Chloroplast Ribonucleoproteins Function as a Stabilizing Factor of Ribosome-free mRNAs in the Stroma*

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Post-transcriptional RNA processing is an important step in the regulation of chloroplast gene expression, and a number of chloroplast ribonucleoproteins (cpRNPs) are likely to be involved in this process. The major tobacco cpRNPs are composed of five species: cp28, cp29A, cp29B, cp31, and cp33 and these are divided into three groups (I, II, and III). By immunoprecipitation, gel filtration, and Western blot analysis, we demonstrated that these cpRNPs are abundant stromal proteins that exist as complexes with ribosome-free mRNAs. Many ribosome-free psbA mRNAs coprecipitate with cpRNPs, indicating that the majority of stromal psbA mRNAs are associated with cpRNPs. In addition, an in vitro mRNA degradation assay indicated that exogenous psbA mRNA is more rapidly degraded in cpRNP-depleted extracts than in nondepleted extracts. When the depleted extract was reconstituted with recombinant cpRNPs, the psbA mRNA in the extract was protected from degradation to a similar extent as the psbA mRNA in the nondepleted extract. Moreover, restoration of the stabilizing activity varied following addition of individual group-specific cpRNPs alone or in combination. When the five cpRNPs were supplemented in the depleted extract, full activity was restored. We propose that these cpRNPs act as stabilizing factors for nonribosome-bound mRNAs in the stroma.

Chloroplasts contain their own genes, and chloroplast gene expression is regulated at both the transcriptional and posttranscriptional level (1–3). Quantitative analysis of spinach (4) and barley (5, 6) has revealed that the mRNA levels of several protein-encoding genes in chloroplasts can increase dramatically (from 20- to ~1,000-fold) during plastid differentiation and chloroplast development. The increased abundance of these mRNAs cannot, however, account for the relative transcription rate of these genes (4).

The half-lives of chloroplast mRNAs have been shown to range from 6 h for the mRNA encoding the 83-kDa chlorophyll a apoprotein of photosystem I gene (psaA) to over 40 h for the mRNA encoding the D1 protein of photosystem II (psbA), 1 Refs. 7 and 8). Moreover, the stability of chloroplast mRNAs has been shown to change in response to chloroplast development and varying light conditions. This suggests that the differential accumulation of chloroplast mRNA is regulated primarily at the post-transcriptional level, with mRNA stability then contributing to the mRNA steady-state level. The mechanism underlying mRNA stability, however, is poorly understood.

Like Escherichia coli mRNAs, most chloroplast mRNAs contain an inverted repeat (IR) sequence in their 3′-untranslated region (UTR) that can fold into a stable stem-loop structure. This structure has been shown to be important in determining mRNA stability both in vitro (9–11) and in vivo (11, 12). Several chloroplast proteins, detected by UV cross-linking (13–17) and gel-shift assays (18–20), have been found to bind to the 5′- or 3′-UTRs of mRNAs. These chloroplast proteins could be gene-specific mRNA-binding proteins. In addition, numerous nuclear mutants of Chlamydomonas reinhardtii (17, 21), maize (22), barley (23, 24), and Arabidopsis thaliana (25), have been identified, which fail to accumulate individual chloroplast-encoded mRNAs (or precursor (pre)-mRNAs) despite having normal transcription rates.

We previously isolated five nuclear-encoded chloroplast ribonucleoproteins (cpRNPs) from tobacco, which we named cp28, cp29A, cp29B, cp31, and cp33 according to their sizes in kDa (26, 27). Based on phylogenetic comparison to the cpRNPs from A. thaliana, tobacco cpRNPs can be classified into three groups: cp29A and cp29B in group I, cp28 and cp31 in group II, and cp33 in group III (28). Tobacco cpRNPs have two consensus sequence-type RNA-binding domains and an acidic N-terminal domain. Similar proteins and genes encoding cpRNP homologs have also been found in a variety of other plant species (29) including spinach (30), A. thaliana (28), maize (31), and barley (32).

