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RESEARCH ARTICLE

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RNA-protein complexes that form in the spinach chloroplast atpI 5′ untranslated region can be divided into two subcomplexes, each comprised of unique *cis*-elements and *trans*-factors

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Abstract Control of gene expression in chloroplasts is critically dependent upon post-transcriptional mechanisms, most of which require formation of RNA-protein complexes. The 5′ untranslated regions (5′UTRs) of chloroplast mRNAs have been shown to affect stability and/or translation of the message. These effects are mediated by the binding of specific protein(s) to the 5′UTR. We can detect such 5′UTR-protein complexes in vitro and have previously shown that the same polypeptide(s) bind many spinach chloroplast 5′UTRs (Robida et al. 2002). Here we report investigations on the RNA elements and protein factors involved in formation of these complexes. Comparison of the atpI 5′UTR, which serves as the representative 5′UTR for these experiments, among 12 angiosperms revealed two phylogenetically conserved regions upstream of a putative ribosome binding site. To determine whether the two conserved regions interact to form a single polypeptide-binding site, binding assays were performed with RNAs containing only one of the two. Those experiments revealed that the entire 5′UTR could be separated into two binding sites for chloroplast polypeptides, each containing one of the two conserved regions. Competition binding assays using the individual binding sites established that each was bound by different polypeptide(s). These data support the hypothesis that there are at least two unique polypeptides involved in these 5′UTR-protein complexes, each binding specifically to a different site within the 5′UTR.

Keywords Chloroplast · Gene expression · Post-transcriptional regulation · Spinach · *Spinacia oleracea* · 5′UTR

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Introduction

Chloroplast gene expression fluctuates in a controlled fashion in response to developmental and environmental signals. Although many chloroplast genes are under transcriptional control, proper chloroplast function also depends upon post-transcriptional mechanisms. Crucial post-transcriptional mechanisms include splicing, editing, processing (cleavage), translation, and RNA degradation (Bauer et al. 2001; Chateigner-Boutin and Hanson 2002; Choquet and Wollman 2002; Mayfield et al. 1995; Monde et al. 2000; Rochaix 2001; Sugita and Sugiura 1996). Translation and RNA stability are primarily controlled by interactions between proteins and RNA elements in the 5′ and 3′ untranslated regions (UTRs) of chloroplast mRNAs (Dauvillee et al. 2003; Hirose and Sugiura 2004; Katz and Danon 2002; Klaff et al. 1997; Komine et al. 2002; Nickelsen 2003; Somanchi and Mayfield 2001; Zerges et al. 2003; Zou et al. 2003).

Specific complexes between 5′UTRs and chloroplast polypeptides have been observed in all chloroplasts examined (Fargo et al. 2001; Hauser et al. 1996; Hotchkiss and Hollingsworth 1999; Kim and Mayfield 2002; Klaff et al. 1997; Ossenbuehl et al. 2002; Robida et al. 2002; Shen et al. 2001; Zerges et al. 2003). Elegant experiments in *Chlamydomonas reinhardtii* have detected and analyzed nuclear-encoded 5′UTR binding proteins that affect translation and stability for individual chloroplast mRNAs (Barnes et al. 2004; Drager et al. 1998; Fargo et al. 2001; Hauser et al. 1996; Higgs et al. 1999;

Manuell et al. 2004; Ossenbuehl et al. 2002; Zerges et al. 2003). Although 5′UTR-protein complexes in land plants have been less extensively studied than those from algal systems, they have also been shown to affect translation and RNA stability both in vivo and in vitro (Eibl et al. 1999; Gillham et al. 1994; Hirose and Sugiura 1996; Horlitz and Klaff 2000; Staub and Maliga 1994; Zou et al. 2003). However, despite general functional similarities, factors affecting translation and mRNA stability differ between *Chlamydomonas* and land plant chloroplasts (Nickelsen 2003).

Research in our lab has concentrated on analysis of 5′UTR binding proteins from spinach chloroplasts (Hotchkiss and Hollingsworth 1999). The proteins that we study appear to be nearly universal in their recognition of chloroplast 5′UTRs, binding with similar affinity to 16 out of the 18 chloroplast 5′UTRs that were analyzed (Robida et al. 2002). Thus we hypothesize that these proteins may mediate a mechanism that affects chloroplast gene expression in a global fashion. Given the limited coding capacity of the chloroplast genome, the binding proteins are likely to be nuclear-encoded and thus be involved in a communication pathway between the nucleus and the chloroplast (Barkan and Goldschmidt-Clermont 2000; O'Brien et al. 2003; Sugiura 1992).

