Characterization and RNA-binding Properties of a Chloroplast S1-like Ribosomal Protein*

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Control of translation is an important step in chloroplast gene expression. A first control can be exerted during the initiation complex formation which, in *Escherichia coli*, involves the ribosomal protein (r-protein) S1. A cDNA clone have been characterized which codes for the precursor of the chloroplast r-protein CS1. The mature protein consists of a central core which shows 31.5% amino acid homology to the *E. coli* protein S1. The CS1 is considerably shorter (40 kDa) than the protein S1 (61 kDa). The core fragment contains three degenerated repeats which show homology to both the ribosome- and the RNA-binding domain of S1. RNA-protein CS1 interactions were studied by UV cross-linking and toeprinting. CS1 has been over-expressed in *E. coli*, and after purification its RNA-binding properties were studied in vitro. We conclude that the CS1 exhibits an RNA-binding activity which is actively involved in the chloroplast initiation complex formation. It is shown that the CS1 binds to poly(A) in contrast with S1 which binds strongly to poly(U). These results are interpreted in relation to the presence of poly(A)-rich regions in chloroplast transcripts of higher plants.

Plastid differentiation and chloroplast development are under the dependence of nuclear and chloroplast encoded genes. Elucidation of the control of chloroplast gene expression is important for the understanding of the complex relations coordinating the nuclear and the chloroplast compartment in response to developmental signals. Recent studies have appeared on the problem of differential accumulation of chloroplast mRNAs during leaf development (1, 2), and there are several reports suggesting translational control in chloroplasts based on a lack of correspondence between chloroplast mRNA and protein levels (3–5). Although a class of nuclear gene products, which act as activators of chloroplast mRNA's translation, has been recently identified in *Chlamydomonas reinhardtii* (6, 7), the mechanisms involved in this control are not understood. The chloroplast translational machinery resembles that of bacteria in many respects (8), but apart from some exceptions (8, 9) most of the proteins acting in the regulation of translational initiation and elongation are unknown.

As a prerequisite for a detailed study of the control of translational initiation in chloroplasts we concentrated our work on the characterization of a chloroplast ribosome component equivalent to the *Escherichia coli* ribosomal protein (r-protein) S1 which increases the efficiency of translation initiation by bridging the mRNA to the 30S ribosomal subunit (10, 11). Unlike the other r-proteins, the *E. coli* r-protein S1 is well characterized at the structural and the functional level (for a review see Ref. 10). It is by far the largest r-protein. Its primary sequence reveals the presence of six repeating stretches of internal homology (10, 12), a fact which suggests an evolutionary origin of the protein by repeated duplication of an ancestral unit. Two different domains are responsible for different functions: the NH2-terminal domain is involved in the attachment of the protein to the 30S ribosomal subunit (13), and the large COOH-terminal domain is responsible for the binding of mRNA to the ribosome during the initiation step of translation (10, 14). This latter domain is composed of 4 contiguous and significant homologous repeats and provides an RNA-binding property which is unique among the r-proteins. Our understanding of this property could be improved by sequence data comparison of S1-like proteins from several prokaryotic species. As only the primary structure of S1 from *E. coli* and from two other Gram-negative bacteria (15, 16) is known, the identification and characterization of an S1-like protein in another distantly related prokaryotic organism seems to be a challenging project.

The presence of an S1-like protein in chloroplast ribosomes has been previously postulated on the basis of immunological studies (17, 18). Here we report on the isolation and sequencing of a cDNA clone coding for a S1-like protein (CS1). We analyze the structural features of CS1 and we present the first assays to analyze the function of this protein in translation. The CS1 protein is much smaller than its *E. coli* counterpart and presents chloroplast-specific features. We show that the protein exhibits specific poly(A)-binding activity and is engaged in chloroplast mRNA binding during initiation of translation. The role of CS1 in the control of chloroplast translation is discussed.

MATERIALS AND METHODS

cDNA Cloning, Sequencing, and Prediction of Protein Secondary Structure—Poly(A)$^\dagger$ mRNA was isolated from young spinach plants. A λgt11 cDNA library was constructed and screened with antibodies raised against spinach 30S r-proteins as described (19). A partial CS1 cDNA was identified through sequencing and comparison of the encoded protein with the *E. coli* S1 r-protein sequence (20). Another cDNA library was constructed from 5 µg of poly(A)$^\dagger$ mRNA, in λgt10, with a kit from Amersham according to the supplier's protocol. 4 × 10$^5$ recombinants were screened with the original partial 32P-labeled CS1 cDNA fragment. Selected cDNA clones were sub-

$^\dagger$The abbreviations used are: r-protein, ribosomal protein; SDS, sodium dodecyl sulfate; ORF, open reading frame; bp, base pair(s); HPLC, high-performance liquid chromatography.