In vitro, tobacco cpRNPs have a strong affinity for RNA homopolymers (poly(G) and poly(U)) rather than single-stranded or double-stranded DNA (33, 34). After UV cross-linking chloroplast proteins with several mRNA probes, a subset of proteins of around 30 kDa can usually be detected in the chloroplasts of land plants (15, 16) and green algae (17). This suggests that cpRNPs bind nonspecifically to chloroplast RNAs. Spinach 28RNP, a similar protein to tobacco cp28 and cp31, was reported to be required for the formation of the 3′-end of several mRNAs in vitro (30, 35). Further studies have shown this protein directs correct processing of the 3′-end pre-mRNA by the high molecular weight complex in vitro (36). We recently found that tobacco cpRNPs in vivo bind not only to mRNAs (and pre-mRNAs) but also to intron-containing pre-mRNAs (37). This suggests that cpRNPs are involved in RNA splicing; PAS, protein A-Sepharose, β-gal, β-galactosidase; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; hRNAP, heterogeneous ribonucleoprotein; LS, large subunit; re-cp, recombinant cp.
RESULTS

cpRNPs Are Abundant Stromal Proteins—The amount of cpRNPs in a series of dilute chloroplast suspensions was determined by Western blot analysis. By comparing the intensity of the five cpRNPs protein bands with the intensity of the control recombinant cpRNPs bands, 107 chloroplasts were estimated to contain –20 ng of cp29A, 10 ng of cp28, and 2 ng of cp33 (Fig. 3A). This is equivalent to 105 molecules of cp29A, 51,000 molecules of cp28, and 8,000 molecules of cp33 per chloroplast. The levels of cp29A and cp29B were similar, and the cp28 and cp31 levels were equivalent to the cpRNPs levels previously estimated by single-stranded DNA column chromatography (26, 27). These results indicate that tobacco cpRNPs accumulate at high levels in the chloroplasts of green leaves. In comparison, 109 chloroplasts have been shown to contain 200 ng of the large subunit (LS) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Fig. 1B). This is equivalent to 2.2 × 107 molecules of LS per chloroplast. Rubisco holoenzyme is composed of eight each of the large and small subunits and is thus calculated to be 2.8 × 109 molecules of Rubisco holoenzyme per chloroplast. This is comparable with 5.8 × 108 Rubisco molecules per chloroplast in barley (40).

To compare the relative amount of cpRNPs protein and mRNA in chloroplasts, we quantified the steady-state level of psba mRNA by Northern blot analysis (Fig. 1C). Using in vitro synthesized psba mRNA (1–500 ng) as the control, we detected 120 ng of psba mRNA per 107 chloroplasts. This is equivalent to ~14,000 psba mRNA molecules per chloroplast.

cpRNPs Form Complexes with RNA—In a previous analysis of the sedimentation profiles of stromal extract cpRNPs by sucrose density gradient centrifugation, we showed that most cp28 and cp31 sediments lie between the top of the gradient and the 18 S Rubisco holoenzyme (41). Moreover, we have recently observed that stromal mRNAs and intron-containing pre-tRNAs coprecipitate with cpRNPs (37). These observations suggest that cpRNPs may form complexes with RNAs. If cpRNPs-RNA complexes do exist in the stroma, their size may contribute to the stability of stromal mRNAs.

EXPERIMENTAL PROCEDURES

Preparation of Intact Chloroplasts and Stromal Extracts—Intact chloroplasts were isolated from the green leaves (5–8 cm) of tobacco plants (Nicotiana tabacum var. Bright Yellow 4) as described previously (26). The chloroplasts were lysed in extraction buffer (50 mM Tris-HCl, pH 7.0, 0.5 mM KCl, 2 mM dithiothreitol, 10 mM MgCl2, and 500 units of RNase inhibitor from Takara Shuzo) for 15 min at 4 °C. Stromal extracts were obtained by centrifuging the lysate at 15,000 × g for 10 min, and filtering the supernatant through a Millipore filter (0.22-μm pore size).

