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Specific Binding of Chloroplast Proteins In Vitro to the 3′ Untranslated Region of Spinach Chloroplast *petD* mRNA

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A detailed analysis of RNA-protein complex formation in the 3′ untranslated region of spinach chloroplast *petD* mRNA has been carried out. Five chloroplast proteins that interact with *petD* RNA in this region, which contains an inverted repeat sequence capable of forming a hairpin structure, have been identified. A 33-kDa protein recognizes specifically the double-stranded stem of the hairpin structure; mutations that disrupt base pairing at the base of the stem reduce or eliminate protein binding. A 57-kDa protein recognizes specifically an AU-rich sequence motif that is highly conserved in *petD* genes of different higher plant species. The 57-kDa protein and possibly the 33-kDa protein form stable complexes with *petD* RNA in vitro and may interact with each other. In addition, their interaction with *petD* RNA is highly sensitive to heparin. The three other proteins, of 100, 32, and 28 kDa, display little sequence or structural binding specificity apart from their preference for uridine-rich sequences. They also interact with the 3′ untranslated regions of other chloroplast RNAs such as those of *psbA* and *rbcL*. The functions of these proteins in the regulation of *petD* gene expression, including possible roles in transcription termination and RNA stability, are discussed.

The expression of plastid genes is regulated by complex developmental, organ-specific, and light-dependent mechanisms (14, 15, 25). During chloroplast development, the transcriptional activities of plastid genes play an important role in determining the steady-state level of mRNA accumulation. Although transcription is constitutive, overall transcription activity increases in barley, maize, and spinach chloroplasts following illumination of dark-grown plants (2, 10, 11, 26, 32, 37, 51). However, the relative transcriptional activities of several plastid genes in spinach and barley cannot account for the differential accumulation of their mRNAs (10, 26). It has therefore been suggested that the accumulation of several plastid mRNAs can also be controlled at the level of mRNA stability (10, 12, 26, 37).

The 3′ untranslated regions (UTR) of many plastid and bacterial mRNAs contain inverted repeat (IR) sequences capable of forming hairpin structures. The 3′ IR is required for the stability of certain bacterial mRNAs (7, 27) and *Chlamydomonas* chloroplast *atpB* mRNA (45) in vivo and for the stability of spinach plastid mRNA in vitro (42, 44). The IR most likely functions as a stabilizing element by impeding the progress of processive 3′→5′ exoribonucleases. In addition, it has been proposed that the function of 3′ IR as a stabilizing element for spinach plastid mRNA is modulated by RNA-protein interactions (44), since some plastid mRNAs accumulate to low levels in dark-grown plants despite constitutive transcription and the presence of an IR (10). Therefore, both RNA structure and RNA-protein interactions may influence mRNA accumulation during chloroplast development.

Several recent reports have demonstrated that RNA-binding proteins are present in chloroplasts. In spinach, general and gene-specific RNA-binding proteins were analyzed in vitro by gel shift and UV-cross-linking assays (44). Two RNA-binding proteins were also identified in mustard chloroplast extracts (28). Recently, proteins that contain conserved RNA-binding motifs were purified from tobacco and spinach chloroplasts (22, 36). The existence of RNA-protein complexes (ribonucleoproteins [RNPs]) in vivo can be inferred by the differential sedimentation of native and deproteinized spinach chloroplast mRNA in glycerol gradients (41).

Our investigation of RNA-binding proteins in spinach chloroplasts has focused on the *petD* gene (17), which encodes subunit IV of the cytochrome b_6-*f* complex and is located at the 3′ end of the *psbB* gene cluster. The organization and expression of the *psbB* gene cluster are highly conserved in several plant species (31, 47–49). This gene cluster contains four genes that encode components of two different photosynthetic complexes. These are *psbB*, which encodes the 51-kDa chlorophyll *a*-binding protein of photosystem II; *psbH*, which encodes the 10-kDa phosphoprotein of photosystem II; *petB*, which encodes cytochrome b_6; and *petD*. These genes are cotranscribed into a single precursor mRNA that undergoes extensive processing to generate a complex pattern of 17 to 20 mRNA products (4, 49). It has been reported that in maize nearly all of these mRNAs cosediment with polysomes in sucrose gradients, suggesting that at least one coding region on most transcripts is translated (4). Although these four genes are cotranscribed, they are not expressed coordinately during the light-induced greening of etiolated spinach seedlings. The *petB* and *petD* gene products accumulate in etiolated plants, but the *psbB* gene product can be detected only after 2 h of illumination (18). Therefore, the expression of the *psbB* gene cluster may be regulated by posttranscriptional mechanisms.

It has previously been shown that an RNA derived from the 3′ end of the spinach chloroplast *petD* gene binds to several chloroplast proteins in vitro (44). We have now carried out a detailed analysis of mRNA-protein complex formation in the 3′ UTR of *petD* mRNA. Five proteins have been implicated in RNA-protein complex formation. Of these proteins, two display sequence and structural binding specificities, while the others appear to be general RNA-binding proteins that interact not only with the 3′ UTR but also with the coding region of *petD* mRNA. These proteins may therefore be involved in the formation of mRNPs in

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vivo. In addition to their sequence and/or structural binding specificities, each *petD* RNA-binding protein possesses distinct binding characteristics. The possible functions of the two specific *petD* mRNA-binding proteins in transcription termination and *petD* mRNA stability are discussed.

MATERIALS AND METHODS

Site-directed mutagenesis. Single-stranded (ss) DNA was obtained and mutations were introduced as described previously (44). Most of the base change and deletion mutants were constructed by using *petD* deletion mutant Δ50 ssDNA (44); for the compensatory mutants, however, the template was mutant petD2 or petD7 ssDNA.