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cloned into pUC18 and Bluescript KSII according to procedures described by Sambrook et al. (21) and were sequenced on both strands by the dideoxy chain termination method (22). Computer analysis of data was performed using the CTT2 (Paris) software. Protein secondary structure was predicted according to Chou and Fasman (23).

**NH2-terminal Amino Acid Sequence—** 30 S r-proteins were isolated from crude Ribosomes (Boehringer) were 5'-end-labeled with polyacrylamide gel electrophoresis. The NH2-terminal amino acid sequence has been obtained by microanalysis of the electrophorelated protein using an Immoblon membrane (Millipore) as described (24).

**Isolation of Chloroplast and E. coli 30 S Ribosomal Subunits—** Chloroplast ribosomes were isolated from freshly grown spinach leaves (Spinacia oleracea, var. GBant d'hiver) as described (25). By this method ribosomes should contain their normal amount of IF3 and S1 protein as previously shown (27). 30 S fractions from E. coli and from spinach were then identically processed as follows. After sedimentation at 100,000 × g for 16 h, the t-coupled ribosomes were gently resuspended in buffer B (buffer A but with 1 mM magnesium acetate). Subunits were then separated on 10–40% sucrose gradients and collected as described (26). 30 S subunits were finally resuspended in buffer C (20 mM Tris-HCl, pH 7.6, 10 mM MgCl2, 100 mM NH4Cl, 6 mM β-mercaptoethanol). Subunits were activated at 40 °C for 15 min. Subunits were then stored at −80 °C.

**Preparation of the r-protein CL22 mRNA—** A spinach chloroplast DNA fragment which was homologous to the CS1 initiation start consensus sequence (5'-ACAA-CAATGGGT-3') (30) and is furthermore included in a region identical to the initiation start consensus sequence (5'-ACAACAATGGGT-3') (50) and is furthermore included in a region which is predicted from the nucleotide sequence, thus identifying the translation initiation start codon (34). The CS1 sequence does not contain a poly(A) stretch, although a putative plant adenylation signal (5'-ATAAA-3'), as defined by Joschi (32), is present (underlined in Fig. 1). The entire 3' noncoding region is present as well in the sequence of a second chloroplast r-protein S1. The cDNA clone was used to screen another cosmid library constructed in Xgt10. An incomplete cDNA clone was selected which was homologous to the E. coli rpsA gene coding for the r-protein S1. The cDNA clone was used to screen another library constructed in λgt10. About 160 clones were obtained after the screening of 4 × 105 recombinant phages. After Southern hybridization the three largest cDNA inserts were selected for sequencing. All of them have identical nucleotide sequences and include the initiation codon. The nucleotide sequence of the largest cDNA is reported in Fig. 1. It contains 1481 bp in good correspondence with the size of transcripts revealed by Northern analysis using the cDNA as a probe (not shown). The nucleotide sequence contains an open reading frame of 412 codons. The first methionine codon is surrounded by a favorable Kozak sequence (5'-AAATACCATGGGCTG-3') (30) and is furthermore included in a region which is identical to the initiation start consensus sequence (5'-ACAACAATGGGT-3') of plant genes identified by Lutke et al. (31). Interestingly, the 5'-untranslated region is 87 bases long and contains a pyrimidine-rich sequence including a CT repeat (from base 1 to 37) which is also found in some other nuclear encoded chloroplast r-protein mRNAs. The 3' noncoding sequence does not contain a poly(A) stretch, although a putative plant adenylation signal (5'-ATAAA-3'), as defined by Joschi (32), is present (underlined in Fig. 1). The entire 3' noncoding region is present as well as in the sequence of a corresponding genomic clone (not shown).

The CS1 protein isolated from 30 S ribosomal subunits by monodimensional SDS-polyacrylamide gel electrophoresis was submitted to NH2-terminal amino acid sequence analysis. The 20-residue sequence matches exactly with residues 42–61 predicted from the nucleotide sequence, thus identifying the

[1]Franzetti, P. Carol, and R. Mache, unpublished data.
The region of CS1 which is homologous to its electrophoretic behavior (17). All these data show that the predicted molecular mass of the 370-residue mature product (40.4 kDa) and its pI value (7.5) are both in agreement with the cleavage site (vertical arrow) of the pro-peptide (41 first residues) shows all the features of the processing site of the pre-protein. The terminal sequencing of the isolated pro-peptide indicates the processing site of the precursor protein. The adenylation signal.

