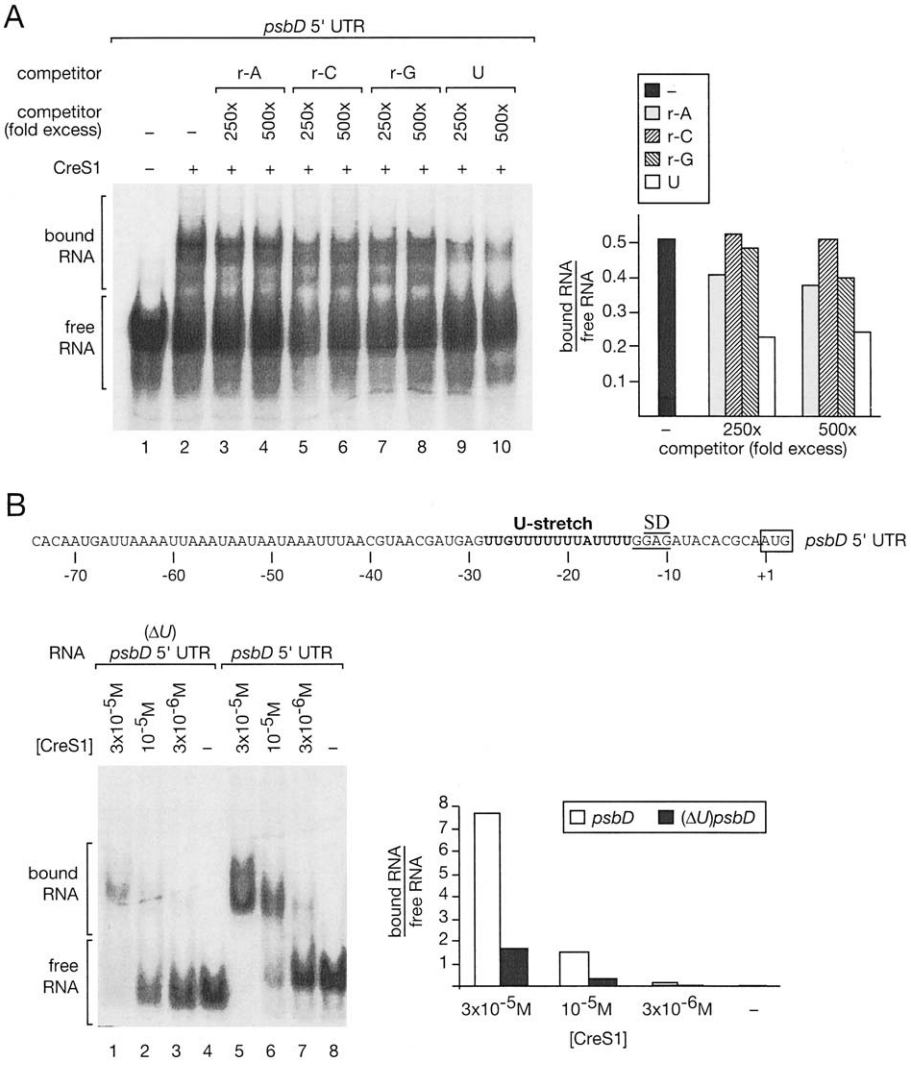

Figure 5. CreS1 protein binds preferentially to U-rich sequences. A. Binding of CreS1 protein to the *psbD* 5′-UTR is preferentially competed by poly(U). Recombinant CreS1 protein was incubated at the fixed concentration of 5×10^{-6} M with the *in vitro* transcribed radioactively labelled *psbD* 5′-UTR. Unlabelled A, C, G or U ribohomopolymers were added as competitors to the binding mixtures at the indicated molar excess. Effects of the competitors on CreS1 binding were estimated from the ratio of bound RNA to free RNA. Quantification of the band intensity was performed with a phosphorimager system (right panel). B. Deletion of a U-stretch upstream of a Shine-Dalgarno (SD)-like sequence affects binding of CreS1 protein to the *psbD* 5′-UTR. RNAs corresponding to the 5′-UTR of either wild-type *psbD* mRNA or to a mutant form (ΔU *psbD*), lacking the stretch of Us (from −25 to −14) near the SD sequence (Nickelsen *et al.*, 1999), were *in vitro* transcribed and radioactively labelled. The RNAs were incubated in the absence or presence of the purified recombinant CreS1 protein at the indicated concentrations. Complexes were fractionated on native polyacrylamide gels (left panel). Quantification of the band intensity was performed with a phosphorimager system. The effect of the deletion in the *psbD* 5′-UTR was estimated from the ratio of bound RNA to free RNA (right panel). The sequence of the *psbD* 5′-UTR is shown in the upper panel with the U stretch in bold, the SD-like sequence underlined and the initiation codon (AUG) framed.

blots. To test if the expression of *CreS1* was dependent on photosynthetic electron flow, dark-adapted cells were treated with DCMU or DBMIB for 5 min before light treatment. These drugs are known to block reduction and oxidation of the plastoquinone pool, respectively (Haehnel and Trebst, 1982). Both inhibitors did not have any significant effect on the light induction of *CreS1* (data not shown). These results suggest that the transient induction of *CreS1* by light is independent of photosynthetic electron flow.