Soluble-chloroplast-protein extracts. Soluble-chloroplast-protein extracts were prepared from leaves of hydroponically grown spinach (*Spinacia oleracea* cv. Marathon Hybrid) by the method of Gruissem et al. (16). Briefly, total soluble proteins were prepared from a clarified chloroplast lysate passed over a DE52-cellulose column (16) and precipitated with ammonium sulfate at 60% saturation. The proteins were dialyzed against buffer E containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.9), 60 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, and 17% glycerol (16). For heparin-agarose chromatography, total soluble proteins in buffer E were loaded onto a heparin-agarose (BRL) column (250 μg of protein per ml) preequilibrated with buffer E at a flow rate of 0.5 ml/min. After being washed with buffer E until there was no further elution at an optical density of 280 nm (flowthrough fraction), bound proteins were eluted with buffer E containing 1 M KCl (bound fraction), dialyzed against buffer E, and then concentrated by using Centricon filtration devices (Amicon). The concentration of each protein fraction was determined by using the Bio-Rad protein assay, and aliquots were stored at −70°C.

Preparation of synthetic RNAs. Synthetic RNAs were prepared as described by Stern and Gruissem (42). Trace-labeled RNAs for competition experiments were synthesized with 8 nM [α-^{32}P]UTP and 0.5 mM unlabeled UTP; transcripts for UV cross-linking were synthesized with 10 μM [α-^{32}P]UTP and no unlabeled UTP.

Oligonucleotide-directed RNase H cleavage. For each oligonucleotide-directed RNase H cleavage reaction, 200 fmol of [^{32}P]RNA and 100 ng of oligonucleotides were mixed in 20 μl of reaction mixture containing 20 mM HEPES (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. The reaction mixture (without enzyme) was incubated at 65°C for 5 min and then at 37°C for 10 min. RNase H (0.5 U) was then added to the reaction mixture, and incubation was continued for another 30 min. RNA cleavage products were then separated by denaturing polyacrylamide gel electrophoresis, and gel slices containing the desired RNA were excised and eluted in 200 μl of 0.3 M sodium acetate–1.25 mM EDTA (pH 8.0)–0.1% sodium dodecyl sulfate (SDS) at 37°C for 1 h or at 4°C overnight. The RNA was then recovered by ethanol precipitation.

UV cross-linking and partial proteolytic peptide mapping of UV-cross-linked proteins. UV cross-linking was carried out by incubating 2 fmol of [^{32}P]RNA with 10 μg of protein in 20 μl of reaction mixture containing 10 mM $MgCl_2$, 3 mM dithiothreitol, 40 mM KCl, 10 mM HEPES (pH 7.9), 0.05 mM EDTA, and 8.5% glycerol. For competition experiments, the competitor was added to the reaction mixture 5 min before the [^{32}P]RNA was added, and the incubation was prolonged for 5 min. After a 10-min incubation at 25°C, the reaction tubes were opened and covered with Saran wrap. The tubes were then placed in a Stratalinker (Stratagene) and irradiated with 18,000 μJ of UV light. After irradiation, 20 μg of RNase A was added, and the reactions were incubated for 30 min at 37°C. A 0.4 volume containing 8% SDS–3 M β-mercaptoethanol in 12% glycerol and 0.05% (each) bromophenol blue and xylene cyanol was then added, and the reaction mixtures were boiled for 5 min and loaded on a 12% discontinuous SDS-polyacrylamide gel. The gel was electrophoresed at 30 mA until the dyes reached the bottom and then dried and autoradiographed.

Partial peptide mapping of UV-cross-linked RNA-protein complexes was carried out according to the procedures of Cleveland et al. (9) and Leibold et al. (21), with modifications. The UV-cross-linking gel used for partial peptide mapping was subjected to autoradiography without drying. The gel slices corresponding to the RNA-protein complexes were excised, rinsed with cold water, and then immersed in 125 mM Tris HCl (pH 6.8)–0.1% SDS–1 mM EDTA for 5 min. The gel slices were then placed into the wells of a 12% SDS–polyacrylamide gel filled with running buffer (25 mM Tris HCl [pH 6.8], 200 mM glycine, 0.01% SDS). Each gel slice was overlaid with 40 ng of *Staphylococcus aureus* V8 protease (Sigma) in 10 μl of 125 mM Tris HCl (pH 6.8)–0.1% SDS–1 mM EDTA–10% glycerol. Electrophoresis was carried out at 30 mA for 4 h. The gel was stained with Coomassie blue to visualize the protein standards, dried, and subjected to autoradiography.

Gel mobility shift assays. The protocol for RNA mobility shift assays was essentially that of Konarska and Sharp (20). Briefly, 2 fmol of [^{32}P]RNA was incubated in 10 μl of reaction mixture containing 10 μg of total soluble chloroplast proteins and the same buffer as for UV cross-linking for 10 min at 25°C. The reaction mixtures were loaded onto 5% polyacrylamide gels in 50 mM Tris-glycine (pH 9.1). Electrophoresis was carried out at 175 V for 3 to 4 h, and the gel was dried and autoradiographed. For competition experiments, nonspecific RNA competitors were added 5 min before the ^{32}P-labeled RNA was added.

RESULTS

Interactions of *petD* 3′ IR RNA with chloroplast proteins in vitro. We have previously shown that in vitro-synthesized *petD* mRNA interacts with several chloroplast proteins (44). The *petD* RNA used in this study contains 84 nucleotides of the 3′ end of the coding region and 117 nucleotides of the 3′ UTR, including an IR capable of forming a hairpin structure that is coincident with the mature mRNA 3′ end. The in vitro-synthesized *petD* RNA (*petD* 3′ IR RNA) therefore corresponds to a putative precursor form of *petD* mRNA, since its 3′ end is exonucleolytically processed in vitro to generate the mature mRNA 3′ end (43). By UV cross-linking, *petD* 3′ IR RNA was shown to bind to three proteins in an extract of total chloroplast soluble protein. On the basis of their migrations in SDS-polyacrylamide gels (44), the sizes of these proteins were estimated to be 55, 28, and 23 kDa. Our refined measurements are 57, 32, and 28 kDa, respectively. When the extract of total soluble protein was further fractionated by heparin-agarose chromatography, only the 32-kDa protein was found in the flowthrough fraction when the column was washed with buffer containing 60 mM KCl. The 57- and 28-kDa proteins were eluted at higher KCl concentrations (the fraction eluted with 1 M KCl is termed the bound fraction) along with four additional *petD* 3′ IR RNA-binding proteins of 100, 66, 45, and 33 kDa (Fig. 1).

