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RESEARCH ARTICLE

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# Proteins are shared among RNA–protein complexes that form in the 5′ untranslated regions of spinach chloroplast mRNAs

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**Abstract** Gene expression in chloroplasts is strongly regulated at the post-transcriptional level. Most post-transcriptional mechanisms require RNA–protein complexes. Here we report an analysis of RNA–protein complexes that form in the 5′ untranslated regions (5′UTRs) of spinach chloroplast mRNAs. Previous studies from our laboratory showed that four ATP synthase 5′UTRs were able to compete with each other for binding by proteins in a chloroplast extract. This implied that at least some of the binding proteins recognized all four of those ATP synthase 5′UTRs. Here, we examine whether the binding proteins are ATP synthase-specific by performing competition-binding assays between an ATP synthase 5′UTR and 5′UTRs from other chloroplast genes. Competition substrates were chosen to represent a wide range of chloroplast mRNAs, including those encoding the photosystems, NADH dehydrogenase, cytochromes and ribosomal subunits, and two previously unexamined ATP synthase subunits. Results from these experiments revealed that, although the ATP synthase-binding proteins do not bind universally to every chloroplast 5′UTR, they do bind to the majority (12/14) of those examined. Thus, these RNA-binding proteins are candidates for factors that link the post-transcriptional expression of many chloroplast genes of disparate function.

**Keywords** Chloroplast · 5′UTR · Gene expression · RNA–protein complexes

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## Introduction

Chloroplasts are semi-autonomous organelles found in all photosynthetic eukaryotes (Bogorad 1981; Hallick 1992). These organelles have a double-stranded circular DNA genome that encodes approximately (depending upon the organism) 100 of the several thousand proteins necessary for chloroplast function (Peltier et al. 2000; Sugiura 1989; Wakasugi et al. 1998). The remainder of the proteins necessary for chloroplast function are encoded by the nucleus and must be translated in the cytoplasm and transported into the chloroplast. Although the chloroplast genome encodes all the tRNAs and rRNAs necessary for translation, most of the polypeptides necessary for function and regulation of gene expression, including most ribosomal subunits and translation factors, are encoded in the nucleus (Smith 1999; Somanchi and Mayfield 1999).

Chloroplast gene expression follows a prokaryotic motif (Gruissem and Tonkyn 1993; Kapoor and Sugiura 1998; Rochaix 1996; Sugita and Sugiura 1996). Factors involved in transcription and translation are similar to those found in prokaryotes; and most genes are co-transcribed as polycistronic RNAs. Messenger RNAs are not capped and, although polyadenylation does occur, it is involved in RNA decay rather than translation/stability (Komine et al. 2000; Monde et al. 2000; Schuster et al. 1999). Some mRNAs have Shine-Dalgarno sequences a few bases upstream of the AUG start codon, but it is not clear how critical those sites are for translation (Fargo et al. 1998; Harris et al. 1994; Sugiura et al. 1998; Zerges 2000). In contrast to prokaryotes, regulation of gene expression in the chloroplast involves additional complexity, because most of the regulatory molecules are encoded by the nucleus and must be transported to the chloroplast (Barkan and Goldschmidt-Clermont 2000; Leon et al. 1998; Mayfield 1990; McCormac and Barkan 1999; Somanchi and Mayfield 1999). These nucleus-encoded chloroplast polypeptides are the foundation for converting

environmental and developmental signals perceived by the nucleus into alterations in chloroplast gene expression.

Gene expression in chloroplasts is strongly affected by post-transcriptional regulatory mechanisms (Eibl et al. 1999; Gruissem and Tonkyn 1993; Kapoor and Sugiura 1998; Rochaix 1996; Sugita and Sugiura 1996). One common feature of gene expression in both unicellular and land-plant chloroplasts is regulation by RNA–protein complexes that form in the 5′ or 3′ untranslated regions (UTRs) of mRNAs. These complexes have been shown to affect translation (5′UTRs) and/or mRNA stability (both 5′ and 3′UTRs; Anthonisen et al. 2001; Barkan and Goldschmidt-Clermont 2000; Higgs et al. 1999; Hirose and Sugiura 1996; Klaff and Gruissem 1995; Klaff et al. 1997; Monde et al. 2000; Yohn et al. 1998a; Zerges 2000).

Research in our laboratory concentrates on the study of 5′UTR–protein complexes that form in mRNAs from the large ATP synthase gene cluster (Hudson and Mason 1988; Stollar and Hollingsworth 1994). This gene cluster encodes four of the six chloroplast-encoded ATP synthase subunits. In order from 5′ to 3′, the genes (and their encoded polypeptides) encoded by the large ATP synthase gene cluster are: *rps2* (small ribosomal protein subunit 2), *atpI* (ATP synthase $CF_o$-IV), *atpH* ($CF_o$-III), *atpF* ($CF_o$-I) and *atpA* ($CF_1$-α; nomenclature as per Hallick and Bottomley 1983). The genes are co-transcribed from multiple promoters and are extensively processed (Hotchkiss and Hollingsworth 1997; Hudson and Mason 1988; Miyagi et al. 1998; Stollar and Hollingsworth 1994). We have shown that specific RNA–protein complexes form in the 5′UTRs of *atpI*, *atpH*, and *atpA* (Hotchkiss and Hollingsworth 1995; Hotchkiss and Hollingsworth 1999). Competition analysis revealed that all four of the ATP synthase 5′UTRs were able to compete with each other for the binding of chloroplast polypeptides, albeit with varying efficiency (Hotchkiss and Hollingsworth 1999). Thus, it appears that many, if not all, of the binding proteins bind all four of these ATP synthase 5′UTRs.

