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RNA-Binding Characteristics of a Ribonucleoprotein from Spinach Chloroplast[1]

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A chloroplast (nuclear-encoded) RNA-binding protein (28RNP) was previously purified from spinach (*Spinacia oleracea*). This 28RNP was found to be the major RNA-binding protein co-purified during the isolation scheme of 3′ end RNA-processing activity of several chloroplastic genes. To learn more about the possible involvement of 28RNP in the 3′ end RNA-processing event, we investigated the RNA-binding properties and the location of the protein in the chloroplast. We found that recombinant *Escherichia coli*-expressed 28RNP binds with apparently the same affinity to every chloroplastic 3′ end RNA that was analyzed, as well as to RNAs derived from the 5′ end or the coding region of some chloroplastic genes. Differences in the RNA-binding affinities for some chloroplastic 3′ end RNAs were observed when the recombinant 28RNP was compared with the "native" 28RNP in the chloroplast-soluble protein extract. In addition, we found that the 28RNP is not associated with either thylakoid-bound or soluble polysomes in which a great portion of the chloroplast rRNA and mRNA are localized. These results suggest that the native 28RNP binds specifically to certain RNA molecules in the chloroplast in which other components (possibly proteins) and/or posttranslational modifications are involved in determining RNA-binding specificity of the 28RNP.

The expression of chloroplast genes is regulated by a variety of mechanisms, one of which is the modulation of RNA stability during chloroplast development. Chloroplast-precursor RNAs undergo a variety of maturation events including *cis*- and *trans*-splicing, cleavage of polycistronic messages, processing of 5′ and 3′ ends, and editing (Mullet, 1988; Gruissem, 1989; Sugiura, 1991; Rochaix, 1992). Most chloroplast genes contain inverted repeats in their 3′ end untranslated region, which are capable of forming stem loop structures and do not serve as efficient transcription terminators. These stem loops are essential for the stability of mRNAs in vivo and in vitro and may function as 3′ end-processing signals (Stern and Gruissem, 1987; Stern et al., 1989, 1991).

To study the molecular mechanisms of 3′ end RNA processing and how mRNA stability is modulated during chloroplast development, the proteins that bind the 3′ end chloroplast RNAs that are involved in these processes must be identified and isolated. Several 3′ end RNPs were identified in the chloroplast extracts of spinach (*Spinacia oleracea*) and mustard using UV-cross-linking and gel-shift methods (Nickelsen and Link, 1989, 1991; Stern et al., 1989; Chen and Stern, 1991). One of these, a nuclear-encoded RNP (28RNP) had been isolated before as the major RNP that co-purified with the 3′ end RNA-processing activity when soluble chloroplast protein extract had been fractionated on anion-exchange and RNA-affinity columns (Schuster and Gruissem, 1991). The deduced amino acid sequence disclosed two similar 80-amino acid RNA-binding domains, as well as an amino terminus acidic domain. In addition, cDNAs of similar RNPs were cloned from tobacco (Li and Sugiura, 1990; Ye et al., 1991; Mieszczak et al., 1992), maize (Cook and Walker, 1992), and *Arabidopsis* (Bar-Zvi et al., 1992; DeLisle, 1993). The expression of these proteins is developmentally regulated, organ specific, and influenced by light. Immunodepletion of the 28RNP from soluble chloroplast protein extract interfered with the in vitro 3′ end RNA-processing activity, suggesting possible involvement of 28RNP in that process (Schuster and Gruissem, 1991).

To learn more about the interaction of 28RNP with chloroplast RNAs, RNA-binding properties and its location in the chloroplast were investigated. We found that recombinant *Escherichia coli*-produced 28RNP binds every RNA that was analyzed with approximately similar affinities. Specificity was found in binding to ribohomopolymers. However, when the affinity of the recombinant *E. coli*-produced 28RNP was compared to that of the "native" protein in the chloroplast protein extract, differences in the binding to some 3′ end RNAs of chloroplastic genes were obtained. These results suggest that the amino acid sequence of the 28RNP produces the basic affinity to RNA and interaction with other proteins and/or posttranslational modifications confer RNA-binding specificity of the 28RNP in the chloroplast.

MATERIALS AND METHODS

Preparation of Synthetic RNAs

Constructions of the plasmids used to transcribe the chloroplast RNAs were previously described (Stern and Gruissem, 1987; Stern et al., 1989). For the experiments described here, the inserts were transferred into Bluescript KS$^+$ (Strategene, La Jolla, CA) so that transcription with T7

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Abbreviations: hnRNP, heterogeneous nuclear ribonucleoproteins; kd, concentration of RNA that gives half-saturation of binding; RNP, RNA-binding protein.

RNA polymerase generated the mRNA-like strand. Synthetic RNAs for the UV-cross-linking and in vitro processing experiments were prepared as previously described with 2.5 μM [α-^{32}P]UTP and 25 μM nonradioactive UTP (Stern and Gruissem, 1987; Stern et al., 1989; Schuster and Gruissem, 1991). Nonradioactive RNAs for competition experiments were synthesized from the same DNA plasmids using the AmpliScribe kit (Epicentre Technologies, Madison, WI) according to the manufacturer's instructions. The amount of RNA (compared to tRNA used as a standard) was quantified by ethidium bromide staining of RNA fractionated on an agarose gel.