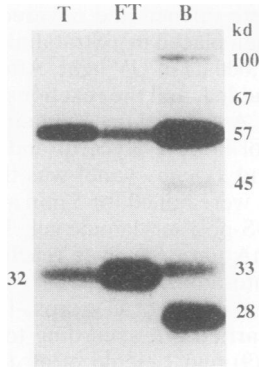

FIG. 1. Cross-linking of *petD* 3′ IR RNA to different chloroplast protein fractions. *petD* 3′ IR RNA was synthesized from the linearized petDΔ50 DNA template. UV-cross-linking assays were carried out with total chloroplast soluble proteins (lane T), flowthrough fraction from a heparin-agarose column washed with buffer E containing 60 mM KCl (lane FT), and bound fraction from a heparin-agarose column from which proteins were eluted with buffer E containing 1 M KCl (see Materials and Methods) (lane B). The proteins were separated in a 12% SDS–polyacrylamide gel. The sizes of UV-cross-linked proteins were calculated by using molecular size standards from Bio-Rad.

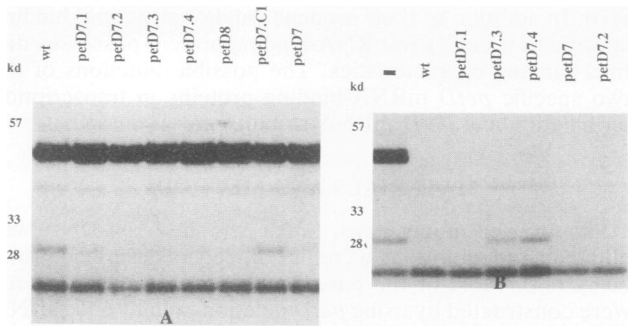

FIG. 3. UV-cross-linking and competition analyses of mutant *petD* 3′ IR RNAs. (A) Seven mutant and wt *petD* 3′ IR RNAs were cross-linked to bound-fraction proteins from a heparin-agarose column in the presence of 100 ng of poly(U). Reactions were analyzed by electrophoresis in a 12% SDS–polyacrylamide gel. The tested wt and mutant *petD* 3′ IR RNAs are indicated above each lane. (B) Competition experiments were carried out using bound-fraction proteins from a heparin-agarose column and ^{32}P-labeled wt *petD* 3′ IR RNA in the presence of 100 ng of poly(U). For each competition experiment, a 500-fold molar excess of trace-labeled RNA competitor was added to the reaction 5 min before the ^{32}P-labeled wt RNA was added. Tested competitor RNAs are indicated above each lane. Lane ■, no competitor.

These additional proteins are most likely present in low concentrations, since they were not detected following UV cross-linking of total soluble proteins. The 66- and 45-kDa proteins were not reproducibly seen in UV-cross-linking experiments; therefore, our analysis has focused on the five proteins of 100, 57, 33, 32, and 28 kDa. Since the 33-kDa protein can be detected only in bound-fraction proteins, we have used the bound fraction to analyze the 33-kDa protein and used the total-soluble-protein extract to analyze the proteins of 100, 57, 32, and 28 kDa.

Definition of binding sites. (i) 33-kDa protein specifically recognizes the hairpin structure. The 3′ IR of *petD* RNA forms a hairpin structure in vitro (Fig. 2) as shown by RNase T_1 mapping (44). Using site-directed mutagenesis, we have introduced deletions into the loop and single- or double-base changes into the stem of the hairpin as shown in Fig. 2. The RNAs synthesized in vitro from these mutant templates are referred to as mutant 3′ IR RNAs. Two mutant 3′ IR RNAs, Δ54 and Δ18, have 3′ end deletions that have been generated by Bal 31 deletion of the template DNA (Fig. 2C; 44).

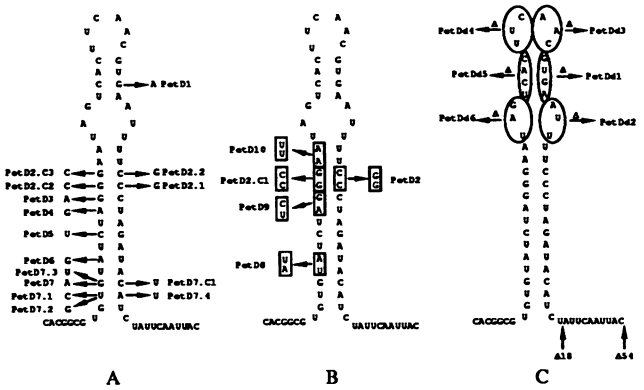

FIG. 2. Site-directed mutagenesis of the *petD* 3′ IR. (A) Single-base-change mutations; (B) double-base-change mutations; (C) deletion mutations (Δ). The 3′ end points of Bal 31 deletion mutants Δ18 and Δ54 (44) are indicated by arrows. petD7.C1 is a compensatory mutant of the petD7 mutant; i.e., it contains the petD7 mutation. petD2.C1, petD2.C2, and petD2.C3 are compensatory mutants of the petD2 mutant; i.e., they also contain the petD2 mutations.

