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RNA-binding activities of the different domains of a spinach chloroplast ribonucleoprotein

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ABSTRACT

An RNA-binding protein of 28 kD (28RNP) has been previously isolated from spinach chloroplasts and was found to be required for 3′ end processing of chloroplast mRNAs. The amino acid sequence of 28RNP revealed two ~80 amino-acid RNA-binding domains, as well as an acidic and glycine-rich amino terminal domain. Each domain by itself, as well as in combination with other domains, was expressed in bacterial cells and the polypeptides were purified to homogeneity. We have investigated the RNA-binding properties of the different structural domains using UV-crosslinking, saturation binding and competition between the different domains on RNA-binding. It was found that the acidic domain does not bind RNA, but that each of the RNA-binding domains, expressed either individually or together, do bind RNA, although with differing affinities. When either the first or second RNA-binding domain was coupled to the acidic domain, the affinity for RNA was greatly reduced. However, the acidic domain has a positive effect on the binding of the full-length protein to RNA, because the mature protein binds RNA with a better affinity than the truncated protein which lacks the acidic domain. In addition, it was found that a stretch of two or three G residues is enough to mediate binding of the 28RNP, whereas four U residues were insufficient. The implications of the RNA-binding properties of 28RNP to its possible function in the processing of chloroplast RNA is discussed.

INTRODUCTION

Chloroplast precursor mRNAs undergo a variety of maturation events including *cis* and *trans*-splicing, cleavage of polycistronic messages, processing of 5′ and 3′ ends and editing (1–3). In addition, modulation of RNA stability during chloroplast development is one of a variety of mechanisms by which chloroplast gene expression is regulated (1,4,5). In order to define the mechanism for the 3′ end processing of chloroplast RNA and to determine how mRNA stability is modulated during chloroplast development, the proteins that bind to RNA 3′ ends have been identified and analyzed (6–11). One of these, a nuclear-encoded 28 kD RNA-binding protein (28RNP), was previously isolated as the major RNA-binding protein that co-purified with RNA 3′ end processing activity, with the final purification being carried out using an RNA-affinity column with the chloroplast *psbA* 3′ untranslated region (UTR) as a ligand (*psbA* encodes the D1 polypeptide of the photosystem II reaction center; 10). The deduced amino acid sequence of 28RNP revealed two similar 80 amino acid RNA-binding domain consensus sequences (CS-RBDs; 12,13), as well as an amino terminal acidic domain. Immunodepletion of the 28RNP from a soluble chloroplast protein extract interfered with the *in vitro* 3′ end maturation of several chloroplast RNAs, suggesting possible involvement of 28RNP in that process (10). cDNAs encoding similar RNA-binding proteins were cloned from tobacco (14–16), maize (17), and Arabidopsis (18,19).

Many of the CS-RBDs proteins contain more than one RNA-binding consensus sequence and an auxiliary domain which can be located at the carboxy or amino terminus of the protein (13). RNA-binding analysis of CS-RBD family protein revealed some diversity in the domains required for binding to nucleic acids. For example, the poly(A)-binding protein contains four CS-RBDs, of which the most N-terminal is not functional in RNA-binding, and the other three differ in their affinities for poly(A) (20). The U1A snRNP contains two CS-RBDs, of which the C-terminal one is not required for specific RNA-binding, while specific binding to its target RNA is determined by eight amino acids conforming to the most conserved octamer motif in the N-terminal CS-RBDs (21). RNA-binding analysis of the tobacco cp29A and cp29B proteins has shown that each RNA-binding domain independently binds to ssDNA, poly(G) and poly(U) with lower affinities than do the two RNA-binding domains combined. In addition, it was found that the acidic domain has no effect, or a slight negative effect, on binding to ribohomopolymers (22).

In this study, we have analyzed the binding of the spinach 28RNP and its different domains to a chloroplast 3′ end RNA, and have also determined the minimal stretch of G nucleotides required for binding. We show that each RNA-binding domain by itself binds the *psbA* 3′ end RNA with a relatively low affinity. A protein composed of the two RNA-binding domains binds the same RNA much more strongly, whereas a protein composed of the first RNA-binding domain and the first 16 amino acids of the second RNA-binding domain (including the most conserved hexamer RNP2 but not the octamer RNP1 conserved sequences), binds the *psbA* 3′ end RNA with a similar or slightly reduced affinity. The acidic domain, which does not bind RNA by itself, has an additive effect on the affinity of other segments of the protein to RNA, because the full-length protein binds RNA with

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about two to three times higher affinity than a protein which lacks only the acidic domain. A minimal stretch of two or three guanosines was found to be sufficient for binding of 28RNP to RNA, however four uridines did not promote binding.