Here, we address the question of whether these binding proteins are ATP-synthase-specific. Competition-binding assays were performed using 5′UTRs of 14 different chloroplast genes to compete with the *atpI* 5′UTR for binding in spinach chloroplast extracts (Table 1). The 5′UTRs were derived from genes encoding several types of polypeptides, including the two other chloroplast-encoded ATP synthase subunits and subunits from photosystems, ribulose bisphosphate carboxylase, NADH dehydrogenase, and ribosomes. Subsets of the mRNAs from which these 5′UTRs were derived also encode introns and are found in varying positions (beginning, middle, end) within a polycistronic transcript or are transcribed solely as monogenic RNAs (Sugita and Sugiura 1996). Since chloroplast 5′UTRs are A/U-rich, competition assays were also performed with polyA and polyU RNAs. We were surprised to discover that the polypeptides involved in the faster of the two ATP synthase 5′UTR–polypeptide

**Table 1** The 5′UTRs of 14 different chloroplast genes used to compete with the *atpI* 5′UTR for binding in spinach chloroplast extracts. When the information was available, the templates were transcribed such that the 5′ end of the transcript was the same as the most prevalent 5′ end found in vivo. The *length* of the RNA is given in nucleotides. The *start/stop* is relative to +1 as the A at the start of the open reading frame. The *gene features* are summarized in Sugita and Sugiura (1996). *ORF* Open reading frame, *PS I* photosystem I, *PS II* photosystem II, *RuBisCo* ribulose bisphosphate carboxylase

| 5′UTR | Length | Start/stop | Gene function | Gene features | % A+U |
|---|---|---|---|---|---|
| *atpI* | 171 | –155 to +16 | ATP synthase $CF_o$-IV | Second in penta-genic cluster | 75 |
| *atpA*[a] | 77 | –54 to +21 | ATP synthase $CF_1$-α | Last in penta-genic cluster | 74 |
| *atpB* | 123 | –100 to +23 | ATP synthase $CF_1$-β | First in di-genic cluster | 77 |
| *atpE* | 159 | –140 to +19 | ATP synthase $CF_1$-ϵ | Second in di-genic cluster, 5′ end overlaps with *atpB* ORF | 61 |
| *atpF*[a] | 90 | –16 to +30 | ATP synthase $CF_o$-IV | Encodes group II intron, fourth in penta-genic cluster | 76 |
| *atpH*[a] | 75 | –54 to +21 | ATP synthase $CF_o$-III | Third in penta-genic cluster | 72 |
| *clpP* | 165 | –150 to +15 | Protease | Encodes group II intron, first in tri-genic cluster | 79 |
| *infA* | 172 | –154 to +18 | Translation initiation factor | Ninth in dodeca-genic cluster | 66 |
| *ndhD* | 129 | –119 to +10 | NADH dehydrogenase subunit ND4 | Start codon edited ACG to AUG, second in di-genic cluster | 66 |
| *petL* | 165 | –150 to +15 | Cytochrome b/f 3.5-kDa subunit | First in di-genic cluster | 74 |
| *psaC* | 177 | –156 to +21 | PS I 9-kDa protein | First in di-genic cluster | 71 |
| *psbA* (short[b]) | 90 | –87 to +3 | PS II 32-kDa protein | Mono-genic | 63 |
| *psbA* (long) | 176 | –173 to +3 | PS II 32-kDa protein | Mono-genic | 65 |
| *rbcL* | 171 | –150 to +21 | Large subunit, RuBisCo | Mono-genic | 72 |
| *rpl22* | 103 | –90 to +14 | Large ribosome subunit protein 22 | Fourth in dodeca-genic cluster | 79 |
| *rpoA* | 168 | –150 to +18 | RNA polymerase α subunit | Last in dodeca-genic cluster | 65 |
| *rpoB* | 165 | –150 to +15 | RNA polymerase β subunit | First in tri-genic cluster | 70 |
| *rps2* | 153 | –145 to +8 | Small ribosome subunit protein 2 | First in penta-genic cluster | 70 |
| *rps11* | 184 | –166 to +18 | Small ribosome subunit protein 11 | Eleventh in dodeca-genic cluster | 75 |

[a] Binding properties of these 5′UTRs were reported by Hotchkiss and Hollingsworth (1999)
[b] Normal length of 5′UTR in vivo (Kim and Mullet 1994)

complexes also bound most (12/14) of the other 5′UTRs examined, although not polyU RNA and only very poorly to polyA RNA. In contrast polypeptide involved in the slower of the two ATP synthase 5′UTR–polypeptide complexes could bind all but one of the RNAs examined and bound to both polyA and polyU. Thus the RNA-binding proteins in the faster complex are candidates for 5′UTR-specific factors that could be used to coordinate expression of a wide range of chloroplast genes.