Preparation of Recombinant *Escherichia coli*-Produced 28RNP

The 28RNP cDNA (minus the transit peptide that mediates transport into the chloroplast where it is cleaved off) was cloned into the pQE31 expression vector (Qiagen, Chatsworth, CA) with the addition of six His residues to the amino terminus of the mature protein. The recombinant protein was produced in SG13009[pREP4] cells by growing to A_{600} = 0.9 on Luria Broth (100 mL) containing ampicillin (100 μg/mL) and kanamycin (25 μg/mL), followed by the addition of isopropyl-β-D-thiogalactopyranoside (2 mM) for 3 h. Bacteria cells were collected by centrifugation, resuspended in 5 mL of sonication buffer (50 mM Na-phosphate, pH 8.0, 300 mM NaCl), and broken using a French press cell operating at 20,000 psi. The membrane fraction was removed by centrifugation for 20 min at 13,000*g* and the soluble fraction containing most of the expressed protein was incubated with Ni-NTA-Agarose for 1 h at 4°C with gentle mixing. The material was applied to a column and washed with 10 volumes of sonication buffer and 10 volumes of washing buffer (50 mM Na-phosphate, pH 6.0, 300 mM NaCl), and the protein was eluted using a linear gradient to 0.5 M of imidazole in a washing buffer, followed by dialysis against buffer E (20 mM Hepes, pH 7.9, 60 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, and 5% glycerol). Protein concentration was determined using the Bio-Rad protein assay kit. To verify that the RNA-binding properties of the protein were not altered by the His residues, other proteins were expressed in the same system and were checked for RNA binding. No RNA binding could be observed using several proteins overexpressed in this way (data not shown). In addition, 28RNP expressed as a fusion protein to the *E. coli* maltose-binding protein or the β-galactosidase had the same RNA-binding affinities as the His tag fusion protein (data not shown). After two cycles of the Ni_2^+ affinity column purification, fractionation of the recombinant protein by SDS-PAGE resulted in only one protein band without any RNase activity when incubated with radioactively labeled RNAs (data not shown).

Oligonucleotide-Directed RNase H Cleavage of RNA

Oligonucleotide-directed RNase H cleavage was carried out as described by Cheng and Stern (1991) with the same oligonucleotide (15 mer), which was kindly provided by Dr. Stern (Boyce Thompson Institute, Ithaca, NY). The RNA cleavage products were separated on a denaturing polyacrylamide gel, followed by extraction and ethanol precipitation.

Chloroplast Protein Extract

Chloroplast protein-soluble extract was prepared from young leaves of hydroponically grown spinach (*Spinacia oleracea* cv Viroflay) plants as previously described (Gruissem et al., 1986). The 28RNP was depleted from the extract by a specific 28RNP IgG column (Schuster and Gruissem, 1991). Briefly, 28RNP-specific IgG was bound covalently to protein A coupled to agarose beads using dimethylpimelimidate (Harlow and Lane, 1988). The IgG-protein A beads were incubated with 500 μg of protein extract for 1 h at 4°C in buffer E, followed by centrifugation and washing with buffer E. The bound fraction was eluted with 0.1 M Gly, pH 2.5, and immediately titrated to pH 7.0 using 0.1 volume of 1 M Tris and dialyzed against buffer E.

UV-Cross-Linking and in Vitro Processing

UV-cross-linking of protein(s) to [α-^{32}P]UTP-labeled RNAs was carried out as previously described (Schuster and Gruissem, 1991). Essentially 30 fmol of RNA (240,000 cpm) or the amount indicated in the figure legends were incubated for 10 min in 15.5 μL with protein(s) in a buffer containing 10 mM Hepes, pH 7.9, 30 mM KCl, 6 mM $MgCl_2$, 0.05 mM EDTA, 1 mM DTT, and 8% glycerol. A longer incubation time did not yield higher cross-linking yield of the RNA to protein. In competition experiments, the nonradioactive RNA was added 5 min before the radioactively labeled RNA. Following UV irradiation in a UV-cross-linker (Hoeffer, San Francisco, CA) at 1.8 J, the RNA was digested by 1 μg of RNase A at 37°C for 30 min and the proteins were fractionated by SDS-PAGE. The transfer labeled from the RNA to the proteins were detected by autoradiograph and quantitation was performed by scanning the autoradiogram with a laser-scanner densitometer. In vitro processing of 3′ end RNA in chloroplast protein extract was carried out as previously described (Schuster and Gruissem, 1991).

Fractionation of Chloroplast into Thylakoid Polysomes and Soluble Fractions

Intact chloroplasts were isolated from young spinach leaves on Percoll gradients and were disrupted osmotically in buffer containing 10 mM Tris, pH 7.9, 1 mM EDTA, and 5 mM DTT. An equal volume of buffer containing 50 mM Tris, pH 7.9, 10 mM $MgCl_2$, 2 mM DTT, 100 μg/mL chloramphenicol, 0.5 mg/mL heparin, and 30% glycerol was added and the thylakoids with bound polysomes were pelleted at 10,000*g* for 10 min. Soluble polysomes were pelleted at 300,000*g* for 3 h through 0.5 and 1.75 M Suc cushions (Barkan, 1988). Unpelleted material was taken as the soluble fraction of the chloroplast.