The 33-kDa protein has previously been shown to interact with the *petD* 3′ IR RNA but not with 3′ IR RNA corresponding to the spinach chloroplast *rbcL* or *psbA* gene (44). It was also shown that a 1-nucleotide change at the base of the stem of the *petD* hairpin (petD7 mutant; Fig. 2A) diminished the binding of the 33-kDa protein to 3′ IR RNA (44). To define more specifically the 33-kDa protein-binding site, we have introduced a series of mutations near the petD7 mutation site that disrupt each base pair at the base of the stem (mutants petD7.1, petD7.2, petD7.3, and petD7.4), a double mutation (mutant petD8), and also one compensatory mutation (mutant petD7.C1) that restores the base pairing in the petD7 mutant. The mutant 3′ IR RNAs were tested in UV-cross-linking assays, and Fig. 3A shows that all of the mutant 3′ IR RNAs tested, except the compensatory mutant petD7.C1, failed to label the 33-kDa protein. The lack of label in the 33-kDa protein could result from a lack of binding or a failure to covalently bind ^{32}P label during UV cross-linking. Therefore, competition experiments were performed. Figure 3B shows that the affinities of the petD7, petD7.1, and petD7.2 mutant 3′ IR RNAs for the 33-kDa protein are not completely abolished, as they are capable of competing with wild-type (wt) 3′ IR RNA at high concentrations (500-fold molar excess), whereas the petD7.3 and petD7.4 mutant 3′ IR RNAs do not compete with wt 3′ IR RNA, indicating a complete lack of affinity for the 33-kDa protein. This result appears to reflect subtle differences in RNA structure in the *petD* 3′ IR RNA mutants. Taken together, these results indicate that the secondary structure in the *petD* 3′ IR, specifically the first 5 base pairs, is essential for the binding of the 33-kDa protein in vitro.

The binding of the 33-kDa protein to *petD* 3′ IR RNA requires not only the secondary structure of the hairpin but also sequences downstream. For example, Δ18 mutant 3′ IR

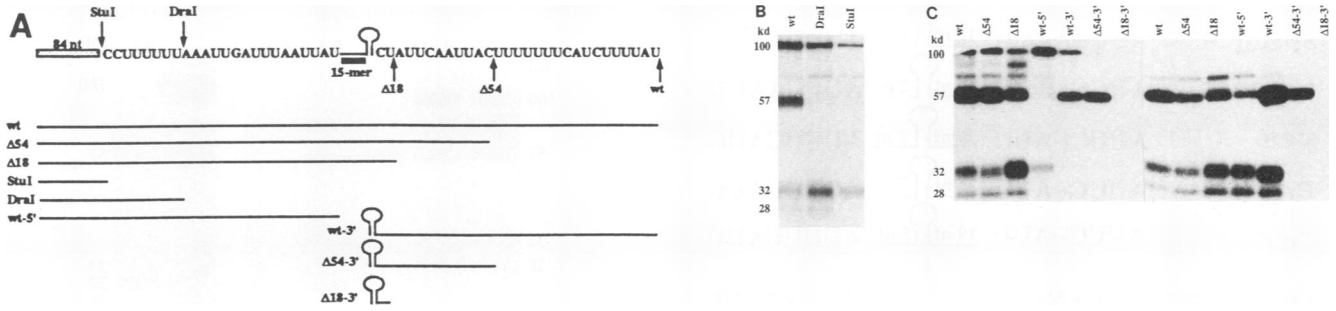

FIG. 4. UV-cross-linking analysis of deletion mutant *petD* 3′ IR RNAs. (A) The upper part of the panel shows wt *petD* 3′ IR RNA. The bar on the left represents the 3′ 84 nucleotides of the *petD* coding region. *Stu*I and *Dra*I restriction sites are indicated, and an oligonucleotide complementary to the 15 nucleotides immediately preceding the hairpin structure is indicated by a solid bar. The *petD* 3′ hairpin structure is shown, and the end points of deletion mutants Δ18 and Δ54 and of wt *petD* 3′ IR RNA are indicated by arrows. The lower part of the panel shows the RNA substrates used in UV-cross-linking experiments. *Stu*I and *Dra*I RNAs synthesized from *Stu*I- or *Dra*I-linearized DNA templates, respectively; wt-3′ and wt-5′, RNAs generated from RNase H cleavage of wt 3′IR-RNA hybridized with the 15-mer; Δ54-3′ and Δ18-3′, RNAs generated by RNase H cleavage of IR Δ54 and Δ18 3′ IR RNAs hybridized with the 15-mer. (B) wt 3′IR-RNA and *Dra*I and *Stu*I RNAs were UV cross-linked to total soluble chloroplast proteins in the absence of poly(U). Reactions were analyzed in a 12% SDS–polyacrylamide gel. (C) UV-cross-linking assays were carried out with total soluble chloroplast proteins in the absence of poly(U) (left) and in the presence of 100 ng of poly(U) (right). The tested *petD* RNAs are indicated above each lane.

RNA (Fig. 2C) does not bind the 33-kDa protein in UV-cross-linking experiments, whereas Δ54 mutant 3′ IR RNA (Fig. 2C) does bind the 33-kDa protein (44). This indicates that all or part of the sequence AUUCAAUUAC, which lies immediately downstream of the hairpin and between the deletion end points of Δ18 and Δ54, is also required for 33-kDa protein binding in vitro. However, the binding of the 100-, 57-, 32-, and 28-kDa proteins is not strongly affected by any of the 25 mutations in the hairpin structure. An exception is mutant petD7.4, which even at high concentrations did not prevent binding of the 57-kDa protein to wt 3′IR-RNA (Fig. 3B), indicating a possible pleiotropic effect of this mutation.

(ii) 57-kDa protein recognizes AU-rich sequence motif. Since mutagenesis of the *petD* 3′ IR affects the binding of the 33-kDa protein but not that of other *petD* 3′ IR RNA-binding proteins, the binding sites for the other proteins must be located either upstream or downstream of the hairpin. To determine the binding sites of the other proteins, we tested RNAs corresponding to regions upstream or downstream of the hairpin in UV-cross-linking experiments.