MATERIALS AND METHODS

Oligonucleotides

1. 28metRNP 5'-TAGCTTCATATGTGTGTTGCTCAAACCTCAGAATGG-GAG-3'
2. RND1 5'-GCGAATTCGCTAAATTGTTTGTGGG-3'
3. RND2 5'-CTTGGAGGACTTCTTCGACTGAGCTCGC-3'
4. RND3 5'-GCGAATTCTCGTGCAGAGTGTATG-3'
5. RND4 5'-CGAGGAACCCCTAGTACTGAGCTCGC-3'
6. AD−R2 5'-CACTCTGCACGAAGGAGGTTCAGAGAACCC-3'
7. dAC 5'-d$(AC)_{10}$-3'

Preparation of *E.coli*-produced 28RNP

The oligonucleotide 28metRNP was used together with the T7 promoter primer to amplify a 28RNP cDNA lacking the transit peptide that mediates transport into the chloroplast. The amplification product was cloned into the *Hin*dIII and *Xho*I sites of the Bluescript SK+ vector (Stratagene Inc.) to yield the 28RNPmet plasmid, which was used thereafter for the generation of deletion mutants. Plasmid 28RNPbam was created from 28RNPmet by digestion with *Sma*I and *Nde*I, repair with the Klenow fragment, and religation. For expression in *E.coli*, the *Bam*HI/*Xho*I insert of 28RNPbam was cloned into the *Bam*HI/*Sal*I sites of the expression vector pQE31 (Qiagen Inc.). Thus, six histidines and several additional residues were added to the amino terminus of the mature protein. The recombinant protein was produced in SG13009[pREP4] cells by growing to OD_{600}=0.9 on LB medium (100 ml) containing ampicilin (100 μg/ml) and kanamicin (25 μg/ml) followed by the addition of IPTG (2mM) 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 were broken using 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 (Bio-Lab. Inc.) for 1 h at 4°C with gentle mixing. The material was applied to a column, washed with 10 volumes of sonication buffer and 10 volumes of washing buffer (50 mM Na-phosphate pH 6, 300 mM NaCl) and the protein was eluted using a linear gradient to 0.5 M of imidazole in a washing buffer followed by dialyzes 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 (Bio-Rad Inc.). To verify that the RNA-binding properties of the recombinant protein were not altered by the histidine residues, other proteins were expressed using the same vector system and checked for RNA-binding. No RNA- binding was observed using several proteins over-expressed in that way (data not shown). In addition, 28RNP expressed as a fusion protein to the *E.coli* maltose-binding protein, or to β-galactosidase, had the same RNA-binding affinity as the histidine-tagged fusion protein (data not shown). After two cycles of Ni_2+ affinity column purification, fractionation of the recombinant protein by SDS−PAGE revealed a single protein species; this preparation lacked detectable ribonuclease activity when incubated with radiolabeled RNAs (data not shown). To express 28RNP as an *E.coli* maltose-binding protein fusion, the *Xba*I/*Xho*I insert of the 28RNPmet plasmid was cloned into the *Xba*I/*Sal*I sites of the pMal-cRI vector (New-England Biolabs, Inc.). Expression and purification of the fusion protein on an amylose column were performed according to the manufacturer's instructions.

Preparation of 28RNP deletion mutants

The DNA fragments for expression of the deletion mutants were amplified using the 28RNPmet plasmid as a template (see Fig. 1 for a schematic description of the proteins). AD DNA was generated using the oligonucleotides RND2 and the T3 primer, R1 was generated with RND1 and RND4, AD−R1 with RND4 and the T3 primer, R2 with RND3 and the T7 primer, and R−R with RND1 and the T7 primer. The amplification products were cloned into the *Eco*RI/*Xho*I sites of the Bluescript vector, followed by digestion with *Bam*HI/*Xho*I and cloning of the inserts into the *Bam*HI/*Sal*I sites of the pQE30 expression plasmid (Qiagen Inc.). Plasmids for expression of R1−RNP2 and AD−R1−RNP2 were generated by digestion of the plasmids for the expression of R−R and 28RNP, respectively, with *Nco*I and religation. The plasmid for expression of AD−R2 was created by oligonucleotide- directed deletion, using the AD−R2 oligonucleotide and 28RNPbam single-stranded DNA as a template. The proteins were expressed in bacteria and purified as described above for 28RNP.