## Materials and methods

### Plant material

Spinach (*Spinacia oleracea* Bloomsdale longstanding) seeds were germinated in vermiculite and transferred to hydroponic tanks. Healthy, mature leaves approximately 8–10 cm in length were used in this study.

### Generation of templates for RNA transcription

DNA templates for in vitro transcription of unlabeled and uniformly radiolabeled RNA substrates were prepared by polymerase chain reaction as described by Hotchkiss and Hollingsworth (1999). Oligonucleotides used in the amplification reactions are given below. All oligonucleotides were purchased from the CAMBI Nucleic Acids Facility at SUNY Buffalo. Sequences are written 5′ to 3′. The first sequence of a pair is the 5′ primer, which begins with a T7 RNA polymerase promoter (italics), followed by a sequence identical to the 5′ end of the sequence to be amplified. The second is the primer complementary to the 3′ end of the sequence to be amplified: *atpI*: (1) *TAA TAC GAC TCA CTA TAG GG*G ATT TTG AAT CTC AAA AAC T, (2) ATG ATA GAA CAT TCA TAT TGT CCT C, *atpB*: (1) *TAA TAC GAC TCA CTA TAG GG*T ATT TAA TTA ATC GAT CAG CT, (2) GAA GTA GTA GGA TTG ATT CTC AT, *atpE*: (1) *TAA TAC GAC TCA CTA TAG GG*C AGA AAC AAT TAG AGG GT, (2) ACA CAA AGA TTT AAG GTC AT, *clpP*: (1) *TAA TAC GAC TCA CTA TAG GG*T TTA TTT ATT CTG TCT TCT TA, (2) AAC ACC AAT AGG CAT TAA, *infA*: (1) *TAA TAC GAC TCA CTA TAG GG*A ATT GGC GGA G, (2) CCA TTT TTG TTC TTT CAT, *ndhD*: (1) *TAA TAC GAC TCA CTA TAG GG*T CCA TTT GAA TCC ATA TTA T, (2) AAG AAT TCA TGA TAA AGA CAA, *petL*: (1) *TAA TAC GAC TCA CTA TAG GG*T GAC ATC TAT AAT GTA ATA GTT AAT, (2) AGT TAG AGT AGA CAT GAA GGA, *psaC*: (1) *TAA TAC GAC TCA CTA TAG GG*T ATT TTG GAC CTC TTT TC, (2) AAT CTT AAC TGA ATG TGA CA, *psbA*: (1) *TAA TAC GAC TCA CTA TAG GG*A ATA ACA ATC TTT CAA TTT C, (2, short) CAT GGT AAA ATC TTG GTT TA, *psbA*: (1) *TAA TAC GAC TCA CTA TAG GG*C AAT TCA CTT CCA TTA TTC A, (2, long) CAT GGT AAA ATC TTG GTT TA, *rbcL*: (1) *TAA TAC GAC TCA CTA TAG GG*T ATT AAC GAA CCA TTT TGA, (2) AGT CTC TGT TTG TGG TGA, *rpl22*: (1) *TAA TAC GAC TCA CTA TAG GG*A TAA GTC TCG TCG TTA AGT T, (2) TTA AAA AAC CCC ATA AAG T, *rpoA*: (1) *TAA TAC GAC TCA CTA TAG GG*A TAC TAT TAA GTT TCG TGC G, (2) TAT TTT CTC TCG AAC CAT, *rpoB*: (1) *TAA TAC GAC TCA CTA TAG GG*T AAT ACA TCC CAT ATA TGG AG, (2) TCC ATC CCG TAG CA, *rps2*: (1) *TAA TAC GAC TCA CTA TAG GG*T AAC AAA TAG AAA GGA ATT A, (2) CTT GTC ATT TCT CCC CA, *rps11*: (1) *TAA TAC GAC TCA CTA TAG GG*A ACA ACG ACA AGG ATA ATA AT, (2) TGG TAT AGG TTT TGC CAT.

Transcript sizes, their location relative to the start of translation, gene function, and gene features are reported in Table 1.

### In vitro transcription, isolation, and quantitation of RNAs

Uniformly radiolabeled RNAs were generated by in vitro transcription with $\alpha$-$^{32}$P-UTP (Green and Hollingsworth 1992). RNAs were purified using either spin columns (Maniatis et al. 1982) or gel purification from 5% polyacrylamide/7 M urea gels. For the latter, an excised gel fragment was crushed in 0.5 M NaCl 20 mM EDTA, 100 mM Tris-HCl, pH 8, and the RNA eluted. Radiolabeled RNAs were quantified by scintillation counting (counts per minute per mole), and normalized to the specific activity of the UTP and the number of Us in the transcript. Unlabeled RNAs were quantified by comparison with standards stained with ethidium bromide in a polyacrylamide/urea gel using GelMeasure software (Tim Heuser, available upon request) or by absorbance at 260 nm. Molar concentrations of polyA and polyU RNA (Sigma, St. Louis, Mo.) and yeast RNA (Roche, Indianapolis, Ind.) were determined by absorbance and normalized to the average length of the RNAs in the mixture, as reported by the suppliers.