Here we report results of an investigation on the *cis*-acting elements and *trans*-acting factors involved in spinach chloroplast 5′UTR-protein complexes. Binding assays using deletion variants of the atpI 5′UTR revealed that there are separable *cis*-acting elements, each containing a sequence that is highly conserved among angiosperms. Competition binding analysis was used to demonstrate that each of the separable elements is bound by different chloroplast polypeptide(s).

Materials and methods

Plant growth conditions and chloroplast isolation

Spinacia oleracea was grown hydroponically under standard greenhouse conditions. Hydroponic media consisted of Peter's Professional Hydrosol 5:11:26 (Scott), supplemented with 8 mM $CaNO_3$. Chloroplasts were isolated on Percoll (Sigma) step-gradients from leaves 8 to 10 cm in length (Orozco et al. 1985).

Template generation

Templates for transcription reactions were generated via polymerase chain reaction (PCR). The PCR template was pJB6, which contains the entire large ATP synthase gene cluster from spinach chloroplasts (Stollar and Hollingsworth 1994). All 5′ primers began with a T7 RNA polymerase promoter (underlined in the sequences that follow). Prior to inclusion in transcription reactions, all templates were extracted with phenol/chloroform and concentrated by ethanol precipitation.

Wild-type 5′UTR

5′ primer:TAATACGACTCACTATAGGGGATTTTGAATCTCAAAAACT (corresponding to bases −155 through −136, relative to A in the ATG start codon as +1)

3′ primer: ATGATAGAACATTCATATTGTCCTA (corresponding to the complement of +15 through −8)

5′ Partial

5′ primer:TAATACGACTCACTATAGGGGATTTTGAATCTCAAAAACT (corresponding to bases −155 through −136, relative to A in the ATG start codon as +1)

3′ primer: AAATTTTCTAATTCGGATACTGATTAATTAC

(corresponding to the complement of bases −112 through −80)

3′ Partial

5′ primer:TAATACGACTCACTATAGGGTTAATTAAAGTAGACAAGTCG

(corresponding to bases −80 through −60)

3′ primer: ATTGTCCTCTTACAGAAATATAACTT

(corresponding to the complement of −1 through −8)

Transcription of binding substrates

RNAs were generated by in vitro transcription from T7 RNA polymerase promoters. Transcription mixtures for radiolabeled RNAs included 50-ng PCR-generated template, 40 mM Tris–HCl pH 7.5, 10 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 0.5 mM each ATP, GTP, CTP, and UTP, 120 μCi $\alpha-^{32}$P-UTP (3000 Ci/mmole, MP Biomedicals), and 25 units T7 RNA polymerase. Conditions for large-scale transcriptions were similar to the radiolabeled reaction, but had 200 ng PCR-generated template, 10 mM DTT, 4 mM each NTP, 210 mM $MgCl_2$, and 125 units T7 RNA polymerase. Radiolabeled transcriptions were incubated at 37°C for 1 h. Large-scale transcriptions were incubated at 37°C for 4 h. All RNAs were purified by isolation from 5% polyacrylamide 7 M urea gels.

Chloroplast extract preparation

Soluble protein extracts were prepared from spinach chloroplasts as previously described (Hotchkiss and Hollingsworth 1999; Robida et al. 2002). Briefly, gradient-purified chloroplasts were lysed and the supernatant from a 25% ammonium sulfate precipitation of the stromal proteins was subjected to chromatography over

DEAE-Sephadex. The flow-through of that column was subjected to a second ammonium sulfate precipitation (31%). The precipitate was collected and re-suspended in 20 mM HEPES pH 7.9, 60 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol. This mixture was passed over heparin-agarose (BioRad). The flow-through contained the binding proteins used in these studies. Protein concentration of the fractions from the heparin-agarose flow-through was determined by a Bradford assay (Sigma), using bovine serum albumin as the standard.