Preparation of synthetic RNAs

Construction of the plasmid used to transcribe the *psbA* 3' end RNA was previously described (11,23). For the experiments described here, the insert was transferred into Bluescript KS+ (Stratagene Inc.) so that transcription with T7 RNA polymerase generated the mRNA-like strand. RNA for UV-crosslinking experiments was prepared with 2.5 μM [α-^{32}P] UTP and 25 μM non-radioactive UTP (10,11,23).

UV-crosslinking

UV-crosslinking of protein to [α-^{32}P] UTP-labeled RNAs was carried out as previously described (10). Essentially, 30 fmol of RNA (240 000 c.p.m.), or the amount indicated in the figure legends, were incubated in 15.5 μl with 50 ng of protein in a buffer containing 10 mM HEPES pH 7.9, 30 mM KCl, 6mM $MgCl_2$, 0.05 mM EDTA, 1 mM DTT and 8% glycerol. Following 1.8 Joules of UV irradiation in a UV-crosslinking apparatus (Hoefer Inc.), the RNA was digested by 1 μg RNase A at 37°C for 30 min and the proteins were fractionated by SDS−PAGE. The label transferred from the RNA to the proteins was detected by autoradiography, and quantitation was performed by scanning the autoradiogram with a laser-scanner densitometer (Cliniscan 2, Helena Laboratories).

Analyzing the number of guanosines required for binding of the 28RNP

The oligodeoxynucleotide dAC (which does not bind the 28RNP) was end-labeled with γ-[^{32}P] ATP and polynucleotide kinase. About fifteen guanidine ribose residues were added to the end-labeled dAC oligodeoxynucleotide using terminal transferase (US Biochem. Inc.) and GTP (2 mM). Only four U residues could be added when UTP was used instead of GTP. An alkaline ladder was generated by boiling the RNA for 10 min in carbonate buffer (100 mM), followed by separation in denaturing 15% polyacrylamide gels, elution of the radioactive bands and removal of the salts by spun column dialysis or ethanol precipitation. Each of these molecules was analyzed for binding to the recombinant

purified 28RNP by the gel shift technique. 200 ng of the protein were incubated with labeled dAC+Gn (n = 0−15) for 30 min at 25°C, and electrophoresed in a 6% acrylamide (acrylamide/bis-acrylamide 80:1) gel in Tris−glycine (each 50mM) buffer. The gel was electrophoresed at 200 volts for 1.5 h, dried and autoradiographed.

RESULTS

Preparation of 28RNP deletion mutants

The 28RNP is a three-domain nuclear-encoded chloroplast RNA-binding protein, as described below. *E.coli*-expressed 28RNP binds many chloroplast RNAs as well as poly(U) and poly(G) with similar affinities, but to poly(C) and poly(A) with very low affinities (Lisitsky *et al.*, submitted). In order to evaluate the contribution of each domain to the affinity of the protein for RNA, the RNA-binding properties of each domain were studied, expressed either alone or in different combinations. As shown in Figure 1 (construct 1), 28RNP consists of an acidic domain (AD), including the 54 N-terminal amino acids; the first RNA-binding domain (R1; amino acids 55 to 133); and the second RNA-binding domain (R2; amino acids 145 to 233). The complete coding region, and each of eight other constructs shown in Figure 1, was expressed in *E.coli* cells, purified, and tested for affinity for *psbA* 3′ end RNA by UV-crosslinking. Figure 2A shows a Coomassie Blue-stained gel in which the different 28RNP variants were separated. The acidic domain (AD) that, according to its calculated molecular weight, should migrate at about 6 kD, migrated anomalously in SDS gels and appeared at about 20 kD. This effect was observed with every protein that included the acidic domain (28RNP, AD−R1, AD−R2, AD−R1−RNP2; Fig. 1). In this respect, the acidic domain of 28RNP resembles the carboxyl terminal charged domain of the 70K U1 snRNP that confers a similar anomalous migration in SDS gels (24).

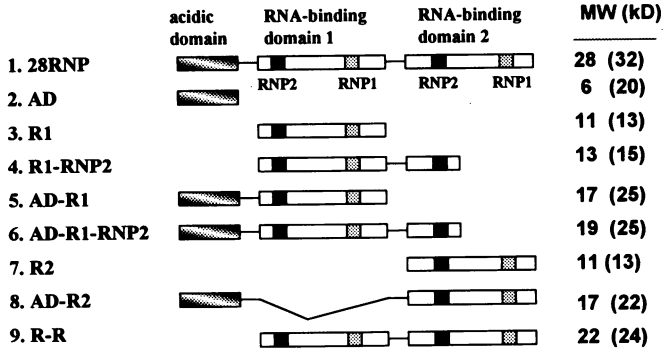

Figure 1. Domains of the 28RNP. Schematic representation (not to scale) of structural domains in the 28RNP, indicating the acidic and glycine-rich domain (AD) and the two RNA-binding domains (R1 and R2). The most highly conserved amino acid sequences within the RNA-binding domains, RNP2 and RNP1 (termed RNA-CS2 and RNA-CS1 in some references) are indicated. Both of the RNA-binding domains, as well as combinations of the 28RNP domains (as shown in the figure), were expressed in bacterial cells, purified, and analyzed for RNA-binding. The molecular weights of the proteins as calculated from the amino acid sequences are indicated, as well as their relative molecular masses as calculated from their migration in SDS−polyacrylamide gels (in parentheses).