### Chloroplast soluble-protein extract

Soluble-protein extracts were prepared from spinach chloroplasts as described by Hotchkiss and Hollingsworth ( 1999). The protein concentration of the resulting fractions was determined by a Bradford assay (Sigma).

### Gel mobility shift assays

Gel mobility shift assays were essentially as described by Hotchkiss and Hollingsworth ( 1999), with a few minor modifications. In each reaction, 10 fmol radiolabeled RNA were mixed with unlabeled specific competitor RNA (from 0- to 10,000-fold excess). A mixture consisting of 10 μg chloroplast extract and 10 μg yeast RNA in 10 mM Hepes (pH 7.9), 10 mM $MgCl_2$, 40 mM KCl, 0.05 mM EDTA, 3 mM dithiothreitol, and 9% glycerol was added to the specific RNAs for a total final volume of 10 μl. After incubation for 10 min at 22 °C, the mixture was subjected to electrophoresis at 375 V, 4 °C, for 6–10 hours in 0.5× TBE (500 mM Tris, 500 mM boric acid, 10 mM EDTA). The 10-min incubation time was sufficient for the binding reaction to reach equilibrium.

## Results

Our original experiments to assess the formation of RNA-protein complexes in spinach chloroplast extracts found that these complexes form in the 5′UTRs of *atpI*, *atpH*, and *atpA* (Hotchkiss and Hollingsworth 1995; Hotchkiss and Hollingsworth 1999). These three 5′UTRs also compete with each other for binding by chloroplast proteins. Although *atpF* 5′UTR-protein complexes were not detected in those experiments, the *atpF* 5′UTR did compete with the *atpI* 5′UTR for protein binding, although with lower efficiency than the others. This implied that at least some, if not all, of the binding proteins interacted with all four 5′UTRs. In contrast to competition with 5′UTR-derived RNAs, an interior fragment from the *atpH* open reading frame competed for only the slower-mobility complex of the two *atpI* 5′UTR-protein complexes. Thus, the binding protein(s) involved in the faster-mobility complex appeared to be specific for sequences and/or structures found in all four of the ATP synthase 5′UTRs.

The question then became whether these binding proteins were specific for ATP synthase 5′UTRs or whether their binding substrates were more universal. To address that question, competition assays were performed with 5′UTRs from a wide variety of chloroplast mRNAs. To simplify the analysis, the 5′UTR from *atpI* was used as the radiolabeled binding substrate in all the competition-binding assays. A variety of chloroplast 5′UTRs were analyzed for their ability to compete with the *atpI* 5′UTR for the binding proteins. They encoded additional subunits of ATP synthase (*atpB*, *atpE*), RNA polymerase subunits (*rpoA*, *rpoB*), ribosomal protein subunits (*rps2*, *rps11*, *rpl22*), photosystem components (*psaC*, *psbA*), a cytochrome b/f complex component (*petL*), an NADH dehydrogenase subunit (*ndhD*), a translation initiation factor (*infA*), a protease (*clpP*), and the large subunit of ribulose bisphosphate carboxylase (*rbcL*) (Sugiura 1989). Including those reported previously, we analyzed 18 5′UTRs for binding to chloroplast polypeptides (Table 1). Some of these are monocistronic (*psbA*, *rbcL*, *clpP*), while the rest are co-transcribed with other(s) as part of a multigenic cluster (Sugita and Sugiura 1996). Some have increased transcript abundance in response to light (*psbA*, *rbcL*), while the transcript abundance of others does not alter in response to light (*atpI*, *H*, *F*, *A*, *B*, *E*) (Chun et al. 2001; Dubell and Mullet 1995; Green and Hollingsworth 1992; Klein and Mullet 1990; Mayfield et al. 1995). Two of these 5′UTRs are upstream of genes that encode group II introns (*clpP*, *atpF*). Every competition reaction also contained $10^4$-fold excess yeast RNA as a non-specific competitor. Since many chloroplast 5′UTRs contain several stretches of A- and/or U-rich sequences, we also performed competition analyses with polyA and polyU RNAs.

Each of the 5′UTRs was first analyzed by electrophoretic mobility shift assay (band-shift assay) to determine whether it was specifically bound by chloroplast polypeptides. Competition with unlabeled RNA identical to the radiolabeled binding substrate (i.e., self-competition) was performed to determine whether the binding was specific. The binding capacity of the extract always exceeded the quantity of radiolabel, requiring a minimum of 1,000-fold excess of self-competitor to completely compete with the radiolabeled RNA. As judged by the quantity of unlabeled RNA required for complete self-competition, it appeared that the chloroplast extracts used in these experiments had a similar binding capacity, within a factor of five, for each of the 5′UTRs. All the 5′UTRs could form specific complexes that were stable enough to be detected by band-shift assay (Fig. 1). However, although shifted bands could be detected for the spinach chloroplast *psbA* 5′UTR, they were neither intense nor entirely reproducible. Experiments from the laboratories of Christopher and Klaff have shown that, for optimal detection, the *psbA* 5′UTR–protein complexes must be stabilized via ultraviolet cross-linking prior to analysis (Klaff and Gruissem 1995; Shen et al. 2001). Because none of the 5′UTRs from the large ATP synthase gene cluster can be covalently bound to their binding proteins under standard UV-crosslinking conditions (J. Johnson, data not shown), the assays used here did not involve cross-linking. This is probably the reason for the less distinct signals for the *psbA* 5′UTR–protein complexes.