Binding assays

When the identity of an RNA binding protein is unknown, formation of RNA-protein complexes is often assessed by UV-cross linking assays (Shen et al. 2001). However, the binding proteins that we study fail to cross-link with RNA under a wide variety of conditions (data not shown). Because of this, we hypothesize that the UV-activated bases in the 5′UTR are distant from any amino acid side chains with which they might react. Thus we assess binding of chloroplast polypeptides to RNAs in vitro by gel mobility shift assays. Binding assays and competition assays were performed as reported previously (Hotchkiss and Hollingsworth 1999; Robida et al. 2002). Briefly, 10 fm of radiolabeled binding substrate was incubated with 10 μg of chloroplast extract at room temperature for 10 min. Prior to the incubation with binding substrate, the chloroplast extract was mixed with 10 μg non-specific RNA [either total yeast RNA (Roche Applied Science, IN, USA) or *E. coli* tRNA (Sigma, MO, USA)]. Binding reactions also included 10 mM $MgCl_2$, 3 mM DTT, 40 mM KCl, 10 mM HEPES pH 7.9, 50 μM EDTA, and 9% glycerol. After incubation, reactions were loaded directly onto 6% native polyacrylamide gels and subjected to electrophoresis at 375 V. Gels were dried and exposed to a phosphor screen (Molecular Dynamics).

Depending upon the particular isolation, the chloroplast extracts used for these binding assays bound roughly 10 pm of 5′UTR per 10 μg of extract. Since only 10 fm of radiolabeled substrate was present in the assays, this meant that even self-competition required roughly 5000-fold excess of competitor for complete competition. A more efficient use of extract would be to dilute it and use considerably less in the assay, so that competition could be seen at less than tenfold excess of specific competitor. However, every dilution condition attempted resulted in a complete loss of binding activity. Thus these binding assays were performed in conditions of substantial protein-excess.

Competition assays

Competition assays were essentially the same as the binding assays, except that the radiolabeled binding substrate was mixed with unlabeled specific competitor RNA prior to addition of chloroplast extract. All competition assays also contained 10,000-fold excess of non-specific RNA, as noted previously.

Software

Sequence alignments were accomplished using Multalin at http://prodes.toulouse.inra.fr/multalin/multalin.html (Corpet 1988). Consensus levels were set for high = 90% and low = 70%. Sequences were obtained from GenBank (accessed through http://www.ncbi.nlm.nih.gov).

Results

The atpI 5′UTR

To simplify the analysis, we use a single 5′UTR as the baseline in all our experiments. It is derived from the spinach chloroplast *atpI* gene, which encodes the CF_o-IV subunit of the ATP synthase complex and is the second gene in the chloroplast large ATP synthase gene cluster (Hudson et al. 1987; Stahl et al. 1993; Stollar and Hollingsworth 1994). Affinity of the binding proteins and the stability of the complex formed with atpI 5′UTR are similar to that for other spinach chloroplast 5′UTRs (Robida et al. 2002). Thus insights into complex formation discovered with the atpI 5′UTR should be applicable to those formed with 5′UTRs from many other chloroplast genes.

5′UTR sequence alignment

The primary sequences of chloroplast atpI 5′UTRs from 12 angiosperms were compared to determine whether there was conserved primary structure. As illustrated in Fig. 1, the entire 5′UTR is well conserved among these plants. Overall, the spinach chloroplast atpI 5′UTR has a 52% match to the consensus sequence derived from all 12 species. Two regions have especially high sequence conservation. The first conserved region (Con1) comprises bases −111 through −96, relative to the start codon of the open reading frame as +1. Con1 has a 69% match to the consensus. Con2, from −56 through −22, has an 83% match to the consensus. Given these high identity values when compared among a dozen diverse angiosperms, we hypothesized that these regions are important for recognition by binding proteins.

The conserved regions are in separable binding sites

To determine whether Con1 and Con2 interact to form a protein-binding site, binding assays were performed with RNAs containing only one of the two conserved regions.