We first investigated the binding properites of the 28RNP domains by UV-crosslinking to *psbA* 3′ end RNA. This RNA was chosen for these experiments because it is a typical chloroplast 3′ end RNA including inverted repeats that can form a stem loop structure (23), and also because the initial purification of the 28RNP was accomplished using a *psbA* 3′ RNA affinity column (10). The results shown in Figure 2B reveal that the RNA-binding domains, individually or in combination, bind RNA and that the acidic domain does not. In addition, proteins composed of the acidic domain and either single RNA-binding domain did not bind RNA (Fig. 2B; AD−R1, AD−R2). These data suggested that the acidic domain dramatically reduces the affinity for RNA of an RNA-binding domain and apparently also reduces the affinity of the whole protein (compare R−R to 28RNP). However, as shown in below, caution should be exercised when interpreting UV-crosslinking experiments as a protein−RNA affinity test.

Defining RNA-binding affinities by RNA saturation experiments

A more precise way to define the affinities of the proteins containing the 28RNP domains for RNA is by saturation experiments, in which the UV-crosslinking assay is performed in the presence of increasing amounts of RNA. The binding constant (kd) is defined as the concentration of RNA that gives half saturation of binding (25). Using this method, the kds of the 28RNP domains that could be crosslinked to *psbA* 3′ end RNA (Fig. 1B) were determined. The results, presented in Figure 3 and Table I, showed that the complete protein had the highest affinity for the *psbA* 3′ end RNA. The protein composed of the

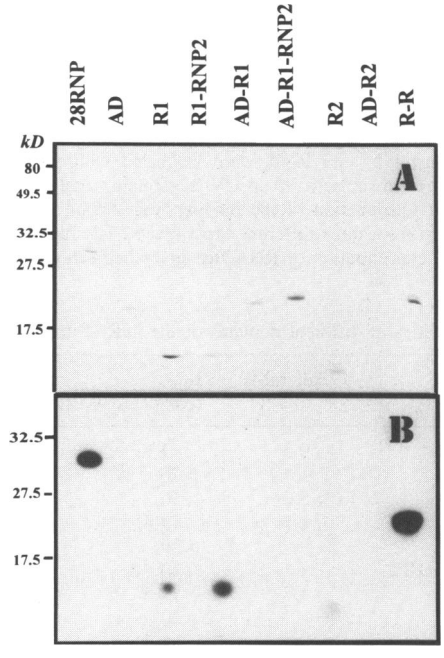

Figure 2. RNA-binding of the different 28RNP domains. The different domains of the 28RNP, as shown in Figure 1, were expressed in *E.coli*, purified and analyzed for binding to *psbA* 3′ end RNA by UV-crosslinking. 50 ng of each protein was UV-crosslinked to 30 fmols of *psbA* 3′ end RNA. **A.** Coomassie-stained SDS−polyacrylamide gel. **B.** Autoradiogram of the UV-crosslinking experiment.

two RNA-binding domains (R−R), although giving the strongest signal in UV-crosslinking experiments (Fig. 2B and inset of Fig. 3), bound RNA with less affinity than the 28RNP. The proteins composed of one RNA-binding domain (R1 and R2) had even less affinity for RNA. These results raised the possibility that the acidic domain reduces the affinity of a protein containing a single RNA-binding domain, but increases the affinity of proteins containing two RNA-binding domains. However, the different observations made using UV-crosslinking and saturation binding assays raised questions regarding the negative UV-crosslinking results using AD, AD−R1 and AD−R2 (Fig. 2B). One possibility is that these proteins do bind RNA, but owing to differences in the distances of the labeled [^{32}P]-UTP to the amino acids that can be crosslinked by UV light, no label-transfer from the RNA to the protein was observed. In order to clarify this issue, RNA-binding competition experiments were carried out.