Once binding of the various 5′UTRs was established, competition-binding analysis was performed to establish whether the 5′UTRs were binding the same or different proteins as the *atpI* 5′UTR. For each analysis, 10 fmol of radiolabeled *atpI* 5′UTR was mixed with increasing concentrations (from 0- to 10,000-fold excess) of a single competitor, incubated with chloroplast extracts and subjected to band-shift analysis. The results from these experiments are shown in Fig. 2.

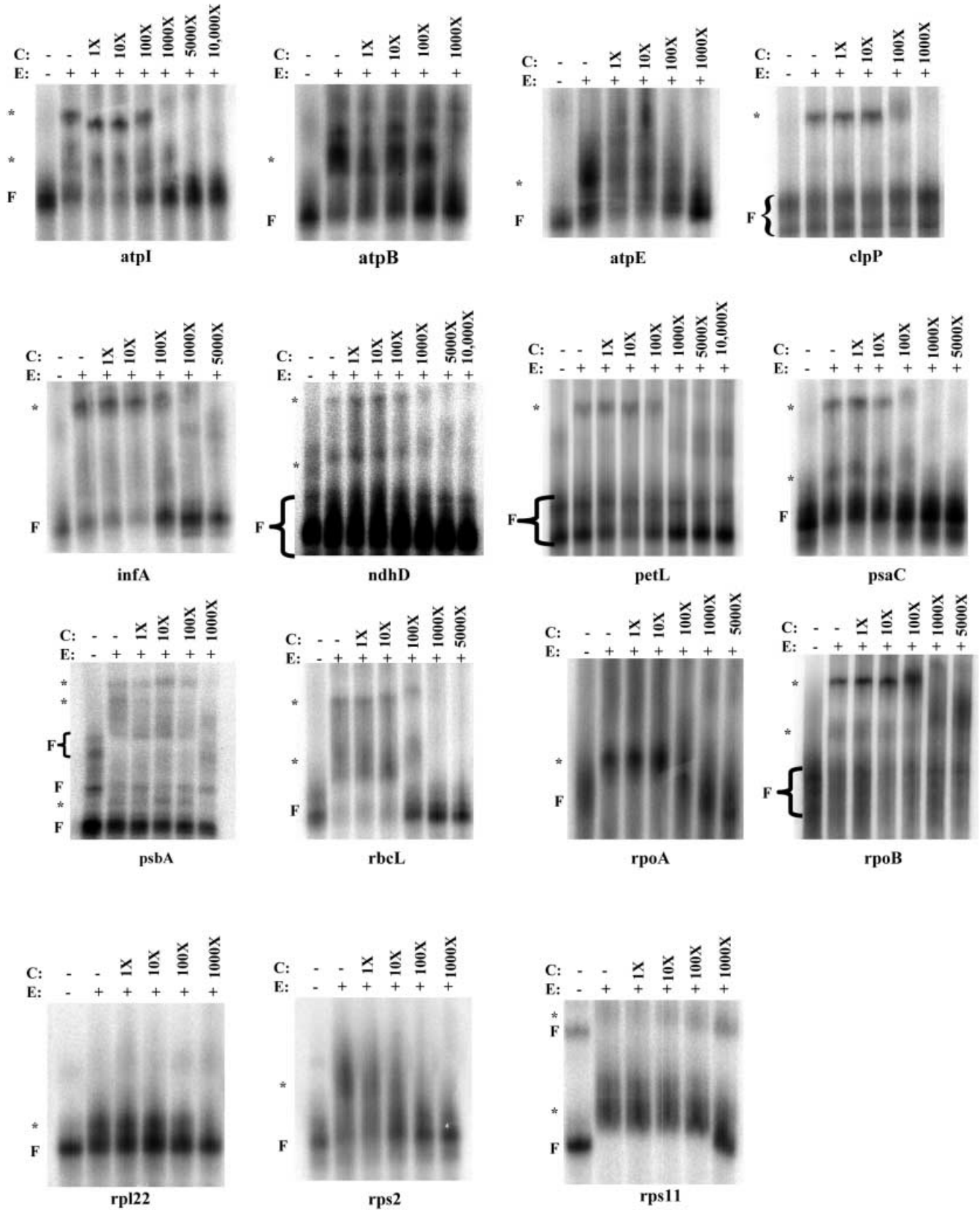
The various 5′UTRs displayed a 10-fold range of quantity required to completely compete with the *atpI* 5′UTR (Fig. 2). Six (*clpP*, *petL*, *psaC*, *rbcL*, *rpoB*, *rps11*) competed with *atpI* for complex formation at 1,000-fold excess, which was at the same fold-excess required for *atpI* self-competition. Five others (*atpE*, *infA*, *ndhD*, *rpoA*, *rps2*) required 5,000-fold excess to completely compete with *atpI* for binding, while one (*rpl22*) required 10,000-fold excess. Competition analysis with *atpB* 5′UTR as competitor resulted in a supershift, probably due to complementarity between *atpB* and *atpI* 5′UTRs (see Discussion). Only *psbA* could not completely compete with *atpI* 5′UTR at a concentration10-fold higher than that required for *atpI* self-competition.