RNA Blot and Protein Immunoblots

Isolation of RNA or proteins, fractionation on denaturing agarose or polyacrylamide gels, and detection with ran-

domly primed *psbA* gene probe or 28RNP-specific antibodies, respectively, were performed as previously described (Schuster and Gruissem, 1991).

RESULTS

Involvement of the 28RNP with the in Vitro 3′ End-Processing Activity

The 28RNP has been isolated as an RNP that co-migrated with the purification scheme of the in vitro 3′ end-processing activity of chloroplast mRNAs. In addition, it was purified by an RNA affinity column that was constructed using an RNA corresponding to the 3′ end of the chloroplast gene *psbA* (encoding the D1 protein of PSII). The deduced amino acid sequence of the nucleus-encoded 28RNP revealed that it can be divided into two RNA-binding domains as well as a novel Gly-rich acidic domain and a transit peptide. The transit peptide directs the protein into the chloroplast and is thereafter cleaved off. Immunodepletion of the protein from a chloroplast-soluble protein extract resulted in interference of the 3′ end-processing activity (Schuster and Gruissem, 1991; and Fig. 1). The in vitro 3′ end-processing product that is identical in size with the mature 3′ end of the corresponding RNA in the chloroplast (Stern and Gruissem, 1987) did not accumulate when the precursor RNA was incubated with an extract that has been immunodepleted of the 28RNP (Fig. 1A). However, the RNA 3′ end-processing activity in terms of generating the correct size of RNA 3′ end-processing product could be observed in the fraction that was eluted from the immunoaffinity column. In addition, the recombinant 28RNP overexpressed and purified from *E. coli* as described in "Materials and Methods" did not have 3′ end-processing or RNase activities when incubated with the precursor 3′ RNAs (Fig. 1A). The same results were obtained for other 3′ end RNAs that were tested in the depleted extract or the purified recombinant 28RNP. These results suggest that other components of the RNA 3′ end-processing machinery were immunodepleted using specific 28RNP antibodies, perhaps by a protein-protein interaction with the 28RNP.

Since immunodepletion of the 28RNP from the chloroplast-soluble protein extract interfered with the in vitro 3′ end-processing activity of chloroplast RNAs, we checked how this activity was affected when purified recombinant 28RNP was added in excess to the extract. First, the amount of 28RNP in the extract was measured using recombinant 28RNP and specific antibodies (data not shown). We found that there are 70 ng of 28RNP in 20 μg of soluble protein extract (0.35%). Addition of recombinant 28RNP to the chloroplast protein extract interfered with the efficiency of the 3′ end RNA processing (Fig. 1B). Less precursor RNA was converted into a product of the correct size when recombinant 28RNP was added in excess. Inhibition was detected at 3-fold excess but much more was detected at 7- and 14-fold (Fig. 1B).

In a similar experiment, addition of BSA protein instead of the recombinant 28RNP had no effect on the 3′ end

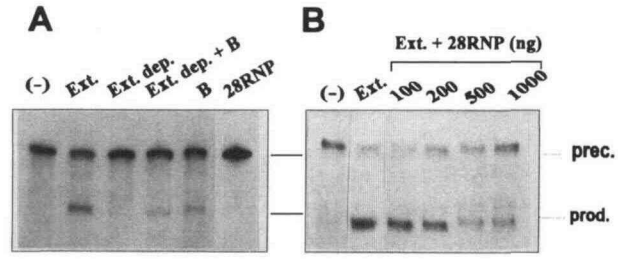

Figure 1. Depletion or addition of 28RNP to chloroplast protein extract interfered with the 3′ end RNA-processing activity. A, Depletion of the 28RNP from chloroplast protein extract. Chloroplast protein extract was chromatographed on a column consisting of specific 28RNP antibodies coupled to agarose. The flow-through and the bound fractions were analyzed for 3′ end-processing activity of the *psbA* RNA as substrate. Each 20-μL reaction contained 20 μg of chloroplast protein extract (containing 70 ng of 28RNP) and 1 fmol of [^{32}P]RNA. The reaction mixture was incubated for 1 h, followed by RNA isolation, fractionation on a denaturing polyacrylamide gel, and autoradiography. The precursor (prec.; 353 nucleotides) and the product (prod.; 262 nucleotides) of the in vitro processing reaction are indicated. Lanes are marked as follows: (−), RNA incubated with no protein; Ext., RNA incubated with protein extract; Ext dep., depleted extract: protein extract chromatographed on 28RNP antibody column (the flow-through); Ext. dep. + B, depleted extract reconstituted with bound fraction: the flow-through and the bound fractions of the 28RNP antibody column were combined together; B, bound fraction: proteins that were bound to the immunoaffinity column (100 ng); 28RNP, recombinant *E. coli* produced 28RNP (70 ng). B, Addition of the 28RNP to chloroplast protein extract. 3′ end processing of *psbA RNA* was performed as described in A with the addition of purified recombinant 28RNP. Lanes are marked as in A and the numbers indicate the amount of recombinant 28RNP that was added to the extract in ng.

RNA-processing reaction. This result could be explained either by the binding of the recombinant 28RNP to the RNA, which, therefore, inhibited the processing event, or by the binding of the 28RNP to essential components of the 3′ end-processing apparatus, which, thereby, prevented them from performing the processing event.