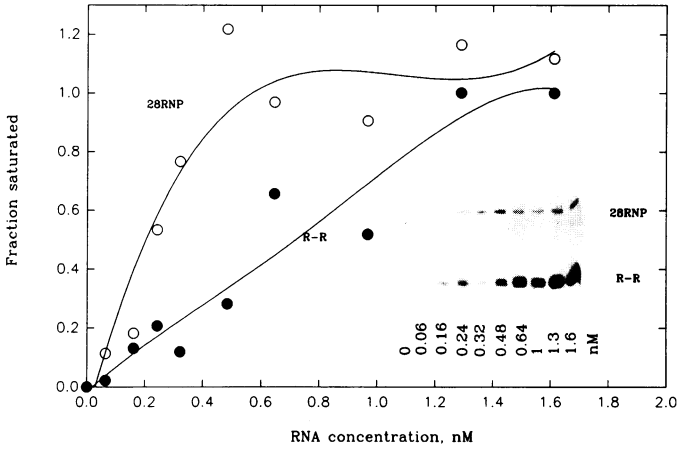

Figure 3. The complete 28RNP binds RNA with a higher affinity than a protein composed of only the two RNA-binding domains. Different amounts of [^{32}P]-labeled *psbA* 3′ end RNA were incubated with purified recombinant proteins (50 ng) and subjected to an UV-crosslinking analysis, as shown in the figure's insert. Quantitation of the binding was carried out by scanning the autoradiographs from three different experiments. The binding affinity, kd, is defined as the concentration of RNA that gives half-saturation of binding.

Table I. Affinities of different domains of the 28RNP for *psbA* 3′ end RNA

Protein	kd, (nM)	I_{50}, (competitor/MBP-28RNP)
1. 28RNP	0.3	1.8
2. AD	–	>20
3. R1	>5.0	>10
4. R1−RNP2	1.1	4.4
5. AD−R1	–	>20
6. AD−R1−RNP2	–	>20
7. R2	4.7	7.5
8. AD−R2	–	>20
9. R−R	0.7	4.4

Affinities of the 28RNP and different domains proteins for *psbA* 3′ end RNA were analyzed in saturation binding crosslinking experiments as shown in Figure 3, and RNA-binding competition experiments as shown in Figure 4. The affinities of the proteins for RNA were defined in the saturation binding experiments as kd (nM): the RNA concentration that gives half-saturation of the UV-crosslinking signal; or I_{50} (competitor/MBP-28RNP): the molar amount of competitor protein as compared to the MBP-28RNP, that inhibits the UV-crosslinking signal by 50%.

RNA-binding competition experiments

For RNA-binding competition experiments, increasing amounts of each 28RNP variant protein were added to the UV-crosslinking assay using *psbA* 3′ end RNA, while the amount of the intact 28RNP was held constant. In order to compare different competition experiments, we defined an I_{50} value as the concentration of a competitor protein which inhibited the binding of RNA to 28RNP by fifty percent in our experimental system (see Materials and Methods). First, self-competition of 28RNP