The *psbA* competition shown in Fig. 2 was performed with a transcript that began at the same 5′ end as that found in vivo for *psbA* mRNAs (Kim and Mullet 1994). That particular RNA is only 90 nt, while *atpI* is 171 nt. Although we have previously shown efficient competition with RNAs as short as 75 nt (*atpH*; Table 1; Hotchkiss and Hollingsworth 1999), we were concerned that the lack of competition could be a length-related phenomenon. To test that, the *psbA* competition experiments were repeated with a 176-nt RNA which included the original competitor sequence, but began 86 nt upstream. The longer transcript was even less successful than the original in competing with *atpI* for binding (data not shown).

Sequence alignment of the 5′UTRs that could compete for complex formation revealed no obvious primary structure conservation (Thompson et al. 1994). However, the 5′UTRs are quite A + U-rich (61–79%), and each

►

**Fig. 1** Proteins in a spinach chloroplast extract bind to spinach chloroplast 5′UTRs. Uniformly radiolabeled 5′UTRs were incubated in the presence or absence of spinach chloroplast extracts with or without unlabeled specific competitor (identical to the binding substrate) and subjected to band-shift analysis. The identity of each 5′UTR is noted *below the image*. *F* Designates the mobility of the unbound RNA(s); and multiple free RNA species are caused by different conformations of the 5′UTR. * Designates the mobility of the RNA–protein complex(es). *E* Denotes the presence (+) or absence (–) of extract in the incubation. *C* Denotes the absence (–) or presence (*x*-fold excess shown *above each lane*) of unlabeled self-competitor in the binding reaction


C: - - 1X 10X 100X 1000X 5000X 10,000X
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atpI
ndhD
rpl22
atpB
petL
rpoA
atpE
psaC
rpoB
clpP
psbA
rps2
infA
rbcL
rps11
F
C:
E:
1X
10X
100X
1000X
5000X
10,000X

◀

**Fig. 2** Competition-binding assays of various 5′UTRs against the *atpI* 5′UTR in spinach chloroplast extracts. Uniformly radiolabeled *atpI* 5′UTR was incubated in the presence or absence of spinach chloroplast extracts, with or without unlabeled competitor RNA, and subjected to band-shift analysis. The identity of each competitor RNA is noted *beneath the image*. *F* Designates the mobility of the unbound *atpI* 5′UTR. * Designates the mobility of the *atpI* 5′UTR–protein complexes. *E* Denotes the presence (+) or absence (–) of extract in the incubation. *C* Denotes the absence (–) or presence (*x*-fold excess shown *above each lane*) of unlabeled competitor in the binding reaction

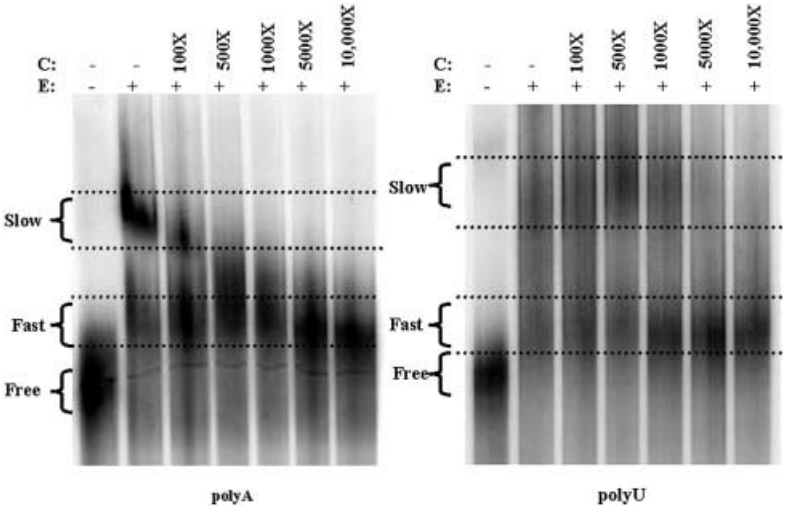
of them has long stretches of A + U sequences and/or polyA or polyU sequences (Table 1). There are many examples demonstrating that A + U-rich regions have important roles as *cis*-acting elements in chloroplast 5′UTR–protein complexes (see Discussion). To test the importance of A/U regions in the formation of these complexes, competition-binding analysis was performed using polyA or polyU RNA to compete with the *atpI* 5′UTR for binding (Fig. 3). PolyA and polyU RNAs efficiently competed for proteins involved in the slower migrating of the two complexes, but not for the faster. Even at 10,000-fold excess, polyU did not compete for proteins involved in the faster complex, while polyA RNA was able to compete for only ca. 25% of them.