Detection of cpRNPs—The number of isolated intact chloroplasts was counted in a hemocytometer by light microscopy. A series of dilute chloroplast suspensions (containing 107–109 chloroplasts) were prepared by diluting samples with extraction buffer. Ten minutes after sample injection, 100 μl of each of the 21 fractions was collected and labeled with protein assay kit (Bio-Rad).

Detection of authentic cpRNPs in the stromal extract was accomplished by single-stranded DNA column chromatography (26, 27). These results indicate that tobacco cpRNPs accumulate at high levels in the chloroplasts of green leaves. In comparison, 109 chloroplasts have been shown to contain 200 ng of the large subunit (LS) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Fig. 1B). This is equivalent to 2.2 × 107 molecules of LS per chloroplast. Rubisco holoenzyme is composed of eight each of the large and small subunits and is thus calculated to be 2.8 × 109 molecules of Rubisco holoenzyme per chloroplast. This is comparable with 5.8 × 108 Rubisco molecules per chloroplast in barley (40).

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Function of Chloroplast Ribonucleoproteins

To determine the size of these proposed cpRNP-RNA complexes, tobacco stromal extracts were separated by size exclusion chromatography through a Superdex 200 PC column (Fig. 2). This column separates proteins in the size range of 10–600 kDa. The 30 S ribosomal subunits (900 kDa) and 70 S ribosomes (2,500 kDa) could be excluded from the column and were collected in void fractions 1–6 (Fig. 2B). The cpRNPs were distributed over a wide range of fractions from 30–600 kDa, and cp29A, cp29B (group I), and cp33 (group III) were also detected in fraction 6 (>600 kDa) (Fig. 2A, frac. no.). The broad cpRNP peaks could not be attributed to overloading the column, because a reduction in the amount of extract loaded did not change the separation profiles (data not shown). The size of the cp28 complexes ranged from 30–600 kDa (fractions 7–14), and the cp31 complexes ranged in size from 30–400 kDa (fractions 8–13). This implies that most of the cpRNPs do not cofractionate with ribosomes.

When the stromal extracts were treated with RNase A prior to size fractionation, the peaks for cp29A, cp29B, and cp28 shifted dramatically to 30 kDa (fraction 12) at the same position as recombinant cp28 (Fig. 2C, his-cp28). This confirms that the proteins detected at 30 kDa are RNA-free cpRNPs. By contrast, the peaks of cp31 and cp33 were detected at about 50 kDa (fraction 11). RNase treatment of the extracts did not change the protein profiles of stromal extracts (data not shown). Overall, these results suggest that cpRNPs form a stable RNA-protein high molecular weight complex with nonribosome-bound RNAs, with cp28, cp29A, and cp29B interacting as monomers and cp31 and cp33 interacting as oligomers.

Most of the Stromal psbA mRNA Binds with cpRNPs—To test whether the cpRNPs are associated with ribosome-free stromal mRNAs, the stromal extracts were subjected to immunoprecipitation using antibodies against group I cpRNP (cp29A), II (cp28 and cp31), and III (cp33), respectively. The amount of mRNA was then determined by Northern blot analysis using gene-specific probes. Approximately 90% of the stromal psbA mRNA coprecipitated with group I and II cpRNPs (Fig. 3), whereas psbA mRNA coprecipitated less with group III cpRNP (cp33) than with group I and II proteins. This indicates that most of the psbA mRNAs are always associated with group I and II cpRNPs but not with group III cpRNP (cp33). Distribution of mRNA into the supernatant and pellet coincides with that of group I and II cpRNPs (Fig. 3). By contrast, the majority of 23 S ribosomal RNA did not coprecipitate with any group of cpRNPs and remains in the supernatant (Fig. 3). This result confirms that most of the mRNA associated with cpRNPs is likely to be ribosome-free. The anti-β-gal antibody did not coprecipitate with psbA mRNA to such a high degree. These observations support previous studies that have shown that most of the stromal psbA mRNA in barley is ribosome-free (42).