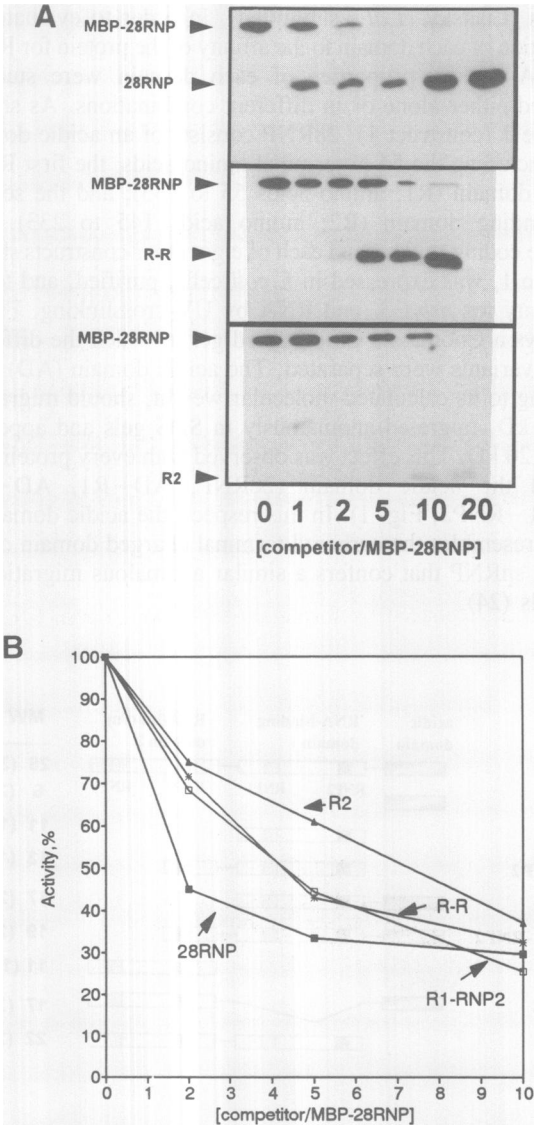

Figure 4. Competition of the different 28RNP domains for binding of the *psbA* 3′ end RNA to the complete 28RNP. (**A**) The 28RNP fused to the maltose-binding protein (MBP−28RNP, 50 ng) was incubated with a molar excess (as indicated at the bottom of the Figure) of the competitor protein, followed by addition of *psbA* 3′ end RNA (30 fmols) and UV-crosslinking. (**B**) Quantitation was carried out by scanning the autoradiographs obtained from at least three experiments. Addition of a 20-fold molar excess of the proteins AD, AD−R1, AD−R1−RNP2 or AD−R2 had no effect on the binding of MBP−28RNP to RNA.

was analyzed. For this experiment, the 28RNP cDNA had to be expressed in bacteria in such a way that the competitor protein would migrate at a different molecular weight than the full-length recombinant 28RNP. To this end, 28RNP was expressed in *E.coli* cells as a fusion protein to the maltose-binding protein (MBP−28RNP), and the fusion protein was shown to have the same RNA-binding properties as full-length *E.coli*-expressed 28RNP (data not shown). Next, competition experiments between MBP−28RNP and different 28RNP variant proteins were performed. Figure 4 shows that in agreement with the UV-crosslinking results shown in Figure 2, the AD, AD−R1 and AD−R2 proteins could not compete the binding of MBP−28RNP to *psbA* 3′ RNA, indicating that these proteins indeed have very low (if any) affinity for this RNA. In agreement with the RNA saturation experiments, MBP−28RNP had the highest affinity for RNA, more than twice that of the R−R and R1−RNP2 proteins, more than four times that of the R2 protein, and at least five times more than that of the R1 protein (Fig. 4B and Table I).

Defining the number of nucleotides required for binding of RNA to 28RNP

In order to define the minimum number of nucleotides required for binding of 28RNP to RNA, we used the deoxyoligonucleotide dAC with between 1 and 15 guanosine ribonucleotides added to its 3′ end. 28RNP binds poly(C) and poly(A) with very low affinities (Lisitsky *et al.*, submitted) and does not exhibit any bound complex with the 28RNP in gel retardation (Fig. 5) or UV-crosslinking (not shown) assays. Therefore, molecules consisting of dAC with the 3′ addition of a different numbers of G residues were analyzed for binding to the 28RNP in the gel retardation assay. As can be seen in Figure 5, a retarded 28RNP/nucleic acid complex could not be detected for dAC and dAC−G1 molecules, but a complex was detected for dAC−G3-11 molecules. Interestingly, the addition of more than 11 guanosines reduced the formation of the bound complex. This inhibition of binding to the 28RNP might be due to the secondary structure of the oligonucleotide that formed when 13 or more guanosines are present. We were unable to find conditions under which more than 4 uridines were efficiently added to dAC instead of guanosines. Even though the addition of 4 guanosines led to the formation of the retarded complex with 28RNP, 4 uridines failed to do so under the same conditions (Fig. 5, lanes 10 and 11). This result indicates that the 28RNP requires fewer guanosines than uridines for binding, and may imply that it has a higher affinity for short runs of guanosines.

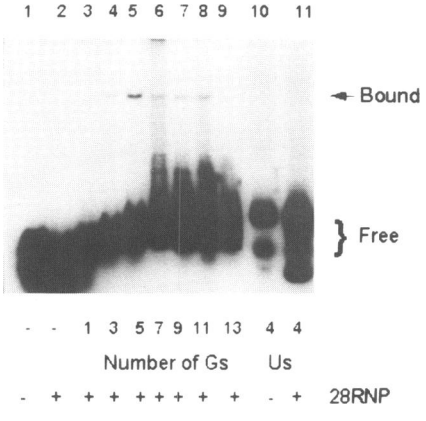

Figure 5. A stretch of two or three guanosines is required for binding of RNA to 28RNP. 1 to 13 guanosine residues were added to the dAC deoxyoligonucleotide by terminal transferase, followed by alkaline hydrolysis as described in Materials and Methods. These molecules were analyzed for binding to the 28RNP by gel retardation. Lane 1: the labeled dAC oligonucleotide incubated without 28RNP. Lane 2: dAC incubated with 28RNP. Lanes 3 to 9: dAC with addition of guanosines as indicated at the bottom of the figure were incubated with 28RNP. Lanes 10 and 11: dAC with addition of four uridines incubated without and with 28RNP, respectively.