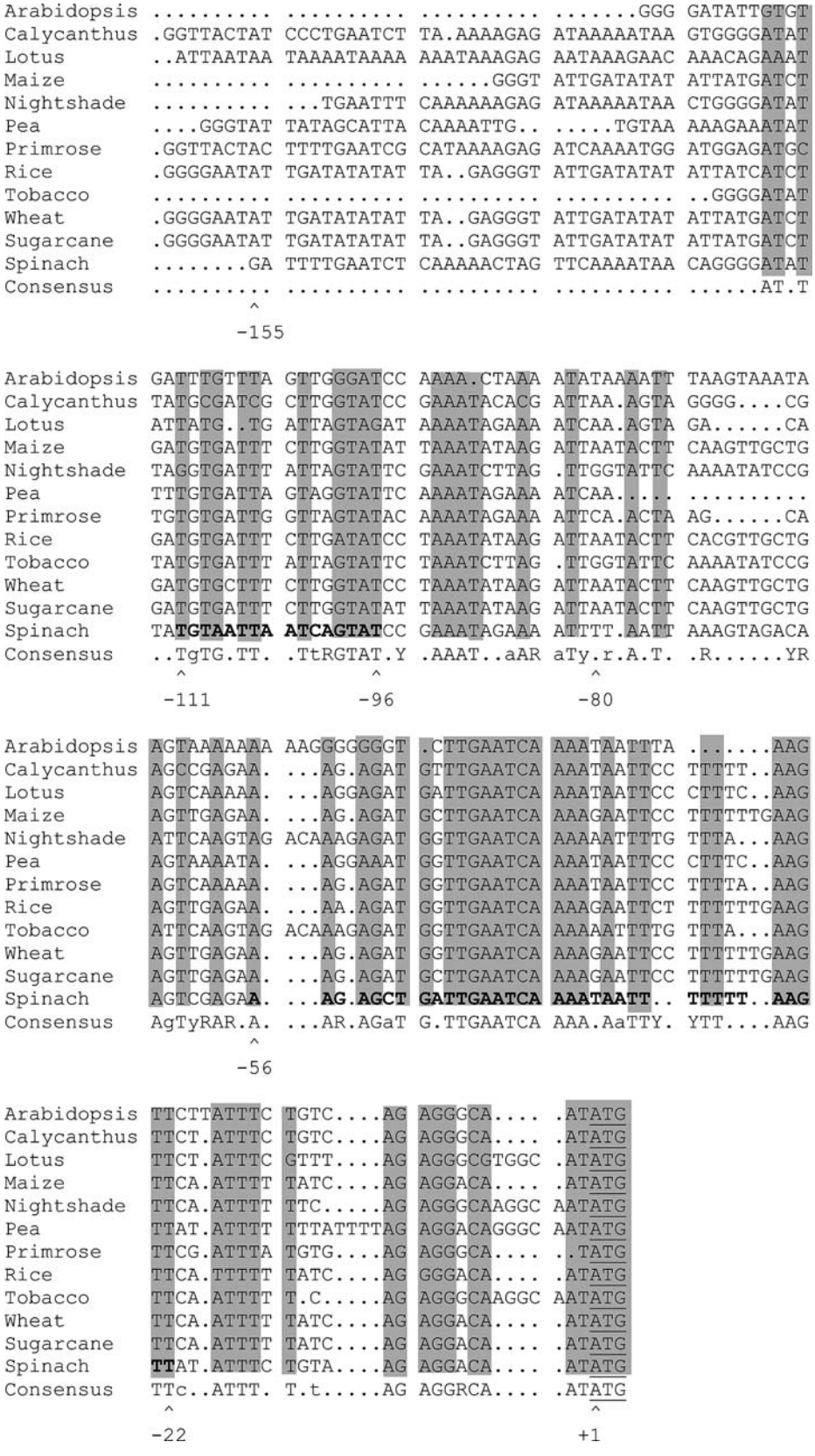

Fig. 1 Comparison of atpI 5′UTRs from 12 angiosperm chloroplasts. 5′UTR sequences were obtained from GenBank. When known (spinach, pea, wheat, tobacco, and maize), the sequence begins at identified 5′ends (Hoglund et al. 1990; Hudson et al. 1987; Huttly et al. 1990; Miyagi et al. 1998; Stahl et al. 1993). Otherwise, 160 nt upstream of the translation start site (*underlined*) were used for comparison. Identity of the plant is noted at left. "*Y*" denotes pyridine and "*R*" purine. Positions with no more than one mismatch among the 12 species are *shadowed*. Two regions in the spinach sequence with highest identity to the consensus are in *bold*. The 5′-most (−111 through −96) is referred to as "*Con1*" and the 3′-most (−57 through −22) as "*Con2*". *Numbering* is relative to the spinach atpI 5′UTR

Two non-overlapping partial RNAs were used for these experiments. "5′ partial" extends from an identified 5′ end of this transcript at −155 through −80 and includes Con1. "3′ partial" covers bases −80 through −1, and encompasses all of Con2. Results of binding assays with these partial 5′UTRs, along with a comparison assay