cpRNPs Stabilize psbA mRNA—To examine the possibility that cpRNPs bind to ribosome-free RNA to protect the RNA from degradation, the effect of cpRNPs on RNA degradation was analyzed using three different stromal extracts: an extract
treated with unrelated serum (control ex), a cpRNP-depleted extract (dep-ex), and a depleted extract supplemented with recombinant cpRNPs (dep-ex + cpRNPs) (Fig. 4). Based on quantification of cpRNPs in the stromal extract (Fig. 1), 3 μg each of cp29A and B, 1.5 μg each of cp28 and cp31, and 0.15 μg of cp33 were supplemented to the depleted extract. Western blot analysis verified that prior to mRNA incubation the depleted extract sample was completely depleted of all five cpRNPs, and appropriate amounts of recombinant proteins were supplemented to the depleted extract (Fig. 4C). The in vitro-synthesized psbA mRNA was then incubated with each extract, and its degradation was monitored (Fig. 4, A and B). The half-life of the full-length psbA mRNA was 6 min in the control extract and 2 min in the depleted extract. Thus, the half-life of the psbA mRNA in the depleted extract was 3-fold shorter than in the control extract. Supplementing the depleted extract with five recombinant cpRNPs, however, lowered the degradation rate back to the level of the control extract. This result was the same as that of an in vitro assay using a shorter psbA mRNA, which lacks 3′-UTR (data not shown).

The protein bands corresponding to the endogenous cpRNPs and the recombinant cpRNPs were detected by UV cross-linking and a 32P-labeled psbA mRNA probe. The cpRNPs interacted directly with the endogenous psbA mRNA probe (Fig. 4C). Overall, the results of this experiment suggest that binding of all or some cpRNPs to mRNA protects the RNA from degradation.

**Different Contributions of Individual Group cpRNPs to mRNA Stability**—To investigate different effects of individual cpRNPs on mRNA stability, we carried out further in vitro mRNA degradation assays using reconstituted stromal extracts. As shown in Fig. 5, depletion of all three groups of cpRNPs reduced mRNA stability levels to 55% of the control extract. When either of three groups of recombinant cpRNP was supplemented to cpRNPs-depleted extract (Fig. 5, control), mRNA stability was increased. Supplementation of the group III cpRNP (cp33) resulted in a drastic increase in mRNA stability rather than group I and II proteins. When the two groups of cpRNPs, in any combination, were supplemented to the depleted extract, mRNA stability was further increased to levels reaching 80–90% of the control extract. These observations indicate that all three groups of cpRNPs are cooperatively involved in stability of psbA mRNA, possibly via individual group-specific cpRNPs. In addition, group III protein (cp33) exhibited the most effective influence on mRNA stability. Supplementation of three groups resulted in full restoration of mRNA stability. This agrees with the previous experiment (Fig. 4).

**DISCUSSION**

The present study has shown that the five cpRNPs are abundant (~3 × 10^5 molecules) and accumulate at one-tenth of the level of Rubisco in tobacco chloroplasts. Their amounts are apparently greater than total molecules of chloroplast mRNAs, including the most abundant psbA mRNA (~14,000 molecules), and perhaps greater than ribosomes. For instance, Rapp et al. (6) estimated that each chloroplast of dark-grown barley seedlings has 1.4 × 10^5 molecules of 16 S rRNA.

We used size fractionation and immunological tools to show that the cpRNPs range in size from 30 to 600 kDa (cp28 and cp31), or to larger (cp29A, cp29B, and cp33) and that most of the cpRNPs bind to ribosome-free RNAs. This size distribution probably reflects the binding of either single or multiple cpRNPs to various RNA species in the stroma. The cpRNPs appear to be part of a higher molecular weight complex that is associated with RNA. RNase treatment of the complex shifts it to a smaller size ~30 kDa. Interestingly, the gel filtration results also suggest that cp28, cp29A, and cp29B interact with RNAs as monomers, whereas cp31 and cp33 may interact as oligomers (~50 kDa). This suggests that the presence of two
distinct forms of cpRNPs may reflect different function(s) for different cpRNPs.