DISCUSSION

Control of gene expression in the chloroplast is regulated by a variety of mechanisms including transcription, RNA stability, translation and at the post- translational level (1−5). RNA processing and the modulation of RNA stability are significant mechanisms by which the expression of several chloroplast genes is regulated during plant development and in response to physiological changes (1−5). The involvement of post-transcriptional regulation predicts the involvement of factors which are involved in the processing and stabilization of RNA in the chloroplast. Evidence for the existence of such nuclear-encoded chloroplast factors has been available for many years with the characterization of mutants which are defective in RNA processing and/or stability due to nuclear mutations (4,5). Indeed, several nuclear-encoded chloroplast-located RNA-binding proteins that are possibly involved in the maturation of chloroplast transcript have been identified, isolated and cDNA cloned in recent years (3,5−11,14−19). One of these, spinach 28RNP, has been shown to be required for *in-vitro* 3′ end processing of several chloroplast RNAs (10). The mature 28RNP consists of three distinct domains: an amino terminal highly acidic and glycine-rich domain, which is followed by a tandem pair of CS-RBDs. Similar proteins have been isolated from other plants (14−19). In order to understand the interaction and contribution of each domain to the affinity of the protein for a 3′ end chloroplast RNA, we studied the RNA-binding affinity of each domain individually and in combination with the other domains.

The acidic domain does not bind RNA independently, and apparently suppresses the binding (probably due to its negative charge) when attached to either the first or second CS-RBD. However, as shown in this work by two different methods, the acidic domain has an additive effect of the affinity for the 3′ end *psbA* RNA, when in the context of the entire protein, since the entire 28RNP binds this RNA with a 2.5-fold higher affinity than a protein composed of only the two CS- RBDs. It should be noted that although such a difference in affinity may appear to be subtle, such a quantitative difference in affinity has recently been demonstrated to account for the ability of another CS-RBD protein, hnRNP A1, to distinguish between its binding site and an unrelated RNA sequence (26). In addition, we have shown that post-translational modification of the 28RNP can modulate its affinity for 3′ end RNAs in a similar fashion, and that the 28RNP is not associated with polyribosomes in the chloroplast and therefore does not bind every RNA sequence presented to the protein (Lisitsky *et al.*, submitted). Taken together, our findings suggest that the effect of the acidic domain on the affinity of the 28RNP to RNA may be important for its function in the chloroplast.

Our finding that the R−R protein gives a stronger signal than the 28RNP in the UV-crosslinking assay, despite its lower affinity

for the same RNA as measured by competition experiments, implies that the acidic domain indeed induces conformational changes affecting the number of uridines that can be crosslinked to RNA and/or protected from ribonuclease digestion. This result is also consistent with the idea that the relative amounts of radioactivity present in UV-crosslinked proteins do not necessarily reflect their relative affinities for RNA.

The acidic domain is poorly conserved among chloroplast CS-RBD proteins (5). This may explain why, in the cases of the tobacco chloroplast cp29A and cp29B CS-RBD proteins, no positive effect of the acidic domain was detected (22). Another explanation for this difference between spinach and tobacco could be that the affinities of the tobacco proteins was measured to ribohomopolymers and ssDNA, and not RNA as in the present work, or that the acidic domains play different roles in the RNA-binding affinities of these proteins. These proteins indeed differ in their affinities for ssDNA, with respect to the other three tobacco CS-RNPs and the spinach 28RNP (22).

Each RNA-binding domain of spinach 28RNP expressed alone binds RNA, although with a very low affinity as compared to the complete protein (at least 15- fold lower), or to the R−R protein (7-fold lower). This may indicate that two RNA- binding domains are required for high-affinity binding to RNA, possibly by binding to the same RNA molecule or to two different molecules. R1 and R2 clearly differ in their affinities for RNA, so it is possible that each RNA-binding domain binds a different RNA sequence and/or in a different manner. It is interesting to note that an addition of 16 amino acids including the spacer between the two RNA-binding domains and the conserved hexamer RNP2 to R1 to form R1−RNP2 protein leads to five-fold increase in affinity for RNA. However, the addition of the acidic domain to form AD−R1−RNP2 eliminated the affinity for RNA. Taken together, these results suggest an importance of each part of the 28RNP in the determination of its overall affinity for RNA.