Two RNAs containing only the 3′ end of the *petD* coding sequence were obtained by T7 transcription of a *Dra*I- or *Stu*I-linearized template (Fig. 4A). To generate an RNA containing only the hairpin and sequences downstream, an oligonucleotide complementary to the 15 nucleotides immediately preceding the hairpin was synthesized and annealed to different 3′ IR RNA substrates, and the duplexes were digested with RNase H. Figure 4A shows the series of RNAs that we have generated and tested in UV-cross-linking assays. The results of these experiments are shown in Fig. 4B and C and summarized in Table 1. From these data, two recognition sites for the 57-kDa protein can be identified. Since the 57-kDa protein binds to wt 5′ RNA but not to *Dra*I or *Stu*I RNAs (Fig. 4B and C), some or all of the 16 nucleotides between the *Dra*I site and the 15-mer used for RNase H cleavage (Fig. 4A) are essential for 57-kDa protein binding. Furthermore, when the 3′ portions of RNase H-cleaved Δ54 and Δ18 3′ IR RNAs were tested in UV-cross-linking reactions, only the Δ54-derived RNA (Δ54-3′ RNA; Fig. 4B and C) bound to the 57-kDa protein. This indicates that some or all of the 8 nucleotides between the Δ18 and the Δ54 deletion points constitute a second binding site for the 57-kDa protein. To confirm that the same 57-kDa protein binds at the two sites, we partially digested the two UV-cross-linked RNA-protein complexes with *S. aureus* V8 protease and examined the digestion products by gel electrophoresis (data not shown). We found that Δ54 3′ RNA–57-kDa protein and wt 5′ RNA 57-kDa protein complexes yield identical polypeptide fragments of 37 and 28 kDa. This demonstrates that the same protein interacts at two sites in *petD* 3′ IR RNA that are separated by the hairpin.

Both of the sequences that contain 57-kDa protein-binding sites are AU rich. A comparison of these two sequences reveals a common sequence motif, AUUYAAUU, all or part of which may serve as a binding site for the 57-kDa protein. To determine if the proposed 57-kDa protein-binding site represents an evolutionarily conserved sequence, we compared the 3′ UTRs of chloroplast *petD* genes from spinach (17), tobacco (39), pea (29), maize (33), and rice (19). Indeed, this motif is found twice in all the genes compared, once upstream and once downstream of the hairpin (Fig. 5). The consensus binding site drawn from this comparison is AUUYNAUU.

As shown above, 33-kDa-protein binding requires sequences downstream of the hairpin that overlap with the binding site of the 57-kDa protein. It is not clear whether both proteins can interact independently with the same sequence or whether binding of the 33-kDa protein is depen-

TABLE 1. UV cross-linking of different *petD* RNAs to chloroplast proteins[a]

Protein	Cross-linking with RNA:			Presence of RNase H digestion products					
				IR proximal			IR distal		
	wt	Δ54	Δ18	wt-5′	*Dra*I[b]	*Stu*I[b]	wt-3′	Δ54-3′	Δ18-3′
100 kDa	+	+	+	+	+	+	+	−	−
57 kDa	+	+	+	+	−	−	+	+	−
32 kDa	+	+	+	+	+	+	+	−	−
28 kDa	+	+	+	+	+	+	+	−	−

[a] +, binding of protein to RNA; −, no binding.
[b] Linearized by this restriction endonuclease.

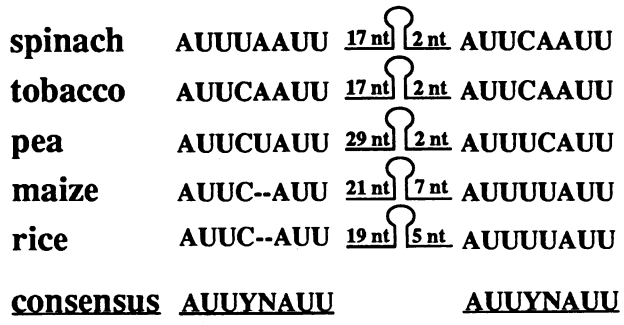

FIG. 5. Comparison of 3′ UTR of *petD* genes from spinach, tobacco, pea, maize, and rice chloroplasts. The distances of the putative binding sites for the 57-kDa protein from the hairpin structure of each gene are indicated in nucleotides (nt). Sources of sequence data were as follows: spinach (17), tobacco (39), pea (29), maize (33), and rice (19). In the consensus sequence, Y is a pyrimidine, and N is A, U, or C.

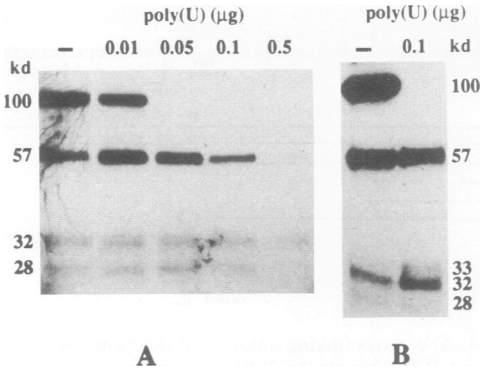

FIG. 6. Effect of amount of poly(U) on *petD* 3′ IR RNA-protein interactions. UV cross-linking was carried out using wt *petD* 3′ IR RNA and total soluble chloroplast proteins. (A) Lane ■, no poly(U); other lanes, different amounts of poly(U) as indicated were added to each reaction before ^{32}P-labeled wt 3′ IR RNA was added. (B) No poly(U) (■) or 0.1 μg of poly(U) was added to the UV-cross-linking reaction using bound-fraction proteins from a heparin-agarose column.

dent on prior binding of the 57-kDa protein to RNA. However, the binding of the 57-kDa protein is clearly independent of the binding of the 33-kDa protein, since all petD7 mutant 3′ IR RNAs failed to label the 33-kDa protein while their binding for the 57-kDa protein was unaffected, with the possible exception of mutant petD7.4 (Fig. 3B).

(iii) Three *petD* 3′ IR RNA-binding proteins have multiple binding sites. As shown in Fig. 4B and C and summarized in Table 1, 3′ IR RNAs derived from RNase H-directed cleavage of wt but not Δ54 or Δ18 3′ IR RNAs interact with the 100-, 32-, and 28-kDa proteins. This indicates that part or all of the uridine-rich sequence between the Δ54 3′ IR RNA 3′ end and the wt 3′ IR RNA 3′ end constitutes a binding site for these proteins. Upstream of the hairpin, these proteins interact with *Stu*I and *Dra*I RNAs and wt 5′ RNAs (Fig. 4B and 4C). Since *Stu*I RNAs RNA contains only *petD* coding sequences (Fig. 4A), we conclude that RNA-protein interactions occur within the *petD* coding region as well as in the 3′ UTR. Since the *petD* coding region is generally less uridine rich than the 3′ UTR and since no contiguous stretch of uridines like that at the 3′ end of wt 3′ IR RNA is found, it appears that a long uridine-rich sequence is not an absolute requirement for 100-, 32-, or 28-kDa-protein binding in vitro. In fact, the interactions between these RNA-binding proteins and *petD* 3′ IR RNA suggest that no single sequence or structural motif is required for protein binding. This is consistent with our previous finding that the 32- and 28-kDa proteins also interact with 3′ IR RNAs derived from the spinach chloroplast *psbA* and *rbcL* genes (44).