The Affinities of the Recombinant 28RNP to RNAs

The binding affinities of several chloroplast 3′ RNAs to 28RNP were analyzed in competition UV-cross-linking experiments using radioactive RNA corresponding to the 3′ end of the *psbA* gene and excess of nonradioactive RNAs. To compare the affinities of the protein to different RNAs, we defined an I_{50} value, which is the amount of competitor RNA required for inhibiting the binding of the 28RNP to *psbA* 3′ end RNA by 50%, in our binding assay system (see "Materials and Methods"). When the affinities of RNAs corresponding to the 3′ end of the chloroplast genes *psbA*, *petD* (encoding the subunit IV of the b_6f complex), and *rbcL* (encoding the large subunit of the ribulose-1,5-bisphosphate carboxylase enzyme) were determined, we found that they were similar (Fig. 2; Table I). In addition, the RNA corresponding to the 5′ end of the *psbA* gene that included an inverted repeat capable of forming a stem loop structure

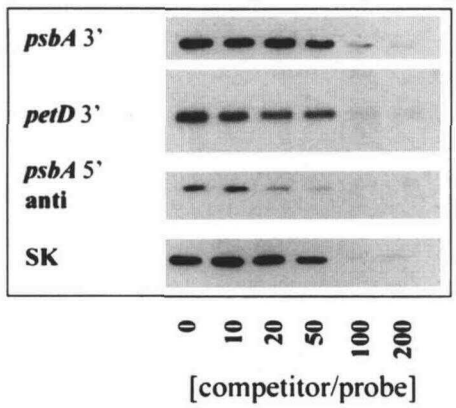

Figure 2. Binding of different RNAs to the 28RNP. Purified recombinant 28RNP was cross-linked to *psbA* 3′ end [^{32}P]RNA with the addition of excess nonradioactive competitor RNAs as indicated. *psbA* 3′ and *petD* 3′, RNAs corresponding to the *psbA* 3′ end (same as the probe) and *petD* 3′ end genes, respectively. *psbA* 5′ anti, RNA corresponding to the antisense RNA of the 5′ end of the *psbA* gene. SK, RNA that was transcribed using T7 RNA polymerase from the pBluescript KS plasmid linearized with *Pvu*II.

also had similar binding activity (Table I). Consequently, the antisense RNAs were transcribed from the 3′ end of the above genes. Although the antisense RNAs have different nucleotide sequences, they are capable of forming the stem loop structure. Competition binding analysis of these RNAs revealed that the I_{50} values of the *petD* and *rbcL* antisense RNAs were somewhat higher then the sense 3′ end RNAs, indicating less affinity to the 28RNP (Table I). In addition, the recombinant 28RNP bound with the same affinity to RNA sequences derived from the Bluescript polylinker sequences that showed no special secondary structure (using Wisconsin GCG FOLD) (Fig. 2; Table I). These results showed that recombinant 28RNP binds RNA molecules independently of the nucleotide sequences, and therefore, the possibility arose that other determinants, such as uridine-rich sequences or stem loop structures, are involved in conferring the RNA-binding affinity.

The *petD* 3′ end RNA is rich in uridine nucleotides in the sequence that does not participate in the formation of the stem loop structure (Fig. 3A). To analyze whether the 28RNP binds specifically to the uridine-rich sequence, to the stem loop structure, or to the general RNA sequence, the *petD* 3′ end RNA was divided into several RNA molecules (Fig. 3, A and B): RNA that contained the 84 nucleotides of the *petD*-coding region with an additional 8 nucleotides from the untranslated region was transcribed from the DNA template that was linearized with *Dra*I. Other RNA types are Δ54, a deletion mutant that lacks 49 nucleotides from the 3′ end of the *petD* 3′ RNA, and Δ24, which lacks 87 nucleotides from the 3′ end of the *petD* 3′ RNA and extends 182 nucleotides to the end of the first sequence of the inverted repeats (Chen and Stern, 1991). In addition, the oligonucleotide complementary to the 15 nucleotides immediately preceding the hairpin was used to generate the 3′ end RNAs (see "Materials and Methods" and Fig. 3A). The deletion mutant *petD* RNAs were analyzed for binding the 28RNP by the UV-cross-linking and competition assays. The results presented in Figure 3C and Table I reveal that each part of the *petD* 3′ end RNA, including or excluding stem loop, the coding region, and the uridine-rich sequences, was capable of binding the recombinant 28RNP with similar affinities.

Binding of the 28RNP to Ribohomopolymers

Many proteins composed of the RNA-binding domains are known to specifically bind ribohomopolymers of some nucleotides but not others. The poly(A)-binding protein binds poly(A), poly(G), and poly(U) but does not bind, or binds only with low affinity, poly(C) (Burd et al., 1991). The heterogeneous nuclear RNPs C1 and C2 bind poly(U) and poly(G) but not poly(A) and poly(C) (Dreyfuss et al., 1993). Similar RNPs from maize and tobacco have been shown to bind poly(U) and poly(G) much better than poly(A) and poly(C) (Li and Sugiura, 1991; Ludevid et al., 1992). We examined the binding affinity of spinach 28RNP to the ribohomopolymers and found that it binds to poly(U) and poly(G) with even higher affinities than to the RNAs corresponding to the 3′ end of the chloroplast genes (Fig. 4; Table I). The affinities of the recombinant 28RNP to poly(C) and poly(A) were found to be 100 and 40 times less, respectively, than to poly(U) and poly(G). These were the only RNA molecules we could find that bound the recom-