Comparison of r-protein CS1 with its E. coli Homologue—The region of CS1 which is homologous to E. coli S1 is restricted to a central core encompassing residues 51–301. Hence, there is a NH2- and a COOH-terminal extension restricted to a central core encompassing residues 51-301.

**FIG. 1.** Nucleotide sequence of the cDNA clone for the spinach r-protein CS1. Nucleotide sequence and deduced amino acids are shown. The arrow indicates the processing site of the precursor protein. The 20 amino acid residues which have been obtained by NH2-terminal sequencing of the isolated protein are underlined. Dotted line, putative adenylation signal.
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one hand, and each repeat of the ribosome-binding domain (D1, D2) or of the RNA-binding domain (R1 to R4) of S1 on the other hand, is reported in Fig. 2A. The highest degree of homology (43% identity and 64% similarity) is found between R1 located at the beginning of the RNA-binding domain and a fragment of the same length (84 residues) which we term unit 3 (U3), located at the end of the CS1 central core. This unit is repeated in two degenerated forms referred to as units 1 and 2 (U1 and U2) as shown in Fig. 2B. The three CS1 units cover the entire homologous central core of CS1. A region of homology (Fig. 2A) was also found between the S1 ribosome-binding domain (D1 and D2) and the CS1 central core. This region encompasses part of unit 1, the entire unit 2, and part of unit 3. In Fig. 2B, the repetitive units of CS1 (U1 to U3) and those of S1 (R1 to R4) are aligned for comparison. We can observe the strong conservation of two motifs between R1 and U3 (underlined amino acid residues in the consensus sequence). These motifs include the highly conserved Gly residues (indicated by vertical arrows in Fig. 2B) which are known to play a key role in the boundary of structural elements. They also include the following sets of 4 conserved amino acid residues: YGAF, GLLH, DRER, and RVSL.

Several β-sheet secondary structures can be predicted using the Chou and Fasman parameters (bold lines in Fig. 2B). Interestingly, these β-sheets are predicted at similar positions in each repetitive element of either S1 or CS1. Percentages of conserved Gly residues within the homologous strings; highly conserved stretches are underlined. Bold bottom lines, β-sheets found at identical position within the homologous repetitive elements. Numbering of amino acid residues as in Fig. 2.

![Fig. 2. Comparison of the chloroplast CS1 and of the E. coli S1 r-proteins. A, schematic representation of homologous domains. Striped boxes correspond to repetitive elements contained in the RNA-binding domain of S1 and to their CS1 homologues; grey boxes correspond to the ribosome-binding domain of S1 and to the homologous region in CS1; black boxes correspond to chloroplast-specific extensions. The CS1 central core is divided into 3 units (U1 to U3) homologous to the internally homologous repeats of S1. Percentages of homology are indicated under the CS1 units. Numbering of amino acid residues starts at the NH2 terminus of the mature protein. B, alignment of repeating homologous sequences of S1 with those of CS1. The four R1 to R4 87-amino acid repeats (upper) are those determined by Wittmann-Liebold et al. (8); the R1 stretch is taken as master sequence. Conservative replacements (shadowed) and identical residues (bold) are boxed. Gaps (-) were introduced in the sequences to maximize homology. Conserved Gly residues are indicated by vertical arrows. Bottom line, conserved residues within the homologous strings; highly conserved stretches are underlined. Bold bottom lines, β-sheets found at identical position within the homologous repetitive elements. Numbering of amino acid residues as in Fig. 2.](image-url)
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![Image of a chloroplast and E. coli UV cross-linking experiment](image)

**FIG. 3. Interaction of CS1 within the initiation complex with the chloroplast CL22 mRNA.** UV cross-linking of chloroplast and E. coli 30 S ribosomal subunits to CL22 mRNA. Stained gel and autoradiogram of the protein-RNA complex from chloroplasts (left) or E. coli (right), after separation of r-proteins by 12% SDS-polyacrylamide gel electrophoresis. The arrowheads indicate the position of S1 or of CS1 in the gel. Lane 0, nonirradiated control sample; lane 1, UV-treated sample; lane 2, as in lane 1 except that the tRNA$^{32}$P is omitted; lane 3, as in lane 1 except 20 mM EDTA are added. The position of the labeled material in the chloroplast sample corresponds to that of CS1 as confirmed by immunoreaction.