Distinctive binding characteristics of *petD* 3′ IR RNA-binding proteins. To characterize further the interactions of chloroplast proteins with *petD* 3′ IR RNA, we have carried out UV-cross-linking assays under different reaction conditions. To test the effect of monovalent cation concentrations on the stability of *petD* 3′ IR RNA-protein complexes, we varied the concentration of KCl from 60 to 400 mM and the concentration of NaCl from 10 to 500 mM. We found that the concentration of KCl had no significant effect on protein binding to *petD* 3′ IR RNA (data not shown); a similar result was obtained for the mustard chloroplast proteins that bind to RNA sequences downstream of the *trnK* gene (28). However, *petD* 3′ IR RNA-protein interactions appear to be destabilized at high NaCl concentrations, since the binding of all proteins to *petD* 3′ IR RNA was strongly reduced in 0.5 M NaCl (data not shown).

To test the effect of divalent-cation concentrations on *petD* 3′ IR RNA-protein complex formation, we treated UV-cross-linking reactions with EDTA, either 5 min before or 5 min after [^{32}P]RNA was added to the reaction mixture. Pretreatment with 25 mM EDTA eliminated binding of the 100-kDa protein, appeared to increase labeling of the 32- and 28-kDa proteins, and resulted in the appearance of several new labeled proteins of approximately 40 to 45 kDa (data not shown). However, if EDTA was added after the 3′ IR RNA, the only effect was a slight increase in labeling of the 32- and 28-kDa proteins (data not shown). From these data we conclude that the interaction of the 100-kDa protein with *petD* RNA requires Mg^{2+} (the principle divalent cation in our reaction mixture) and that once formed, the RNA-protein complex is stable and either tightly binds or no longer requires Mg^{2+} ions. These results also suggest that high Mg^{2+} concentrations are not required for binding of the 57-, 32-, and 28-kDa proteins. We previously reported that Mg^{2+} is required for binding of the 33-kDa protein (43).

A high affinity for the ribonucleotide homopolymer poly(U) has been reported for several RNA-binding proteins, such as the A1, C, and D heterogeneous nuclear RNPs (for reviews, see references 3 and 46). Here, we have carried out a series of UV-cross-linking experiments using poly(U) as a competitor. Figure 6A shows that the 100-kDa protein has a relatively high affinity for poly(U), since its binding to *petD* 3′ IR RNA could not be detected in the presence of 50 ng of poly(U), whereas 57-, 32-, and 28-kDa-protein binding occurred except when 500 ng of poly(U) was added to the reaction. The sensitivity to poly(U) shown by the 100-kDa protein cannot be interpreted simply as a preference for binding uridine-rich sequences, since the 100-, 32-, and 28-kDa proteins all bind preferentially to uridine-rich sequences and the 57-kDa protein recognizes a U-rich motif (Fig. 4 and text), yet they all have different sensitivities to poly(U). Interestingly, the addition of 100 ng of poly(U) to the reaction inhibits binding of the 100-kDa protein but enhances binding of the hairpin-binding 33-kDa protein (Fig. 6B). Therefore, poly(U) may stabilize the interaction of 3′ IR

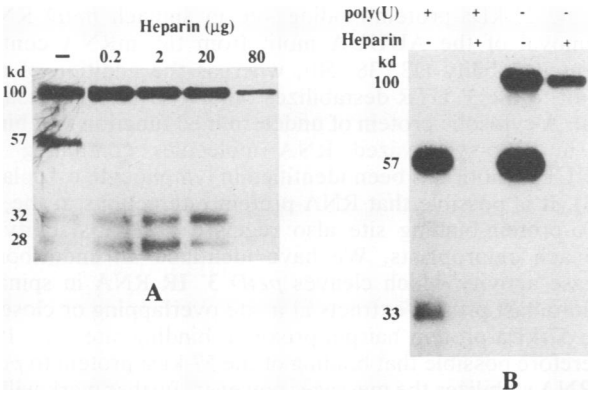

FIG. 7. Effect of heparin on *petD* 3′ IR RNA-protein interactions. (A) UV cross-linking was carried out using wt *petD* 3′ IR RNA and total soluble chloroplast proteins. The tested amounts of heparin were as follows: lane ■, no heparin added; other lanes, heparin (as indicated) was added to each reaction before the ^{32}P-labeled wt 3′ IR RNA was added. (B) UV cross-linking was carried out using wt *petD* 3′ IR RNA and bound-fraction proteins from a heparin-agarose column in the presence (+) or absence (−) of 100 ng of poly(U) or 0.2 μg of heparin.

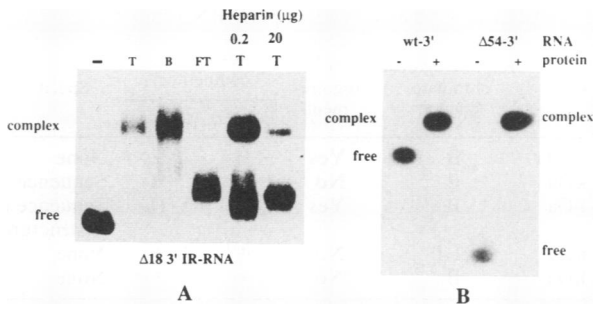

FIG. 8. Gel mobility shift assays of *petD* 3′ IR RNA. (A) Deletion mutant Δ18 3′ IR RNA was incubated with different chloroplast protein fractions and analyzed in a 5% polyacrylamide–50 mM Tris-glycine nondenaturing gel. Lane ■, no protein added; lane T, 10 μg of total soluble chloroplast proteins; lane B, 5 μg of bound-fraction protein from a heparin-agarose column; lane FT, 28 μg of flowthrough-fraction protein from a heparin-agarose column. The last two lanes show the assays using 10 μg of total soluble protein in the presence of 0.2 or 20 μg of heparin. (B) wt-3′ and Δ54-3′ RNAs were used for gel mobility shift assays. Lanes −, no protein added; lanes +, 10 μg of total soluble chloroplast proteins was added to the reaction; complex, RNA-protein complexes; free, unbound RNA.