Table I. *Binding of recombinant 28RNP to different RNAs*

Binding of the recombinant 28RNP to different RNAs was measured by scanning autoradiographs of several UV-cross-linking competition experiments such as those shown in Figures 2 and 4 between the *psbA* 3′ end RNA and the different RNAs. Fifty nanograms of 28RNP was incubated with 30 fmols of *psbA* 3′ end RNA (2 nM) and competitor RNAs. The RNAs were described before (Stern et al., 1989; Chen and Stern, 1991) and schematic presentation of the deletion mutants of *petD* 3′ end RNA is shown and described in Figure 3A. *petD*(Δ24) is a deletion mutant that lacks 87 nucleotides from the 3′ end of the *petD* 3′ RNA, and its 182-nucleotide-long RNA extends to the end of the first sequence of the inverted repeats; hence this RNA lacks this structure. SK, RNA that was transcribed using T7 RNA polymerase from the pBluescript KS plasmid linearized with *Pvu*II.

RNA	I_{50}
	nM
petD 3′ sense	43
petD 3′ (*Dra*I)	48
petD 3′ (Δ54)	35
petD 3′ (Δ24)	48
petD 3′ antisense	70
psbA 3′ sense	50
psbA 3′ antisense	35
psbA 5′ sense	35
psbA 5′ antisense	46
rbcL 3′ sense	58
rbcL 3′ antisense	93
poly A	380
poly G	10
poly C	960
poly U	10
SK	35

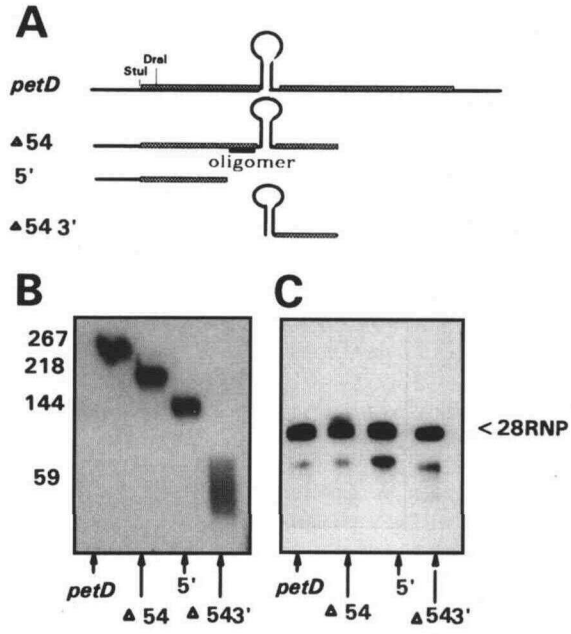

Figure 3. Binding of the 28RNP to deletion mutants *petD* 3′ end RNAs. A, Schematic representation of the RNA substrates used in the UV-cross-linking experiments. The line on the left represents the 3′ 84 nucleotides of the *petD*-coding region. *Stu*I (located exactly at the 3′ end of the translated region) and *Dra*I restriction sites are indicated, and the oligonucleotide complimentary to the 15 nucleotides immediately preceding the hairpin structure is indicated by a solid bar. The *petD* 3′ hairpin structure and the end point of the Δ54 (49 nucleotides 5′ to the end of *petD* RNA) are shown. Uridine-rich sequences are marked with hatched boxes. B, The deletion mutant *petD* 3′ RNAs were fractionated by denaturing PAGE and autoradiography. The length of the RNAs in nucleotides is shown on the left. C, UV-cross-linking binding analysis of the recombinant 28RNP to the deletion mutants of the *petD* 3′ end RNAs.

binant 28RNP with very low affinities. These experiments were supported also by binding experiments of the recombinant 28RNP to deoxyoligonucleotides, which were composed of 20 repetitions of C and A or G and T. It was found that the protein strongly binds the deoxyoligonucleotide with G and T but not with C and A and that a minimal two-G stretch is essential for binding (Lisitsky et al., 1994). Owing to the general RNA-binding activity of the recombinant 28RNP, the possibility arose that, if that is the case also with the mature protein, then the 28RNP should be localized with the most RNA-rich fractions of the chloroplast. Therefore, we next investigated the distribution of the 28RNP within the chloroplast.