The 3′ untranslated regions of chloroplast mRNAs are highly enriched in uridine-rich sequences. The 28RNP does not bind poly(A) and poly(C), but has a high affinity for poly(U) and poly(G) (Lisitsky *et al.*, submitted). This phenomenon allowed us to design an experimental system in which we could measure the minimal number of guanosine residues required for binding to 28RNP. We found that three guanosines were sufficient to promote RNA binding, but that four uridines were not. This may also be reflected in the observation that the affinity of the 28RNP for poly(G) is slightly higher than for poly(U) (Lisitsky *et al.*, submitted). A tract of at least five consecutive uridines has been shown to mediate specific binding of hnRNP C proteins to RNA (27). Several other chloroplast RNA-binding protein target sequences were mapped onto U rich sequences in the 3′ mRNA untranslated regions (7,28). A computer search of the tobacco chloroplast genome revealed numerous three guanosine tracts and many stretches of uridines. These guanosine and uridine tracts are dispersed over the entire genome including protein coding regions, introns, and 5′ and 3′ untranslated regions. Therefore, in order to better understand the actual binding site(s) and mechanisms by which the 28RNP binds to its target RNA, other components of the 3′ end processing machinery, as well as the role of post-translational modification(s) of those proteins, should be uncovered. Experiments are currently underway to address these questions.

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REFERENCES

1. Gruissem, W. (1989) *Cell* **56**, 161−170.
2. Sugiura, M. (1991) in *Cell Culture and Somatic Cell Genetic of Plants*. (Bogorad, L. ed.) New York: Academic Press, Inc., pp.125−137.
3. Rochaix, J.-D. (1992) *Ann. Rev. Cell Biol.* **8**, 1−28.
4. Mullet, J. E. (1988) *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **39**, 475−502.
5. Gruissem, W. and Schuster, G. (1993) in *Control of Messenger RNA Stability*, (Belasco J. and Brawerman G. eds) Academic Press, pp. 329−365.
6. Adams, C. C. and Stern, D. B. (1990) *Nucleic Acids Res.* **18**, 6003−6010.
7. Chen, H-C. and Stern, D. B. (1991) *Mol. Cell. Biol.* **11**, 4380−4388.
8. Nickelsen, J. and Link, G. (1989) *Nucleic Acids Res.* **17**, 9637−9647.
9. Nickelsen, J. and Link, G. (1991) *Mol. Gen. Genet.* **228**, 89−96.
10. Schuster, G. and Gruissem, W. (1991) *EMBO J.* **10**, 1493−1502.
11. Stern, D. B., Jones, H. and Gruissem, W. (1989) *J. Biol. Chem.* **264**, 18742−18750.
12. Kenan, D. J., Query, C. C. and Keene, J. D. (1991) *Trends Biochem. Sci.* **16**, 214−220.
13. Bandziulis, R. J., Swanson, M. S. and Dreyfuss, G. (1989) *Genes Develop.*, **3**, 431−437.
14. Li, Y. and Sugiura, M. (1990) *EMBO J.* **9**, 3059−3066.
15. Ye, L., Li, Y., Fakami-Kobayashi, K., Go, M, Konishi, T., Watanabe, A. and Sugiura, M. (1991) *Nuc. Acids Res.* **19**, 6485−6490.
16. Mieszczak, M., Klahre, U., Levy, J. H., Goodall, G. J. and Filipowicz, W. (1992) *Mol. Gen. Genet.* **234**, 390−400.
17. Cook, W. B. and Walker, J. C. (1992) *Nucleic Acids Res.* **20**, 359−364.
18. DeLisle, A. J. (1993) *Plant Physiol.* **102**, 313−314.
19. Bar-Zvi, D., Shagan, T., Schindler, U. and Cashmore, A. R. (1992) *Plant Mol. Biol.* **20**, 833−838.
20. Nietfeld, W., Mentzel, H. and Pieler, T. (1990) *EMBO J.*, **9**, 3699−3705.
21. Scherly, D., Boelens W., Dathan N. A., Van Venrooij, W. J. and Mattaj, I. (1990) *Nature* **345**, 502−506.
22. Ye, L. and Sogiura, M. (1992) *Nucleic Acids Res.* **20**, 6275−6279.
23. Stern, D. B. and Gruissem, W. (1987) *Cell* **51**, 1145−1157.
24. Querry, C. C., Bentley, R. C. and Keene, J. D. (1989) *Cell* **57**, 89−101.
25. Marciniak, R. A., Garcia-Blanco, M. A. and Sharp, P. A. (1990) *Proc. Natl. Acad. Sci. USA.* **87**, 3624−3628.
26. Burd, C. G. and Dreyfuss, G. (1994) *EMBO J.* **13**, 1197−1204.
27. Wilusz, J. and Shenk, T. (1990) *Mol. Cell. Biol.* **10**, 6397−6407.
28. Nickelsen, J. and Link, G. (1991) *Plant J.* **3**, 537−544.