RNA and the 33-kDa protein, since the 33-kDa protein was only weakly labeled in the absence of poly(U) (Fig. 6B).

Since most *petD* 3′ IR RNA-binding proteins can be trapped on heparin-agarose columns (44), we tested the effect of adding increasing amounts of heparin in UV-cross-linking experiments. Figure 7A shows that the binding of the 57-kDa protein to *petD* 3′ IR RNA was strongly reduced following the addition of 0.2 μg of heparin to the reaction mixture, whereas binding of the 100-, 32-, and 28-kDa proteins was affected only by the highest amount of heparin tested (80 μg). To test the effect of heparin on binding of the 33-kDa protein, we have carried out UV-cross-linking assays in the presence or absence of poly(U) by using bound-fraction proteins. Figure 7B shows that the binding of the 33-kDa protein is best detected in the presence of poly(U) and the absence of heparin. The addition of 0.2 μg of heparin inhibits the binding of the 33-kDa protein in both the presence and the absence of poly(U). The similar sensitivities to heparin of both 57- and 33-kDa-protein binding is striking and correlates with our other data suggesting that 33-kDa protein binding might depend on prior binding of the 57-kDa protein. Heparin has been used frequently in the study of transcription and splicing complexes as a competitor to dissociate components that interact with RNA nonspecifically, in order to facilitate the identification of specific components of these RNA-protein complexes. Since we have shown that the 57- and 33-kDa proteins bind to specific sequences and/or structures in *petD* 3′ IR RNA whereas the 100-, 32-, and 28-kDa proteins bind RNA nonspecifically and are relatively insensitive to heparin, experiments in which heparin is used to dissociate nonspecific RNA-protein complexes must be interpreted with caution.

***petD* 3′ IR RNA-binding proteins form complexes with *petD* RNA in vitro.** To demonstrate that the *petD* 3′ IR RNA-binding proteins form complexes with *petD* RNA in vitro, different *petD* 3′ IR RNAs were used in gel shift assays under the same conditions as those used for UV cross-linking. Figure 8 shows that Δ18 3′ IR RNA, which corresponds to a mature form of *petD* 3′ IR RNA, forms complexes with total soluble chloroplast proteins or with bound-fraction proteins from a heparin-agarose column. On the other hand, only a small mobility shift occurred when the flowthrough fraction from the heparin-agarose column was used. Since the only protein in this fraction that binds *petD* 3′ IR RNA, as detected by UV cross-linking, is the 32-kDa protein, this result suggests that the 32-kDa protein may require the presence of other proteins to form a complex with *petD* 3′ IR RNA. Addition of poly(U) has no effect on the retarded band using the total- or bound-protein fractions when they are tested under conditions that affect only the 100-kDa-protein binding in UV-cross-linking assays (Fig. 6; data not shown). However, the Δ18 RNA-protein complex is sensitive to heparin treatment, since the complexes were dissociated by heparin at the same concentrations that diminish only the 57- and 33-kDa-protein binding to *petD* 3′ IR RNA in UV-cross-linking assays (Fig. 7 and 8). This suggests that the 57-kDa protein is required for complex formation with *petD* 3′ IR RNA, since the 33-kDa protein does not bind to Δ18 3′ IR RNA. In addition, RNAs containing either of the two binding sites for the 57-kDa protein form complexes with the 57-kDa protein. Both Δ18 3′ IR RNA, which contains only the upstream binding site, and wt 3′ and Δ54 3′ RNAs, which contain only the downstream binding site, exhibit mobility shifts (Fig. 8B). Taken together, these results demonstrate that the 57-kDa protein is required for complex formation with *petD* 3′ IR RNA and that protein-protein interactions may occur between different *petD* 3′ IR RNA-binding proteins.

DISCUSSION

We have carried out a detailed analysis of RNA-protein interactions in the 3′ region of the spinach chloroplast *petD* transcript. Using UV-cross-linking and gel shift assays, we have identified five *petD* RNA-binding proteins, and each has different RNA recognition sequences and distinct binding characteristics. Table 2 illustrates how these proteins can be divided into two groups on the basis of our results. Three proteins of 100, 32, and 28 kDa do not require specific sequences to bind RNA, although they exhibit a preference

TABLE 2. Characteristics of *petD* RNA-binding proteins

Protein	Heparin-agarose chromatography[a]	Mg^{2+} requirement	Response to competitor[b]		Specificities
			Poly(U)	Heparin	
100 kDa	B	Yes	H	+	None
57 kDa	B	No	+	H	Sequence
33 kDa	B	Yes	+	H	Sequence and structure
32 kDa	FT	No	+	+	None
28 kDa	B	No	+	+	None

[a] B, bound-fraction proteins; FT, flowthrough-fraction proteins.
[b] H, highly sensitive to competitor; +, competition occurred at high level of competitor.

for uridine-rich sequences. RNA-binding proteins such as the A1, C, and D heterogeneous nuclear RNPs have also been shown to bind preferentially to uridine-rich sequences (for reviews, see references 3 and 46). Two chloroplast proteins that bind in a Mg^{2+}-dependent manner to RNA sequences located 3′ to the mustard chloroplast *trnK* gene have also been suggested to interact with uridine-rich sequences (28). It is likely that the 100-, 32-, and 28-kDa spinach chloroplast RNA-binding proteins have general functions. For example, they may participate in mRNA maturation or in the formation of chloroplast mRNPs. Previous studies showing that most heterogeneous nuclear RNPs remain tightly bound to ssDNA columns upon heparin treatment correlate with our data showing that the cross-linking of the 100-, 32-, and 28-kDa proteins to *petD* 3′ IR RNA is unaffected by the addition of heparin. Two recent reports have described four chloroplast RNP proteins from tobacco with sizes of 28, 30, 31, and 32 kDa (22) and a chloroplast RNP from spinach of 28 kDa (36). Two of the proteins that we have identified are similar in size to the tobacco chloroplast RNP proteins and may be homologous.