Localization of the 28RNP in the Chloroplast

During translation, mRNAs in the chloroplast are associated with two populations of polysomes: soluble or thylakoid bound. Most of the mRNAs in the chloroplast are bound to either of these polysome fractions. Since the recombinant 28RNP binds with high affinity to almost every RNA that has been tested, we designed an experiment to analyze whether the native 28RNP is associated in the chloroplast with polyribosomes, where most of the rRNAs, as well as translated mRNAs, are present. Isolated chloroplasts were fractionated into soluble fraction, soluble polysomes, and thylakoid-bound polysomes. Staining the rRNAs of these fractions revealed equal distribution between the thylakoids and soluble polysomes and only a minor amount in the nonpolysomal soluble fraction (Fig. 5A). The distribution of the *psbA* RNA between these fractions was analyzed by probing with the *psbA* gene-specific probe (Fig. 5B). The *psbA* is a chloroplast-encoded gene that

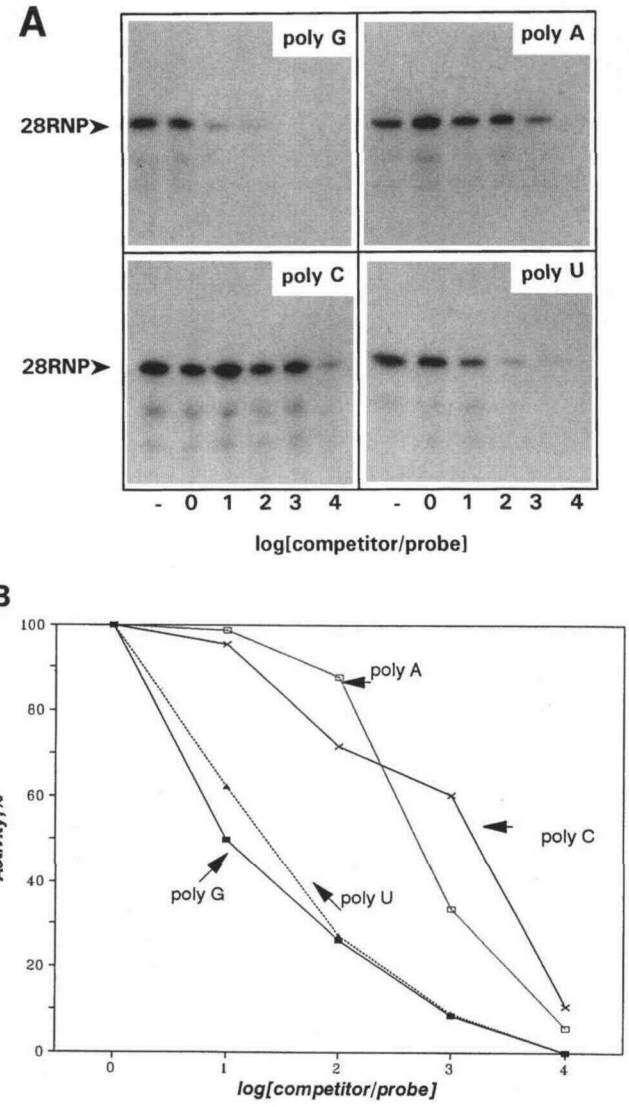

Figure 4. Binding of ribohomopolymers to the 28RNP. Recombinant 28RNP was incubated with *psbA* 3′ end RNA with the addition of poly(G), poly(A), poly(C), or poly(U) as competitors. Different amounts of ribohomopolymer as indicated were added to each reaction before the ^{32}P-labeled *psbA* 3′ end RNA was added. A, Autoradiogram of the UV-cross-linking analysis. B, Quantitation of the binding of the 28RNP to ribohomopolymers by scanning autoradiographs like the one presented in A of at least three different experiments. I_{50} was defined as the concentration of competitor that inhibits the binding to the *psbA* 3′ end RNA by 50% and is shown in Table I.

encodes the D1 reaction center protein of PSII and is translated by thylakoid-bound ribosomes (Kim et al., 1991). As was described before (Klein et al., 1988), most of the *psbA* RNA in the chloroplast could be detected in the soluble polysome fraction (that accommodated also RNA associated with small polysomes like monomers and dimers) and the thylakoid-bound ribosome fraction (Fig. 5B). The 28RNP, however, as probed with specific antibodies and by UV-cross-linking to 3′ end *psbA* RNA, was found primarily in the soluble fraction of the chloroplast (Fig. 5, C and D). Therefore, the 28RNP is not associated with polysomes and is primarily located in the soluble fraction of the chloroplast where only a small fraction of *psbA* RNA is localized. This is in agreement with the hypothesis that the 28RNP is involved in the 3′ end processing of RNA, which is a rapid and short-time process in vivo, as was shown in experiments in which 3′ end RNA precursors were electroporated into intact chloroplasts (G. Schuster, P. Klaff, and W. Gruissem, unpublished data; Adams and Stern, 1990).

Differences in RNA Binding between the Recombinant and the Native 28RNP

The similar affinities to different RNAs that were observed using the recombinant purified 28RNP raised the possibility that additional proteins and/or posttranslational modifications are involved in determining the specificity of binding. This is the case, for example, with the U2-B″ protein of the spliceosome, which specifically binds its target U2 RNA only in the presence of another protein, U2-A′, that is itself not an RNP (Scherly et al., 1990a). Therefore, we wanted to know whether the affinity of the native 28RNP in the chloroplast protein extract to 3′ end RNAs is the same in comparison to the recombinant purified protein. To this end, we performed RNA saturation UV-cross-linking assays in which increasing amounts of RNA were UV-cross-linked to the native 28RNP in the soluble protein extract and compared it to the same experiment using the bacterial expressed recombinant purified protein. The affinity of binding is measured from the RNA concentration that gives half-saturation of binding (Marciniak et al., 1990). Using these experimental conditions with the chloroplast protein extract, we identified three UV-cross-linked proteins of 24, 28, and 33 kD (Fig. 6, A and B). The 28-kD (middle) band gave the strongest UV-cross-linked signal and was identified as 28RNP by probing with specific antibodies (not shown). The 24-kD protein is an RNP similar in structure to the 28RNP in terms of binding to single-strand DNA and amino acid sequence composed of a C terminus acidic domain followed by two 80-amino acid RNA-binding domains (Gruissem and Schuster, 1993; S. Abrahamson, G. Schuster, and W. Gruissem, unpublished data). The lower cross-linked band was identified as the 24RNP by probing with specific antibodies. We obtained differences of approximately 3-fold between the

