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ATP synthase 5′ untranslated regions are specifically bound by chloroplast polypeptides

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Abstract Expression of the large ATP synthase gene cluster in spinach (*Spinacia oleracea*) chloroplasts is regulated at the post-transcriptional level. RNA stability and the translational efficiency of some chloroplast transcripts have been shown to be regulated through RNA-protein interactions in the 5′ untranslated region (5′ UTR). In this report we show that spinach chloroplast extracts contain polypeptides that specifically interact with the 5′ UTRs of three of the four genes in the large ATP synthase gene cluster. A subset of binding polypeptides may be gene-specific, although at least one appears to be a more general chloroplast RNA-binding protein. We hypothesize that these RNA-protein interactions may affect the expression of this gene cluster from two perspectives. The first would be at a gene-specific level, which could serve to control the stoichiometry of ATP synthase subunits. The second would be a more global effect, which may adjust the abundance of the entire ATP synthase complex in response to environmental or developmental cues.

Key words ATP synthase · Chloroplast · RNA-binding proteins · Translation

Introduction

Multi-subunit complexes in chloroplasts and mitochondria are generally encoded by a combination of nuclear and organellar genes. Thus, efficient organelle biogenesis and function require the coordinated expression of both genetic systems. (Ellis 1984; Herrmann et al. 1992; Barkan et al. 1995). The mechanisms by which eukaryotes coordinate the expression of nuclear and organellar genes are long-standing and fundamental questions in eukaryotic molecular biology.

The proton-translocating ATP synthase/ATPase is a multi-subunit complex ubiquitous and essential to the function of all chloroplasts, mitochondria and prokaryotes (Strotmann and Bickel-Sandkotter 1984; Merchant and Selman 1985; McCarty et al. 1988; Ort and Oxborough 1992). In the chloroplast, the ATP synthase holo-complex is composed of two sub-complexes, CF_0 and CF_1. CF_0 has four subunits (I–IV) and is embedded within the thylakoid membrane, where it mediates the transfer of protons, generated by photosynthesis, across the membrane. CF_1 is composed of five subunits (α–ε) and uses the energy generated by proton translocation through CF_0 to phosphorylate ADP (Strotmann and Bickel-Sandkotter 1984; Merchant and Selman 1985; McCarty et al. 1988). The nine individual subunits of this complex are encoded by six chloroplast and three nuclear genes, thus providing a model for the study of coordination of nuclear and organellar gene expression (Hennig and Herrmann 1986; Hudson and Mason 1988; Sugiura 1989).

Research in our laboratory has focused on expression of the large ATP synthase gene cluster in spinach chloroplasts. This cluster encodes, in order, the small ribosomal protein subunit 2 (rps2), and four ATP synthase subunits: CF_0-IV (*atp*I), CF_0-III (*atp*H), CF_0-I (*atp*F) and CF_1-α (*atp*A) (see Fig. 1) (Hudson et al. 1987; Stollar and Hollingsworth 1994; for nomenclature see Hallick and Bottomley 1983). Transcription of this cluster originates upstream of both *rps*2 and *atp*H, terminating downstream from *atp*A (Hudson et al. 1987; Stollar and Hollingsworth 1994). The two primary transcripts are extensively processed, resulting in more than 30 mRNAs, all of which are bound by polysomes. (Stollar and Hollingsworth 1994, Stollar et al. 1994). After processing, the stoichiometry of complete, and therefore potentially translation competent, open reading frames (ORFs) within the total mono- and

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poly-cistronic ATP synthase RNA population becomes 2:11:3:1 (Hotchkiss and Hollingsworth 1997). In contrast, the ATP synthase polypeptides encoded by these RNAs accumulate in a 1:6–12:1:3 ratio (Strotmann and Bickel-Sandkotter 1984, McCarty et al. 1988).