![Image of UV cross-linking experiment results](image)

**FIG. 4. Dependence of S1 and CS1 binding to RNA on the presence of translational start signals.** The 30 S subunits were mixed with sense (S) and antisense (AS) CL22 mRNAs. Lanes 1 and 2, nonirradiated samples; lane 3, UV-treated 30 S mRNA complexes; lane 4, as in lane 3 but with antisense mRNA; lane 5, as in lane 4 except that the tRNA$^{32}$P is omitted; lane 6, as in lane 4 except 20 mM EDTA are added.

(see Fig. 6). Hence we conclude that, in this experiment, the cross-link of the CS1 with the chloroplast CL22 mRNA is mainly due to a specific recognition of the mRNA probably involving the translation initiation region.

**Characterization of RNA-binding Properties of the Isolated r-protein CS1.**—As a preliminary step to studying the CS1 RNA-binding properties, the protein had to be purified in a sufficient amount. The protein encoded by the CS1-cDNA was overexpressed in E. coli as a glutathione S-transferase fusion protein and the CS1 protein was purified as described under "Materials and Methods." The isolated CS1 r-protein maintains an RNA-binding property for CL22 mRNA as observed by UV cross-linking (not shown). The RNA-binding activity between CS1 and the sense CL22 mRNA was tested by filter retention assays. The RNA-protein complex is dependent on the protein concentration and is stable even in the presence of tRNA in excess as nonspecific competitor (Fig. 5A). An association constant ($K_a$) of 2.10$^m$ M$^{-1}$ was calculated from a Scatchard analysis of the data. UV cross-linking experiment showed that the RNA-binding property of the CS1 protein was weakly expressed against antisense CL22 mRNA (not shown). These results suggest that purified CS1 binds the CL22 mRNA in a specific relation to the translational initiation region of the CL22 mRNA. To test this hypothesis, initiation complex stability in the presence of an increasing concentration of CS1 was examined by toeprinting assays (Fig. 6). The chloroplast initiation complex blocked reverse transcription at a site situated at position +15 from the initiation codon. The addition of free CS1 to the reaction mixture first results in an increase in the toeprint signal. This is probably due to the fact that during the preparation small subunits are partially devoid of CS1 proteins. Then, higher CS1 concentration led to the disappearance of the toeprint signal reflecting an inhibition of the initiation complex formation that can be explained by CS1 competition with the 30 S ribosomal subunit for mRNA binding. Thus we obtained indirect evidence that free CS1 binds to the mRNA either at the same site as does CS1 when it is a part of the 30 S ribosomal subunit or at a site situated in the region covered by the small ribosomal subunit.
polymers show that poly(A) competes efficiently with CL22 presence of 0.125 to poly(A) and not to the other homopolymers. We conclude that CS1 preferentially binds to the poly(A) stretch. Then, the purified CS1 r-protein was tested for its RNA homopolymer binding activity. Fig. 5C shows that CS1 binds efficiently to the initiation complex. Lane 0, primer extension without addition of 30 S subunits; lanes 1–6, toeprinting in the presence of 0.125 μM of 30 S chloroplast ribosomal subunits and 1.25 μM trRNA<sup>Met</sup>. Concentrations of CS1 added are indicated over the lanes. Lanes T, G, C, and A contain sequencing reaction of the CL22 clone using the same oligonucleotide as for the primer extension reaction. The arrowhead indicates the ribosome dependent stop signal situated at position +15. CS1 inhibits the toeprint signal at a concentration of 1 μM or more.

**DISCUSSION**

This paper describes the cDNA cloning, purification, and functional analysis of a chloroplast r-protein (CS1) homologue to the E. coli r-protein S1. We have shown that CS1 is encoded in the nucleus and that its gene (rpsl) codes for a chloroplast r-protein synthesized in the cytoplasm and transported to chloroplasts. The mature protein contains NH<sub>2</sub>- and COOH-terminal extensions in addition to a S1 homologous central core. It is surprising that in spite of the extensions, the CS1 mature protein (370 residues) is much shorter than the S1 protein (557 residues). The homologous region of CS1 (259 residues) is evidently shorter, having less than half the size of its bacterial counterpart. About two-thirds of the total number of chloroplast ribosomal proteins are nuclear encoded, and more than 13 of these proteins have been characterized by their primary structure (34). From these data, E. coli-like chloroplast r-proteins can be classified into two functional groups, one containing proteins of the same size as their bacterial counterparts and the second containing larger sized proteins due to the presence of chloroplast-specific extensions (8). CS1 is the first example of an E. coli-like chloroplast r-protein with ribosomal-specific extensions which is smaller in size than its E. coli homologue.