Protein levels of CreS1 and of the other 30S ribosomal proteins were also determined by immunoblotting with specific antisera (Figure 6B). These levels remain constant in the dark and under the light conditions tested, probably because either protein synthesis and/or turnover are regulated.

Discussion

As a first step towards understanding the role of S1 in the initiation of chloroplast translation, we have cloned the gene encoding the chloroplast homologue of the bacterial S1 protein of *Chlamydomonas*, CreS1, and examined its RNA-binding properties and its expression pattern. Sequence alignment of CreS1 with spinach CS1 indicates that CreS1 contains three RNA-binding domains (Figure 1; Yamaguchi, 2002). Both CS1 and CreS1 represent truncated versions of bacterial S1 that contains six repeated modules with the two N-terminal domains binding the ribosome and the four C-terminal domains binding RNA (Subramanian, 1983). CS1 and CreS1 have the highest sequence identity and similarity at the level of the third RNA binding domain. Interestingly, this domain is required for binding of CS1 to mRNA (Shteiman-Kotler and Schuster, 2000).

The strong sequence conservation between bacterial S1, CS1 and CreS1 suggests that these proteins may share the same RNA-binding properties. Contradictory results concerning the RNA-binding preference of S1 and CS1 have been reported. Some reports indicate that S1 has no strict sequence specificity and binds poly(U), poly(C) and poly(A) (Subramanian, 1983, 1984; Kalapos *et al.*, 1997). In contrast, experiments performed by Boni *et al.* (1991) indicate that S1 may interact with AU-rich sequences of bacterial transcripts. CS1 has been shown to be either a poly(A) (Franzetti *et al.*, 1992a) or a poly(U)-binding protein (Shteiman-Kotler and Schuster, 2000). However, toeprinting experiments suggest that CS1 may interact with an A stretch in the translation initiation region of spinach chloroplast transcripts in the presence of the 30S ribosomal subunit (Franzetti *et al.*, 1992a). To study the RNA-binding properties of CreS1, we purified recombinant CreS1 from bacteria (Figure 4A) and tested its affinity to several RNA targets using RNA mobility shift assays. As RNA targets, we used the 5′-UTRs of the chloroplast *psbA* and *psbD* transcripts that both contain U stretches and putative SD sequences. The latter are required for translation of *psbA* (Mayfield *et al.*, 1994), but not of *psbD* mRNA (Nickelsen *et al.*, 1999). Together, these sequences may act as a platform for the interaction of CreS1 and the chloroplast 30S subunit with the mRNA. We found that CreS1 binds with similar affinity to the *psbA* and *psbD*5′-UTRs, as expected for a general translation factor (Figure 4B). However, the dissociation constant of the CreS1-RNA interaction was in the micromolar range, indicating a low binding affinity. We cannot exclude that this low binding affinity of CreS1 to the RNA targets, already observed for bacterial S1 (Subramanian, 1983; Kalapos *et al.*, 1997) and CS1 (Franzetti *et al.*, 1992a), is due to the *in vitro* assay. It is possible that *in vivo* other ribosomal proteins and ancillary factors improve the strength and the specificity of CreS1 binding to the mRNA. It is interesting that Psrp-7, one of the newly identified components of the 30S ribosomal subunit of *Chlamydomonas*, also contains two S1 domains. It was proposed that this protein could facilitate mRNA recognition by the chloroplast S1 protein (Yamaguchi *et al.*, 2002, 2003).

To better define the target site of CreS1 on the chloroplast mRNAs in *Chlamydomonas*, we performed binding experiments in the presence of competitors. These studies revealed that CreS1 binds the 5′-UTR of the AU-rich chloroplast *psbA* and *psbD* transcripts significantly better than the nuclear *PsaD* transcript that is GC-rich (Figure 4C). Moreover, poly(U) competed the binding of CreS1 to *psbD* 5′-UTR more efficiently than other ribohomopolymers (Figure 5A). We also tested the affinity of the CreS1 protein to a mutant *psbD* 5′-UTR *(ΔU psbD)* with the deletion of a stretch of Us near the SD sequence. It was previously shown that translation of the *psbD* transcript is affected by this deletion (Nickelsen *et al.*, 1999). Binding of CreS1 was decreased fourfold (Figure 5B), confirming that this stretch of Us is part of the target site. Because of the limited effect of the deletion on the binding of CreS1 to *psbD* 5′-UTR,