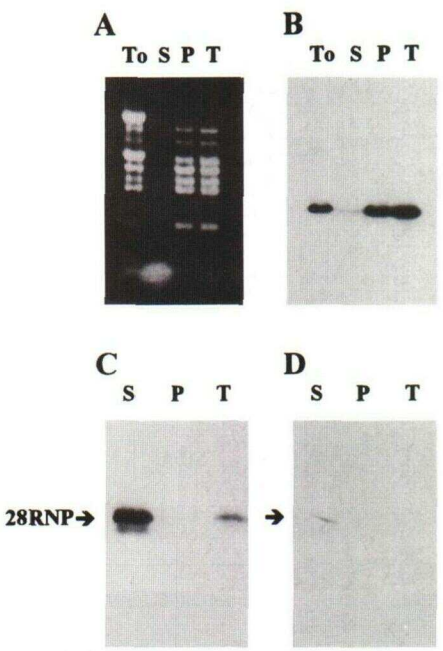

Figure 5. Distribution of the *psbA* RNA and 28RNP between the thylakoids, polysomes, and soluble fractions of the chloroplast. Intact chloroplasts were disrupted osmotically and fractionated into thylakoid fraction (T), polysome fraction (P), and soluble fraction (S). Equivalent amounts of extracted RNA or proteins were fractionated, stained to visualize rRNAs and tRNAs (A), probed with the *psbA* gene-specific probe (B), UV-cross-linked to 3′ end *psbA* RNA (C), and immunoblotted with 28RNP-specific antibodies (D). To, Total leaf RNA.

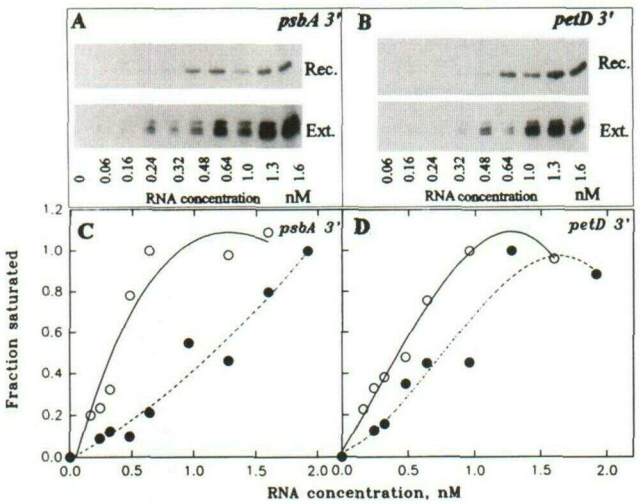

Figure 6. Saturation-binding curves of the recombinant and native 28RNP to *psbA* and *petD* 3′ end RNAs. Different amounts of [^{32}P]*psbA* (A and C) or *petD* (B and D) 3′ end RNA were incubated with either chloroplast-soluble protein extract or a purified recombinant 28RNP and subjected to UV-cross-linking analysis (A and B). Rec., Recombinant 28RNP. Ext., Chloroplast protein extract (the 28RNP is the middle band from the three cross-linked bands). Quantitation of the binding was done by scanning the autoradiographs of at least three different experiments and best fit analysis of the graphs. ○, Quantitation of the binding of the recombinant 28RNP. ●, Quantitation of the binding of the native 28RNP in the chloroplast protein extract.

Table II. *Differences in binding of recombinant 28RNP and native 28RNP in the chloroplast protein extract to chloroplast RNAs*

Saturated binding curves of binding of different RNAs to recombinant 28RNP or chloroplast-soluble protein extract was performed as shown in Figure 6. The RNA molecules are described in "Materials and Methods." In the case of binding of the *psbA* 3′ end and *rbcL* 3′ end RNAs to chloroplast protein extract, no saturation had been reached, and therefore the kd is higher than the one shown.

RNA	Recombinant 28RNP (kd)	Native 28RNP in Chloroplast Extract (kd)
	mm	*mm*
psbA 3′	0.3	>1.0
petD 3′	0.5	0.6
rbcL 3′	0.5	>1.2
psbA 3′ antisense	0.3	1.0
psbA 5′	0.5	1.2