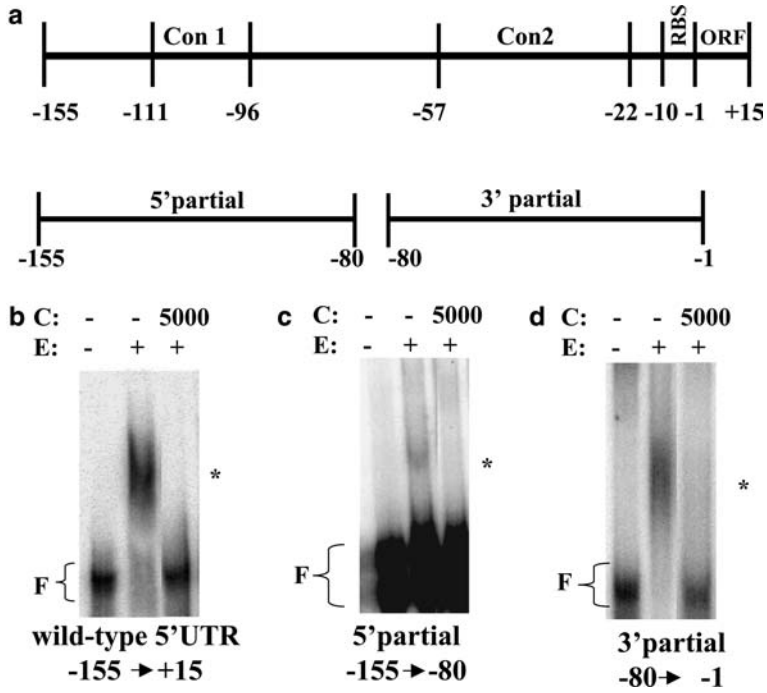

Fig. 2 Binding assays using whole or fragments of the atpI 5′UTR. **a** A diagram of the spinach chloroplast atpI 5′UTR. The regions of the 5′UTR that comprise the 5′ partial and the 3′ partial RNAs are designated below the map. *Con1* and *Con2*: two regions of phylogenetically conserved sequence in this 5′UTR (as defined in Fig. 1). *ORF* open reading frame; *RBS* ribosome binding site (putative). **b**–**d** Images of band shift assays performed with chloroplast extract and radiolabeled RNAs corresponding to nucleotides. **b** −155 to +15 (wild-type 5′UTR), **c** −155 to −80 (5′ partial) and **d** −80 to –1 (3′ partial). *E* designates the presence (+) or absence (−) of chloroplast polypeptides in the binding reaction. *C* designates the absence (−) or fold-excess of specific competitor identical to the binding substrate. Every binding reaction also contained 10,000-fold excess of non-specific RNA. * designates specific RNA-protein complexes and *F* denotes the migration of the assorted conformers of unbound RNA that resolve in these native gels. The sizes of the images were adjusted such that they were all the same. If analyzed on the same gel, the RNAs and complexes do not have the same mobility

with the entire 5′UTR, are shown in Fig. 2. Both of the partial 5′UTRs are specifically bound by chloroplast proteins. Thus, there are at least two individual elements within the atpI 5′UTR that can be bound by chloroplast proteins.

Although both partial RNAs were specifically bound by chloroplast polypeptides, one clear difference between them was stability of the complexes formed. Less than half of the 5′ partial RNA was bound in the absence of specific competitor. This was a distinct contrast to the situation with 3′ partial RNA, where over 90% of the RNA was bound.

The two binding sites do not efficiently compete with each other for binding by chloroplast polypeptides

Competition binding assays were used to determine whether the same or different proteins bound the two partial 5′UTRs. For these assays, radiolabeled binding substrate was mixed with increasing concentrations of unlabeled competitor prior to the addition of chloroplast proteins. If the same protein(s) binds two RNAs with similar affinity, competition will occur at the same fold-excess of competitor, regardless of which RNA is used as binding substrate or competitor. In contrast, RNAs that are bound by different proteins do not compete with each other in binding assays.

As explained more thoroughly in Materials and methods, binding proteins in the chloroplast extracts used in these experiments are in substantial excess over the ten femtomoles of radiolabeled binding substrate. A large (roughly 5000-fold) excess of unlabeled competitor is necessary for complete self-competition. Given the quantities of competitor RNA in these assays, it was possible that competition at 5000–10,000-fold excess was actually non-specific due to an overwhelming amount of RNA in the assay. However, identical competition experiments using additional non-specific RNA (*E. coli* tRNA) in place of chloroplast-derived RNAs showed no competition whatsoever (data not shown). Thus the amount of RNA necessary for self-competition reflects the quantity of specific binding proteins and not saturation of the system.