The six evolutionary related elements of S1 (D1, D2, and R1 to R4) are separated into two functional domains (see Introduction). Interestingly, the three homologous units of CS1 show significant homology with both the RNA and ribosome-binding domains. The functional identity of the three CS1 units is therefore of interest. As the U2–U3 units have the highest homology with the R1–R2 fragment which contains a strong RNA-binding activity (14, 35), we assume that the CS1 units contain the RNA-binding function which is described in this paper. The comparison of the amino acid sequence of the CS1 repeats relative to S1 should be useful for the elucidation of the precise motifs which in these proteins are involved in mRNA recognition. Other RNA-binding proteins which are implicated in RNA biosynthesis and processing have been studied recently (36, 37), and an 80-residue RNA recognition motif has been identified. Within the RNA recognition motif of these proteins two stretches of amino acids are highly conserved (RNP1 and RNP2). Interestingly, S1 or CS1 do not contain evident similarity with either the RNA recognition motif, the RNP1, or the RNP2 stretches. We can speculate that the two conserved β-sheet structural elements which are surrounded by conserved Gly residues are essential for the affinity of CS1 and of S1 to mRNAs. The comparison of the amino acid sequence of R1 and of U3, the most related fragments, allows the detection of several stretches of 4 amino acid residues (see “Results”) which might be essential for RNA binding. An experimental characterization of these RNA-binding elements will be necessary to specify the relationship, if any, between the S1 proteins and the RNA-binding protein family.

We have shown that CS1, either assembled in the 30 S ribosomal subunit or isolated, has mRNA-binding properties. In E. coli, at least three of the four RNA-binding repeats of S1 are necessary to maintain mRNA binding activity (14). Since we showed that CS1 is engaged in the same biological function as S1, all the three homologous chloroplast repeats should also be required for the mRNA binding. The domain of CS1 involved in protein-protein interaction in the 30 S ribosomal subunit cannot be localized at present, but the settlement of this property within the central part has to be considered, although it could also be localized in one or both of the CS1 polypeptide extensions. The CS1 protein contains three repetitive elements instead of six in the S1 and therefore constitutes a simpler model to investigate on the essential elements responsible for the S1-type functions.

A functional property that clearly distinguishes CS1 from S1 is the high binding affinity of CS1 for poly(A) exclusive of the other homopolymers. In contrast, S1 has a very high affinity for poly(U) (10) and cross-reacts with a specific pyrimidine-rich sequence contained in the leader region of many E. coli mRNAs (11).

We demonstrate that the RNA-binding property of CS1 assembled into the chloroplast 30 S ribosomal subunits is associated with the initiation complex formation. The functional significance of this mRNA-binding activity will now be discussed. First, CS1 would have an RNA affinity which would not be sequence- or structure-specific. This property would increase the stability of the mRNA-30 S ribosomal subunit interaction, the correct positioning at the initiation being controled by other factors (Shine-Dalgarno RNA-RNA interaction and initiation factors). A second function of CS1 is suggested by the binding of free CS1 to the CL22 mRNA and by the toeprinting competition assays between 30 S subunits and free CS1. It is possible that CS1 would recognize specific structures that would enable the correct positioning of the ribosomal small subunit on the initiation region. Because of its poly(A) binding activity the CS1 protein might recognize a poly(A) stretch in the translation initiation region. An analysis of the entire tobacco chloroplast genome (38) shows the presence of many poly(A) stretches which could play a role in the efficiency of translation initiation. In particular, the presence of a poly(A)-rich region in the CL22
mRNA translation start region (position +13 to +30, see Ref. 18) could be responsible for the initiation complex inhibition that we report in vitro in the presence of an excess of free CS1 as competitor. Further experiments are necessary to show whether a specific site of interaction exists between CS1 and the L22 mRNA. Since it has been reported that a free pool of CS1 is present in the chloroplast stroma (19), CS1 may also act as a translational repressor by interacting with the poly(A) stretches.

Poly(A)-binding proteins which bind to the 3'poly(A) tail of cytosolic mRNAs in eukaryotic cells have been characterized and were shown to increase the translational efficiency (39). By analogy, the cytosolic precursor of the r-protein CS1 could have a similar function in the translation of cytosolic mRNA.

In conclusion, we have established the presence of an S1-like protein in the chloroplast ribosome. The CS1 protein possesses structural and functional properties which are specific. The protein is a good candidate to be a regulatory factor in chloroplast translation. The identification of CS1 and the characterization of some of its properties open up new possibilities for studying the regulation of chloroplast translation at the initiation stage.

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