We have described two RNA-binding proteins that recognize specific features of *petD* RNA: a 33-kDa protein that recognizes the double-stranded stem of the hairpin structure and a 57-kDa protein that recognizes a consensus sequence, AUUYNAUU. The binding of these proteins to RNA may be dependent on protein-protein interactions, since conditions that prevent binding of the 57-kDa protein always prevent binding of the 33-kDa protein. This may be analogous to the ordered assembly of the U2 small nuclear RNP, where binding of the U2 B″ protein to stem-loop IV of U2 small nuclear RNA requires the presence of the U2 A′ protein (34, 35). We have also shown that binding of the 33- and 57-kDa proteins but not the 100-, 32-, and 28-kDa proteins is strongly inhibited by heparin (Table 2). The different affinities of the 33- and 57-kDa proteins for heparin may prove useful for protein purification. In fact, the 57-kDa protein is eluted from heparin-agarose and poly(U)-Sepharose columns only in 0.4 M KCl, whereas all other *petD* 3′ IR RNA-binding proteins elute at lower KCl concentrations (1).

The 57-kDa protein recognizes a motif, AUUYNAUU, that is highly conserved in the *petD* genes of other higher-plant species. The evolutionary conservation of this sequence may indicate its specific function in the regulation of *petD* gene expression through interactions with chloroplast proteins. In mammalian cells, an AU-rich sequence located in the 3′ UTR of a variety of lymphokine and proto-oncogene mRNAs has been shown to be related to mRNA instability (for reviews, see references 5, 6, and 38). These AU-rich sequences always contain an AUUUA motif identical to part of the 57-kDa-protein-binding site in spinach *petD* RNA. Removal of the AUUUA motif from the mRNA confers greater stability (13, 38, 50), whereas the addition of this motif to the 3′ UTR destabilizes otherwise stable messages (38). A cytosolic protein of undetermined function that binds to in vitro-synthesized RNA molecules containing the AUUUA motif has been identified in lymphocyte cytoplasm (23). It is possible that RNA-protein interactions at the 57-kDa-protein-binding site also regulate mRNA stability in spinach chloroplasts. We have identified an endoribonuclease activity which cleaves *petD* 3′ IR RNA in spinach chloroplast protein extracts at a site overlapping or close to the 57-kDa-protein hairpin-proximal binding site (41). It is therefore possible that binding of the 57-kDa protein to *petD* mRNA stabilizes the message; however, further work will be required to confirm the involvement of the AU-rich sequence and the 57-kDa protein in the regulation of *petD* mRNA stability.

The 33-kDa protein interacts with the stem of the hairpin structure and may consequently stabilize the secondary structure of the hairpin. Any base change that disrupts the first 5 bp of the stem eliminates or reduces binding of the 33-kDa protein to the *petD* 3′ IR RNA precursor. A compensatory mutation that restores base pairing of the stem also restores the binding of the 33-kDa protein. However, this protein does not interact with the mature *petD* 3′ IR RNA Δ18 in vitro, since sequences downstream of the IR constitute part of its binding site, either directly or indirectly via interaction with the 57-kDa protein. It is possible, however, that the 33-kDa protein binds to mature *petD* mRNA in vivo.

We are currently testing the possibility that the 33-kDa protein is involved in transcription termination. In prokaryotes, a GC-rich sequence of dyad symmetry followed by a series of uridine residues constitutes a factor-independent transcription termination signal that directly generates the mRNA mature 3′ end (for a review, see reference 30). On the other hand, rho-dependent termination can produce an mRNA that is processed exonucleolytically to yield the mature 3′ end (24). In chloroplasts, most 3′ UTR contain a sequence capable of forming a hairpin structure that is coincident with the mature mRNA 3′ end. It has been proposed that the 3′ end of spinach chloroplast *petD* mRNA is generated directly by transcription termination at the hairpin structure we have been studying (49). Here, we have shown that a 33-kDa protein binds specifically to the *petD* mRNA 3′ hairpin structure, which is consistent with a possible role for this protein in the transcription termination. For example, binding of the 33-kDa protein to the hairpin of the nascent *petD* transcript may stabilize its secondary structure, eliciting a pause by RNA polymerase, and the uridine-stretch downstream may facilitate dissociation of the transcript from the template in a manner similar to prokaryotic transcription termination. Since the *petD* 3′ IR terminates with only a 50% efficiency in vitro (42), read-through transcripts would most likely mature via the efficient exonucleolytic processing activity that we have previously described for spinach chloroplast protein extracts (42, 43). In previous studies of 3′ IR-directed transcription termination by spinach chloroplast RNA polymerase, we and others have found that only known *Escherichia coli* transcription terminators and some tRNA genes terminate at high (>80%) efficiency; among plastid IRs, only the *petD* 3′ IR terminates at a significant (>10%) efficiency (8, 42). This relatively efficient in vitro termination can be correlated with the presence of uridine-rich sequences 3′ to the IR and with

binding of the 33-kDa protein. This protein does not bind to *psbA* or *rbcL* 3′ IR RNAs in vitro. A possible function for augmented termination at *petD* is that the *rps11-infA-rpoA* operon is transcribed convergently (40) from a weak promoter (10); in the absence of efficient transcription termination, unacceptably high levels of antisense RNA might be generated. Since *petD* 3′ IR mutants unable to bind the 33-kDa protein are available (see Results), the in vitro transcription termination efficiency of wt and mutant 3′ IR can be compared. However, our studies do not rule out functions for the 33-kDa protein such as transcript processing, stability, or interaction with mRNP particles.

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