Competition assays comparing one binding site with another were performed over a $1–10^4$ -fold excess of competitor. Results of competition assays with the two partial 5′ UTRs are presented in Fig. 3. Under the same conditions where self-competition was complete by 5000-fold competitor (Fig. 2), heterologous competition was incomplete even at 10^4 -fold excess competitor. Since heterologous competition was substantially less efficient than homologous for both partial 5′UTRs, it

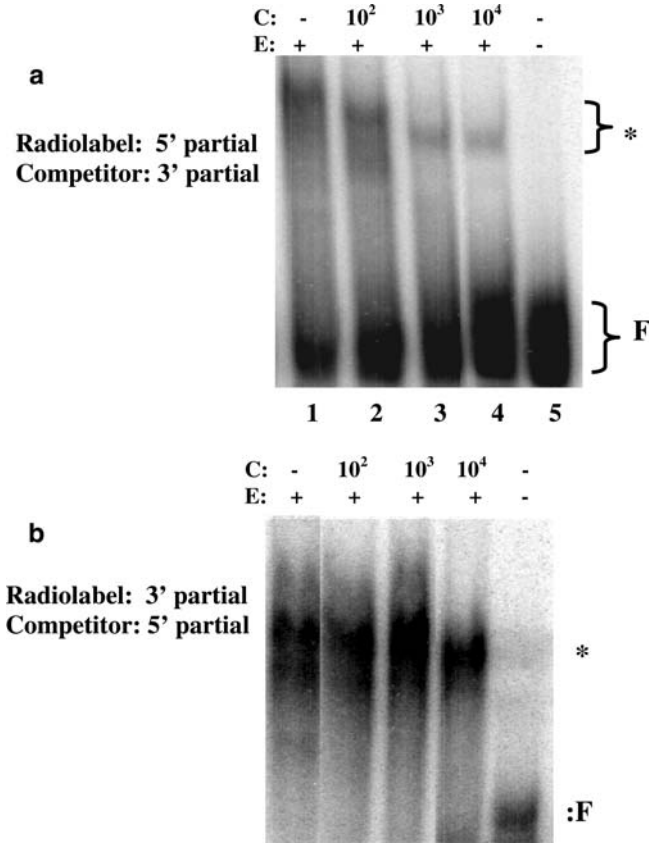

Fig. 3 Competition binding assays using two non-overlapping fragments of the atpI 5′UTR. Assays were performed with ten femtomoles of one radiolabeled binding substrate (**a** 5′ partial or **b** 3′ partial, see map in Fig. 2) and varying fold-excess of the other. Images of the assays after native gel electrophoresis are shown here. Identity of the radiolabel and competitor are given to the left of each image. Every binding reaction also contained 10,000-fold excess of non-specific RNA. "*E*" designates the presence (+) or absence (−) of chloroplast polypeptides in the binding reaction. "*C*" designates the absence (−) or fold-excess of specific competitor. * designates migration of RNA-protein complexes and "*F*" denotes the migration of the assorted conformers of unbound RNA that resolve in these native gels

appeared that a different protein might bind each of them.

Partial 5′UTRs do not completely compete for binding of the entire 5′UTR by chloroplast polypeptides

If the idea that each partial 5′UTR is bound by a different protein is correct, then neither partial RNA should be able to completely compete for all the proteins binding the entire atpI 5′UTR, which has both of the binding sites. Competition experiments were performed to test that prediction. As can be seen in Fig. 4, a faster-migrating atpI 5′UTR-protein complex remained after competition with 10,000-fold excess of either partial RNA. The original, slower-migrating atpI 5′UTR-protein complex was either reduced by 90% (competition with 5′ partial, Fig. 4a) or no longer visible (competition with 3′ partial, Fig. 4b). Comparison of the two competition reactions reveals that the remaining faster-mobility complexes have different mobilities themselves, implying that they are bound by polypeptide(s) of a different mass to charge ratio. Thus it appears that the original slower-mobility complex has at least two different proteins, one specific for each of the binding sites.

Discussion

The sequence alignment in Fig. 1 shows that there is extensive conserved sequence within the 5′UTR of *atpI* genes encoded by 12 angiosperm chloroplasts. This was not unexpected, because sequences of individual chloroplast genes do tend to be highly conserved among land plants (Sugiura 1995; Wakasugi et al. 2001). Similarity still exists when the comparison is extended to algal chloroplasts, but it is not as substantial (data not shown). In contrast, there is very little sequence similarity between 5′UTRs of different genes in the chloroplast, even within a single species. We have previously shown that the same proteins bind the 5′UTRs from 16 of 18 different spinach chloroplast genes (Hotchkiss and Hollingsworth 1999; Robida et al. 2002). Since there are no apparent primary sequence similarities among those 16 5′UTRs, the cis-acting elements recognized by the binding proteins must be found in secondary or tertiary structure, not in primary sequence. Chloroplast 5′UTRs have a high percentage of adenosine and uracil bases (e.g., the spinach chloroplast atpI 5′UTR is 75% A + U.) Because of the high A + U content, many secondary structures of approximately equal stability can be predicted. It is difficult to decide with any confidence which of the predicted structural motifs might be the one that they share in vivo. Experiments are currently in progress to investigate the structural features necessary for binding.