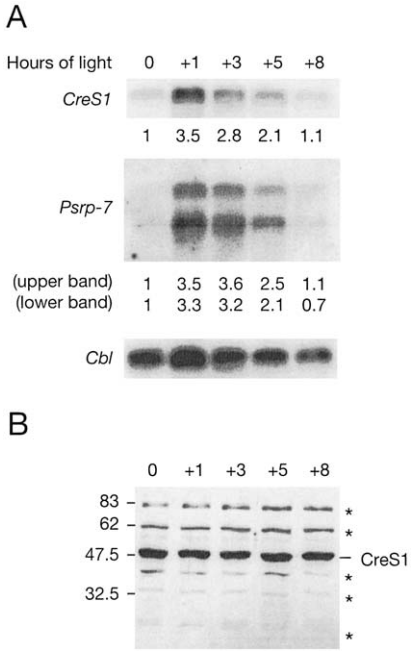

Figure 6. Analysis of *CreS1* gene expression upon light induction. Wild-type cells were dark-adapted for 3 days and then shifted to light for 0, 1, 3, 5 and 8 h. A. Total RNA was isolated and the levels of *CreS1*, *Psrp-7* and C*bl* transcripts were determined by RNA blot analysis with specific probes. The increase of transcripts levels was measured using dark amounts as reference. The values were normalized with *Cbl* transcript levels as loading control. The obtained values are reported below each lane of the blots. Quantification of the band intensity was performed with a phosphorimager system. B. Proteins were extracted from total cells and the levels of CreS1 and 30S ribosomal proteins (indicated with an asterisk) were determined by immunoblotting with specific antisera. The positions of the molecular weight markers are indicated on the left.

other domains of this region are probably involved in its binding.

To confirm that CreS1 is the authentic chloroplast orthologue of the ribosomal S1 protein in *Chlamydomonas*, we determined its localization in *Chlamydomonas* cells. Biochemical purification of sub-cellular fractions and immunoblotting with specific anti-CreS1 antibodies confirmed that CreS1 is localized to the chloroplast (Figure 2). As already observed for CS1 (Alexander *et al.*, 1998), CreS1 was associated to both soluble and membrane chloroplast fractions. The same localization was observed for other proteins of the 30S subunit (data not shown). Earlier studies indicated that the first steps of translation initiation for chloroplast proteins occur in the chloroplast stroma where the 30S subunit first interacts with the transcript (Chua *et al.*, 1976). Whether this complex interacts with the 50S ribosomal subunit before or after association to the thylakoid membrane is not clear. The ribosomes are released in the chloroplast stroma only once they have completed the polypeptide chain. The localization of CreS1 in both chloroplast soluble and membrane fractions is compatible with its general role in the translation of chloroplast transcripts and suggests that CreS1 may accompany the ribosome during the whole translation process.

We analysed the association of CreS1 to ribosomal subunits by sucrose density gradient centrifugation (Figure 3). We observed that only a small fraction of the CreS1 population is present as free protein and that most of CreS1 is associated to large complexes that co-migrate with the 30S ribosomal subunit. During the preparation of this work, a detailed proteomic analysis of the chloroplast small ribosomal subunit of *Chlamydomonas* was reported (Yamaguchi *et al.*, 2002). Among the factors identified, a protein identical to CreS1 was found associated to the 30S ribosomal subunit. These data, together with our results, indicate that CreS1 is an authentic ribosomal protein and support the hypothesis that it is the *Chlamydomonas* orthologue of the S1 protein from bacteria and land plant chloroplasts.

Light is known to profoundly influence chloroplast protein synthesis (Deng and Gruissem, 1987; Malnoe *et al.*, 1988). It was previously found that the association of polysomes to chloroplast membranes is induced by light and correlates with the activation of plastid translation in *Chlamydomonas* (Breidenbach *et al.*, 1988; Chua *et al.*, 1976). However, we were unable to detect any light-induced increase in binding of CreS1 to the chloroplast membranes (data not shown). We also tested whether the association of CreS1 protein to the 30S ribosomal subunit was increased by light. Total extracts of dark-adapted cells (data not shown) or of cells adapted to dark and then shifted to light for 3 h (Figure 3) were fractionated by sucrose density gradient centrifugation. No significant difference in the level of association of CreS1 to the ribosome was detected under these different light regimes. Because CreS1 is part of the chloroplast translational machinery, it was interesting to test if the expression of CreS1 was induced by light. To analyse the light effect, we performed a dark-to-light shift experiment. The level of *CreS1* mRNA increases transiently upon 1 h of light exposure of dark-adapted cells and returns to the dark level after 8 h in the light (Figure 6). Interestingly, this light effect was also observed for the *Psrp-7* transcript, another component of

the 30S subunit, suggesting a general light control of the synthesis of the chloroplast ribosomal machinery in *Chlamydomonas*. In contrast, no difference of *CS1* gene expression was observed between spinach cotyledons grown either in the dark or light (Franzetti *et al.*, 1992b). However, because of the different experimental growth conditions used, we cannot fully exclude that a light response also occurs for *CS1* gene expression.

We also analysed the light effect on protein levels of CreS1 and other 30S ribosomal proteins. In contrast to the increase in the amounts of the transcripts, the protein levels of CreS1 and other 30S ribosomal proteins were constant during the dark-to-light transition, suggesting that either protein synthesis or turnover is regulated. This transient light-induced effect on the ribosomal protein mRNAs without a concomitant increase in protein levels is intriguing. If turnover of the ribosomal proteins is indeed increased during the dark-to-light shift, one interesting possibility is that this process leads to small changes in the ribosome protein composition and possibly to the recruitment of light-specific translation factors. Another possibility is that such a turnover could facilitate post-translational modifications of ribosomal proteins required for adaptation to growth in the light.

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