binding of the 28RNP in the chloroplast extract and the recombinant purified 28RNP to the *psbA* 3′ end RNA (Fig. 6C; Table II). The 28RNP was found to bind 3′ *psbA* RNA with reduced affinity as compared to the binding of the recombinant protein. Similar results were also obtained with the *rbcL* 3′ end and *psbA* 5′ end RNAs but not with the *petD* 3′ end RNA (Fig. 6D; Table II). In the case of binding of the *psbA* 3′ end and *rbcL* 3′ end RNAs to chloroplast protein extract, no saturation had been reached and, therefore, the kd is even higher than the one shown and the differences are even more than 3-fold. Since we observed similar kd values to the *petD* 3′ end RNA with the recombinant 28RNP and the native protein in the chloroplast extract, we concluded that the differences obtained with the other 3′ RNAs were due to different binding properties of the native 28RNP and not degradation of the RNA in the extract or dilution of its available concentration to the 28RNP by other RNPs. The similar RNA-binding affinities obtained with the *petD* 3′ end RNA eliminated also the possibility that the differences obtained with other RNAs are due to competition of RNA that exists in the chloroplast extract. The smaller affinity to the *petD* RNA of the native 28RNP in the extract as compared to the recombinant 28RNP that was always observed in our experiments (Fig. 6; Table II) might be explained from the intrinsic RNA competition. However, the significant differences obtained with the other RNAs could not be explained in that way. Taken together, these data strengthen the hypothesis that other proteins and/or posttranslational modifications are involved in the determination of the RNA-binding affinities and also, perhaps, in the specificity of binding of the 28RNP in vivo to its target RNA sequences.

DISCUSSION

In this work, we have shown that recombinant ribonucleoprotein from spinach chloroplasts binds with similar affinities to every RNA sequence tested. The recombinant protein binds uridine-rich sequences, stem loop structures, and RNA derived from sense and antisense transcripts of 5′ and 3′ untranslated regions, as well as coding regions of chloroplastic genes. The only RNA molecules that bind to the recombinant 28RNP with low affinities were poly(A) and poly(C).

How can these results be explained? Most of the RNPs of the RNA-binding domain family display similar RNA-binding activity (Burd et al., 1991; Kenan et al., 1991; Dreyfuss et al., 1993). Many hnRNPs, as well as the tobacco RNPs, were purified using single-stranded DNA columns and displayed different preferences for ribohomopolymers (Li and Sugiura, 1990; Ye et al., 1991; Dreyfuss et al., 1993). In addition, the chloroplast RNPs from *Arabidopsis* were cloned when cDNA expression libraries were screened with unrelated oligonucleotide probes (Bar-Zvi et al., 1992; DeLisle, 1993). Only a few examples of sequence-specific RNA binding of proteins of the RNA-binding domain family have been reported: The hnRNP C protein binds to a stretch of five uridines around the splice site (Wilusz and Shenk, 1990). Other hnRNPs display preferential binding to sequences found in introns at or near the 3′ splice site (Dreyfuss et al., 1993). It has also been shown that the affinity and specificity of a protein to RNA can be modulated through protein-protein interactions. The U2-B″ protein interaction with another protein, U2-A′, increases the specificity of binding to the U2 RNA. Without binding to the U2-A′ (which is not an RNP by itself), U2-B″ binds RNA with much less specificity (Scherly et al., 1990a, 1990b). The amino acid sequences required for the specific interaction of these two proteins are within the RNA-binding domain of the U2-B″, suggesting that the U2-A′ must be in close proximity to the RNA (Scherly et al., 1990b).

Even though most studies attempting to find specificity of RNA binding of proteins harboring the RNA-binding domain resulted in general binding activity to RNA and single-stranded DNA and a variability in binding to ribohomopolymers, it was assumed that these proteins have specific RNA-binding activity in the cell (Kenan et al., 1991; Dreyfuss et al., 1993). This assumption was made because these proteins have a spectrum of binding activities with higher affinities to certain sequences and low (nonspecific) affinity to others. Therefore, we can interpret the differences in RNA-binding activity that were observed in this work between the recombinant 28RNP as well as the native one in the chloroplast protein extract and suggest that this situation is also the case for the 28RNP in the chloroplast. It is possible that the amino acid sequence of the RNA-binding domain provides the basic affinity to RNA by forming the structure of $\beta1$-$\alpha1$-$\beta2$-$\beta3$-$\alpha2$-$\beta4$ (α = α helix and β = β sheet), as was described from the three-dimensional structure of the RNA-binding domain of the U1 small nuclear RNP A protein (Nagai et al., 1990). The specificity of binding to specific RNA(s) can be obtained by posttranslational modification and/or interactions with other proteins as was described above for the U2 small nuclear RNP proteins.

Proteins of the RNA-binding domain family have been reported to possess phosphorylation and methylation properties (Kenan et al., 1991; Dreyfuss et al., 1993). Indeed, the 28RNP was phosphorylated in experiments in which chloroplasts were incubated with [^{32}P]Pi and when the

recombinant protein was incubated with casein kinase II and [γ-^{32}P]ATP (I. Lisitsky and G. Schuster, unpublished data). On the other hand, several proteins can be immunoprecipitated from a chloroplast-soluble protein extract together with the 28RNP or by a 28RNP affinity column (V. Liveanu and G. Schuster, unpublished data). Our hypothesis that the 28RNP is not a general chloroplast RNP that binds every RNA and single-stranded DNA molecule in vivo is also supported by the fractionation experiments. The results of these experiments clearly indicate that the 28RNP is not associated with the polysome fractions, where most of the chloroplast RNA is localized and therefore the native 28RNP binds RNA in the chloroplast differently from the recombinant bacterial expressed protein, as was shown in this work in analyzing the affinities of these proteins. Experiments to characterize these differences and to learn what confers specificity in RNA binding in vivo are in progress.

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