A question arising from the phylogenetic comparison was whether Con1 and Con2 interact to form one binding element or whether they are in separable binding elements. In Fig. 2, it can be seen that each conserved region is found within a separate binding element. It was interesting to observe the difference in the proportion of the two partial RNAs that could be detected in complexes. Depending upon the preparation (compare Fig. 2c lane 2 and Fig. 3a lane 1), no more than 30% of the 5′ partial RNA was ever found in complexes, while more than 90% of the 3′ partial RNA was found (compare Fig. 2d lane 2 and Fig. 3b lane 1). There are at least three possible explanations for this observation. First, the proteins that bind the 3′ partial RNA might be in substantial excess over those that bind the 5′ partial RNA. If that were true, then competition assays, both with self and with the wild-type 5′UTR, should have been complete at a substantially lower-fold excess of 5′ partial RNA than the 3′ partial RNA. Instead, we observed that the same fold-excess of either partial RNA

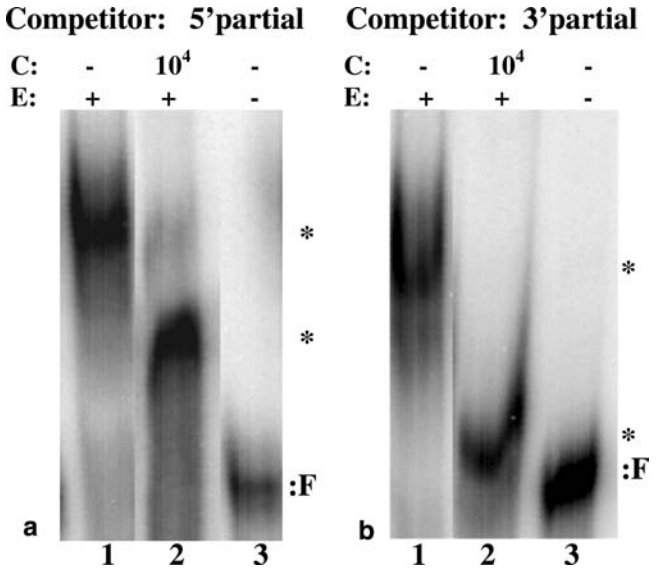

Fig. 4 Competition binding assays using radiolabeled whole atpI 5′UTR competed with unlabeled atpI 5′UTR fragments. Assays were performed with 10 fm of radiolabeled atpI 5′UTR and 10,000-fold excess of either **a** 5′ partial or **b** 3′ partial RNAs (see map, Fig. 2). After incubation, the reaction mixtures were subjected to native gel electrophoresis. Images of the gels are shown here. Identity of the unlabeled competitor is given above each image. "*E*" designates the presence (+) or absence (−) of chloroplast polypeptides in the binding reaction. "*C*" designates the absence (−) or fold-excess of specific competitor. Every binding reaction also contained 10,000-fold excess of non-specific RNA. * designates specific RNA-protein complexes and "*F*" denotes the migration of unbound RNA.

was necessary for competition against itself and wild-type 5′UTR. A second explanation for the data in Fig. 2 might be that the 5′binding proteins only bind a particular conformer of the 5′ partial RNA and that conformer is in low abundance. In that case, it would be surprising that the 5′ partial RNA competes so efficiently with wild-type for binding by chloroplast polypeptides. If only 10–30% of 5′ partial RNA is in a binding-competent conformation, then it would seem likely that competition of 5′ partial RNA for binding of wild-type 5′UTR would require three to tenfold more competitor than required for competition of 3′ partial RNA with wild-type 5′UTR. Instead, the two partial RNAs competed with the wild-type RNA at what appeared to be the same fold-excess. Thus it seems that a third explanation is the mostly likely one: that 5′ partial RNA-protein complexes are considerably less stable than 3′ partial RNA-protein complexes under these assay conditions.