In the present study, the important finding was that cpRNPs contribute to RNA stabilization via direct binding to target RNAs. Chloroplast extracts depleted of all five cpRNPs degraded exogenous psbA mRNA faster than did nondepleted extracts. The IRs of psbA mRNA have previously been shown to act as cis-elements for RNA stability in spinach (9) and C. reinhardtii (11). In this study, however, the rapid degradation of IR-containing exogenous psbA mRNA suggests that the IR of psbA mRNA may only contribute in part to mRNA stability in vitro. Numerous ribonuclease activities have been reported in chloroplasts (36, 43–47). Klaff (48) reported that degradation of psbA mRNA is initiated by endonucleolytic cleavage of psbA mRNA. Once the mRNA has been cleaved internally, the RNA fragments are then efficiently polyadenylated and exonuclease lytically degraded (49, 50). The cpRNPs probably bind to internal sequence(s) targeted for cleavage by endonucleases, thereby protecting these sequences from degradation. Although cp33 exists at a 10-fold lower level than other cpRNPs, it demonstrated a significant effect on mRNA stabilization rather than the more abundant group I and II cpRNPs. This suggests that cp33 may bind initially to mRNAs, and thereby recruit other cpRNPs or unknown components to facilitate the formation of stable cpRNP and mRNA complexes.

Our previous work has clearly shown that several mRNAs (psbA, petD, and rbcL) encoding photosynthetic components and intron-containing pre-tRNAs coprecipitate predominantly with group I and II cpRNPs (37). This suggests that group I and II cpRNPs are involved mainly in the stability of mRNA and/or splicing of pre-tRNAs. Moreover, using an in vitro RNA editing system developed from tobacco chloroplasts, we have observed that only cp31 is required for RNA editing (C→U conversion) of psbL mRNA that encodes the L-protein of photosystem II.2 These results indicate that each cpRNP contributes to a differing extent to RNA stability, RNA cleavage, RNA editing, or RNA splicing.

It is likely that tobacco cpRNPs are general RNA-binding proteins, like nuclear-localized heterogeneous ribonucleoprotein (hnRNP). Both cpRNPs and hnRNPs have strong affinities for poly(G), poly(U), and single-stranded DNA (33, 34, 51), and both are abundant proteins within the chloroplast and nucleus, respectively. In analogy to the function of hnRNP, cpRNP plays a role in various RNA processing before initiation of translation of mature mRNAs. Transcription is believed to occur in nucleoids that are composed of chloroplast DNA and several proteins (52, 53). It is interesting to note that cpRNPs are also detected in tobacco chloroplast nucleoids.3 This suggests that cpRNPs bind to nascent RNAs in the nucleoids.

From the overall results of the present study, we propose a model for the possible role of cpRNPs. The cpRNPs associate with nascent RNAs or pre-RNAs immediately after transcription in the nucleoids, and form RNA-protein complexes in the stroma. These cpRNP-RNA complexes confer stability and ribonuclease resistance to the RNAs. The complexes also act as a scaffold for the specific catalytic machinery involved in RNA maturation, RNA splicing of intron-containing pre-tRNAs, or RNA editing. When the cpRNPs dissociate from fully processed and mature mRNAs, ribosomes then attach to the mRNAs for translation.

The cp31 and cp33 proteins have 64 and 42 residues, respectively, of auxiliary domains in their N terminus with 43% acidic residues (26). The N-terminal regions of these proteins may be functionally significant, because the acidic region is required for protein-protein interaction (54). The N-terminal acidic regions of some cpRNPs are efficiently phosphorylated in or-ganello in a light-dependent manner, and association of cpRNPs with RNAs and their dissociation from RNAs may be regulated by phosphorylation in tobacco4 and spinach (55). To clarify this possibility, further biochemical and molecular analyses need to be carried out.

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