## Discussion

It is apparent from these data that chloroplast proteins bind to the 5′UTRs of many spinach chloroplast mRNAs. One or more of the proteins involved in these complexes must be in common for most of the them, since all but two of the 5′UTRs that we have examined thus far could compete with reasonable efficiency with the *atpI* 5′UTR for complex formation. There was a reproducible and RNA-specific amount of competitor required for each particular 5′UTR to completely compete with the *atpI* 5′UTR, from the same as to 10-fold more than *atpI* self-competition. Until the binding proteins are identified and binding analysis is performed with purified polypeptides, it is difficult to predict why a range of competitor concentrations was observed. Regardless, the fact that most of the 5′UTRs analyzed do compete with the *atpI* 5′UTR for complex formation strongly supports the idea that at least some of the proteins in the complexes are the same.

Two *atpI* 5′UTR–polypeptide complexes were detected in these assays. The ratio of these two complexes varies, depending upon the extract preparation (e.g., compare *atpI* complexes in the *petL* and *psaC* competitions in Fig. 2). Previously reported competition analysis with a more narrow range of competitor concentrations than that shown in Fig. 2 supported the idea that the slower mobility (larger) complex is derived from the faster (smaller) complex (Hotchkiss and Hollingsworth 1999). We hypothesize that the varying ratios of the two complexes in different preparations is due to varying concentrations of the polypeptide(s) that associate with the faster complex to produce the slower. The slower mobility complex appears to be markedly less specific than the faster. In an earlier study, we showed that a fragment internal to the *atpH* open reading frame, corresponding to the 5′ end of an RNA that is abundant in vivo, competes for the slower-mobility complex, but not the faster (Hotchkiss and Hollingsworth 1999). In addition, polyA and polyU both compete for the slower-mobility complex but inefficiently, if at all, for the faster (Fig. 3). In contrast, the faster-mobility complex is more specific, since its binding to *atpI* 5′UTR can be competed only with other 5′UTRs. The proteins involved in the faster complex must require

**Fig. 3** PolyA and polyU RNAs compete only for formation of the slower-mobility *atpI* 5′UTR–protein complex. Binding reactions of radiolabeled *atpI* 5′UTR in spinach chloroplast extracts were performed in the presence of increasing excess of polyA or polyU RNA. All binding reactions also contained 10,000-fold excess of yeast RNA. The identity of each competitor RNA is noted beneath the image. *Free* Designates the mobility of the unbound *atpI* 5′UTR. *Slow*, *Fast* Designate the relative mobilities of the *atpI* 5′UTR–protein complexes. *E* Denotes the presence (+) or absence (–) of extract in the incubation. *C* Denotes the absence (–) or presence (*x*-fold excess shown *above the lane*) of polyA or polyU in the binding reaction



some *cis*-acting element that most of the chloroplast 5′UTRs analyzed thus far have in common.

The *atpI* 5′UTR was chosen as the baseline ATP synthase 5′UTR for these studies. This is because the *atpI*, *atpH*, and *atpA* 5′UTRs all compete with each other for binding, implying that at least some of their binding proteins are shared. Furthermore, affinity of the *atpI* 5′UTR for the binding proteins and the stability of the complex formed were similar to that for *atpH* and *atpA* 5′UTRs (Hotchkiss and Hollingsworth 1999). Thus, RNAs that can compete with the *atpI* 5′UTR for complex formation are likely to also compete with *atpH* and *atpA* 5′UTRs.

The 5′UTRs examined in this and our previous study represent a wide range of different types of chloroplast genes encoding a variety of chloroplast functions. The genes include those that function in reaction centers (*psaC*, *psbA*), electron transport (*petL*), RNA polymerase (*rpoA*, *rpoB*), ATP synthase (*atpA*, *atpB*, *atpE*, *atpF*, *atpH*, *atpI*), ribosomal subunits (*rps2*, *rps11*, *rpl22*), carbon fixation (*rbcL*), translation (*infA*), redox reactions (*ndhD*), and protein degradation (*clpP*; Table 1). The fact that all but two of these 5′UTRs can efficiently compete for binding supports the idea that these 5′UTR–protein complexes may have some kind of near-universal function in the chloroplast.

One of the two less-efficient competitor 5′UTRs was *atpB*, which caused a supershift when incubated with *atpI* in spinach chloroplast extracts. The *atpB* 5′UTR has a 30-nt region with 77% complementarity to the *atpI* 5′UTR. That region is flanked by two additional 22-nt regions of 64–69% complementarity to the *atpI* 5′UTR. It is probable that the supershifts detected in the competition assays of *atpB* against *atpI* are due to base-pairing between their complementary regions at the low temperatures used for the binding (22 °C) and electrophoresis (4 °C). Thus, we cannot rule out the possibility that *atpB* and *atpI* 5′UTRs can be bound by the same proteins.