Once the atpI 5′UTR was shown to have separable protein binding sites, there were three distinct models that could be used to predict the minimum number of proteins involved in the complex. The simplest idea would be a single protein with a binding site(s) that could recognize either of the RNA elements. A second model would also be a single protein, but with two binding sites, each specific for one of the elements in the 5′UTR. The third model would be that in which at least two different proteins are involved in the complexes, each one able to bind uniquely to only one of the elements in the 5′UTR.

The first idea, that there was only one binding protein that recognized both binding sites, was disproved by heterologous competition analysis with the two partial RNAs (Fig. 3). Had there been only one protein with the same binding site for each half of the 5′UTR, results of heterologous competition would have been identical to self-competition experiments. Since self-competition was complete for either RNA by 5000-fold competitor, but heterologous competition was incomplete even at 10^4 -fold excess competitor, the same protein with a single binding site could not be binding both partial RNAs.

The second model, that there was a single polypeptide with two different binding sites, would predict that heterologous competition would result in a change in the mobility of complex, because the original radiolabeled RNA-protein complex would be able to bind the heterologous competitor, forming a new complex of altered mobility. Results shown in Fig. 3 provide some support for this model. The 5′ partial RNA-protein complex in Fig. 3a changes mobility upon incubation with increasing concentrations of 3′ partial RNA (compare Fig. 3a, lanes 1 and 5). However, competition is incomplete and the complex observed in the inverse competition reaction, with radiolabeled 3′ partial RNA, shows no change in mobility upon competition with unlabeled 5′ partial RNA (compare Fig. 3b, lanes 1 and 5).

The third model for the composition of the atpI 5′UTR-protein complex is that there are at least two different binding proteins, each one specific for a different region of the 5′UTR. If that is true, then it would be expected that neither partial 5′UTR could fully compete with the full 5′UTR for complex formation, since at least one protein, the one not specific for the partial 5′UTR being used as competitor, would remain bound to the full-length 5′UTR. In that case, competition of the full 5′UTR with either of the partial RNAs should result in a change in mobility of the full-length 5′UTR-protein complex, from a slow-mobility complex with multiple proteins bound to a faster mobility complex with fewer proteins bound. This is what is observed in Fig. 4. An additional piece of evidence that at least two proteins of different molecular weights bind to the atpI 5′UTR is that the mobility of the atpI 5′UTR-protein complex after competition depends upon the competitor. The complex remaining after competition with 5′ partial RNA has a distinctly slower mobility relative to that remaining after competition with 3′ partial RNA (compare lanes 2 in Fig. 4a and b). Thus it appears that the proteins that remain bound to the atpI 5′UTR after competition with different competitors have a different mass to charge ratio.

The data in Fig. 4 strongly support the idea that there are multiple different polypeptides in these 5′UTR-pro-

tein complexes. However, this does not explain the results in Fig. 3a, where the 5′ partial RNA-protein complex exhibits an altered mobility upon competition with 3′ partial RNA. There are two possible explanations for that observation. First, one of the multiple proteins in the atpI 5′UTR-protein complex might be able to bind both binding sites, but has greater affinity for the 3′ site than the 5′. Alternatively, there could be a polypeptide that binds strongly to the 3′ binding site and weakly to a protein that binds to the 5′ binding site. Both of these explanations are supported by the data in Figs. 3 and 4. Experiments to isolate the polypeptides and distinguish between these two ideas are currently in progress.

Multiple different proteins specifically binding to unique sequences within 5′UTRs is a common occurrence in chloroplasts (reviewed in Barkan and Goldschmidt-Clermont 2000; Manuell et al. 2004; Nickelsen 2003). In contrast to the gene-specific 5′UTR binding proteins identified in algae, the binding proteins analyzed here are more general, binding to 16 of the 18 5′UTRs examined in an earlier study (Robida et al. 2002). Experiments to compare these general 5′UTR binding proteins with other chloroplast RNA binding proteins have been hampered by their inability to cross-link to RNA under many different conditions (Hollingsworth, unpublished). Affinity methods are now being used to isolate and identify the binding proteins.

5′UTR-protein complexes have been shown to affect translation and/or RNA stability in land plant and algal chloroplasts (reviewed in Nickelsen 2003). Utilizing results of the binding assays presented here to predict important *cis*-acting elements, we are currently analyzing chloroplast transformants with variants of the atpI 5′UTR placed upstream of a reporter gene. Preliminary data support the idea that the conserved regions affect both RNA abundance and translation (Merhige and J.O. Baecker, unpublished). Further experiments are planned to more finely resolve the structure of the *cis*-acting elements and to identity the *trans*-acting factors that bind them, to strengthen our understanding of the effects of these widespread complexes on chloroplast function and development.

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