The only poor competitor that could not be explained by complementarity between it and the binding substrate was the 5′UTR of *psbA*. The other 5′UTRs analyzed completely competed with *atpI* for binding when present in 5,000- to 10,000-fold excess. In contrast, competition with the *psbA* 5′UTR was less efficient, competing for a maximum of 75% of the faster complex when in 10,000-fold excess. Thus, these 5′UTR binding proteins bind *psbA* 5′UTR at reduced efficiency compared with the others examined. It is not entirely surprising that the *psbA* 5′UTR does not compete efficiently with the *atpI* 5′UTR for binding by chloroplast polypeptides. The product of the *psbA* gene, the D1 subunit of the photosystem II reaction center, exhibits an extremely fast turnover at the polypeptide level (Edelman and Reisfeld 1980; Kyle et al. 1984). Regardless of the function of the 5′UTR–protein complexes, it would not be completely unexpected to discover that *psbA*, with its strong protein-turnover regulatory component, is not so strongly influenced by whatever near-universal function these complexes may mediate. However, proteins do bind the *psbA* 5′UTR in spinach, tobacco, *Arabidopsis*, and *Chlamydomonas* chloroplasts (Alexander et al. 1998; Fong et al. 2000; Hirose and Sugiura 1996; Klaff and Gruissem 1995; Shen et al. 2001; Yohn et al. 1998a, b). Perhaps there are some *psbA* 5′UTR-binding polypeptides that are unique to that transcript.

Chloroplast 5′UTRs have a high percentage A + U and often have uninterrupted runs of five or more As or Us. A/U-rich sequences are found in the regulatory regions of many chloroplast 5′UTRs. For example, Hirose and Sugiura (1996) showed that A + U-rich sequences affect translation in tobacco chloroplast extracts. In addition, Eibl and colleagues (1999) have shown that an A + U-rich putative stem-loop structure derived from the *psbA* 5′UTR affects translation of a reporter gene in transformed tobacco chloroplasts. There are also examples of A/U-specific binding proteins that bind chloroplast 5′UTRs. Klaff and colleagues have found that the ribosomal subunit S1 protein binds U-rich regions of the *psbA* 5′UTR in spinach chloroplasts (Alexander et al. 1998). Mayfield and colleagues have shown that expression of the *psbA* gene in *Chlamydomonas* is regulated via a redox-controlled mechanism that requires the binding of a polyA binding protein to a polyA region in the mRNA 5′UTR (Fong et al. 2000). To begin to examine the importance of A/U in the formation of these complexes, competition analysis was performed with polyA and polyU RNAs (Fig. 3). Only the slower of the two *atpI* 5′UTR–protein complexes was efficiently competed. PolyU did not compete for proteins involved in the faster complex at all, while polyA competed for those proteins only at the highest excess (10,000-fold) and, even then, inefficiently ($\leq 25\%$; Fig. 3). Thus, it does not appear that A + U-rich regions as primary sequence elements are important recognition signals for binding. However, A + U-rich regions are often involved in extensive secondary structure. There are many secondary structures that can be predicted in these 5′UTRs via modeling programs (Mathews et al. 1998). Although all of the 5′UTRs are predicted to form extensive and stable secondary structures, there are no obvious putative structures that these UTRs share. Experiments are currently in progress to investigate the importance of primary and secondary structural *cis*-acting elements in the formation of 5′UTR–protein complexes.

Given the almost-complete functional identification of the open reading frames in the chloroplast genome, it is likely that these 5′UTR binding proteins are nucleus-encoded (Sugiura 1989). Nucleus-encoded 5′UTR binding proteins have been detected for many algal and land-plant chloroplast transcripts (reviewed by Barkan and Goldschmidt-Clermont 2000). In *Chlamydomonas*, these proteins appear transcript-specific, with a unique set binding to each 5′UTR (Barkan and Goldschmidt-Clermont 2000; Chen et al. 1997; Hauser et al. 1996; Hong and Spreitzer 1998; Kim and Mayfield 1997; Stampacchia et al. 1997; Wu and Kuchka 1995; Yohn

et al. 1998b; Zerges and Rochaix 1994; Zerges et al. 1997). Nucleus-encoded 5′UTR-specific binding proteins have also been discovered in spinach, tobacco, and amaranth chloroplasts (Alexander et al. 1998; Hirose and Sugiura 1996; Klaff and Gruissem 1995; McCormac and Barkan 1999). Despite the plethora of binding data, most binding proteins have not yet been identified (Barkan and Goldschmidt-Clermont 2000).

The widespread presence of these 5′UTR–protein complexes and the involvement of one or more shared proteins imply that the formation of these complexes may mediate chloroplast-wide effects on gene expression. However, the fact that these specific complexes form does not in itself provide us with any information about their function. In both land-plant and algal chloroplasts, combinations of genetic and biochemical studies have revealed that 5′UTR–protein complexes affect some combination of translation and RNA stability (reviewed by Barkan and Goldschmidt-Clermont 2000; Zerges 2000). Experiments in vivo and in vitro have shown that 5′UTRs affect translation of tobacco chloroplast RNAs (Eibl et al. 1999; Hirose and Sugiura 1996; Hirose et al. 1998). Preliminary data from in vivo transformation experiments support the idea that this particular set of complexes may affect RNA stability (Sneddon, Allison, and Hollingsworth, unpublished data). Experiments to probe the function of these complexes are on-going.

Regardless of the outcome of in vivo reporter experiments and in vitro assays, the function of these complexes will need to be unequivocally verified by genetic experiments in which the genes encoding the binding proteins are identified and mutated. Experiments are currently in progress to isolate and identify these 5′UTR binding proteins. Once identified and cloned, the effects of alterations in their gene expression on chloroplast function and biogenesis can be more thoroughly explored.

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