Many chloroplast polypeptides are translated in abundance and quickly turn over if they are unable to assemble into complexes (Gruissem and Tonkyn 1993). However, there are some exceptions to this rule and the ATP synthase polypeptides appear to be among them. In wild-type rye and *Chlamydomonas* chloroplasts, CF_1 subunits exhibit little if any turnover during a 24-h chase period following labeling (Merchant and Selman 1984; Biekmann and Feierabend 1985). In addition, the pool of unassembled subunits is very small, less than 1% of the total subunits. Studies using *Chlamydomonas* mutants revealed that synthesis of α and β are closely coordinated at the translation, rather than the turnover, step (Drapier et al. 1992). These findings are consistent with translation of the ATP synthase subunits in stoichiometric ratios followed by quick assembly of the subunits into stable complexes. Chloroplast mutants in which the synthesis of one or more of the ATP synthase subunits is disrupted have a vastly reduced accumulation of the other subunits as well (Sears and Herrmann 1985; Ketchner et al. 1995; Fiedler et al. 1997). Thus it appears that, like the subunits of other chloroplast complexes, ATP synthase subunits are unstable if unassembled. Given the difference between the stoichiometries of the ATP synthase ORFs and polypeptides, and the apparent synthesis of these subunits in correct ratios for the formation of the holo-complex, we hypothesize that regulation of the expression of this gene cluster has a significant translational component.

Expression of many chloroplast genes has been shown to be translationally regulated (Danon and Mayfield 1994; Gillham et al. 1994; Sugita and Sugiura 1996; Kloppstech 1997; Stampacchia et al. 1997). Regulation of translational initiation has been shown to be mediated by 5′ untranslated region-polypeptide interactions in chloroplasts from *Chlamydomonas* and tobacco. (Staub and Maliga 1994; Mayfield et al. 1995; Hirose and Sugiura 1996; Zerges et al. 1997; Yohn et al. 1998). We examined the ability of 5′ untranslated region-containing transcripts from the large ATP synthase gene cluster to interact with polypeptides in a chloroplast extract. We found that the chloroplast extract contains polypeptides that specifically interact with a subset of these transcripts. These polypeptides are candidates for translational and/or stability regulators of transcripts from the large ATP synthase gene cluster.

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 RNA probes. DNA templates for in vitro transcription were generated with the polymerase chain reaction (PCR). To facilitate subsequent transcription, 5′ primers for each reaction were synthesized with a T7 promoter immediately preceding the sequence to be amplified. Oligonucleotides used in the PCR reactions are as follows (sequences are 5′ → 3′):

*atp*I 5′ TAA TAC GAC TCA CTA TAG GGG ATT TTG AAT CTC AAA AAC T,
*atp*I 3′ ATG ATA GAA CAT TCA TAT TGT CCT C,
*atp*H 5′ TAA TAC GAC TCA CTA TAG GGG ATT GTA TCA TTA ACC ATT T,
*atp*H 3′ CCC CCC GGG GGC AGC AAT CAG TGG ATT CA,
atpH_i 5′ TAA TAC GAC TCA CTA TAG GGG ACA GCC CGA AGC AGA AGG A,
atpH_i 3′ GGA TTA AAC AAA AGG ATT GCG AAA T,
*atp*F 5′ TAA TAC GAC TCA CTA TAG GGG AAG TAG CAA ACA ATT GAA A,
*atp*F 3′ GAA AAC GAA AGA ATC GGT TA,
*atp*A 5′ TAA TAC GAC TCA CTA TAG GGA TTT TTT TGT TTC AAA AAA A,
*atp*A 3′ CGT TCA CGG ATA ATT TTG CTA ATT TC,
*psb*A 5′ TAA TAC GAC TCA CTA TAG GGA ATA ACA ATC TTT CAA TTT C,
*psb*A 3′ CAT GGT AAA ATC TTG GTT TA.

Template DNA for the ATP synthase PCR reactions was pJB6, which contains the entire ATP synthase gene cluster (Stollar et al. 1994). Template DNA for the *psb*A transcript was pMK1. The vector for pMK1 was *Eco*RV-digested pSK^+ (Stratagene). The *psb*A insert fragment in pMK1 was generated from reverse transcriptase-PCR of total spinach chloroplast RNA, using the *psb*A 5′ and 3′ primers listed above. The PCR products were phenol/chloroform-extracted, ethanol-precipitated, and resuspended in diethylpyrocarbonate-treated water. Uniformly radiolabeled RNA-binding substrates were generated by in vitro transcription with ^{32}P-UTP (Green and Hollingsworth 1992). Figure 1 is a map of the large ATP synthase gene cluster and shows the location of the binding substrates in the cluster. Transcript sizes are as follows: 170 nt for *atp*I, 73 nt for *atp*H, 127 nt for *atp*H_i, 109 nt for *atp*F, and 86 nt for *atp*A. The psbA transcripts were 93 nt in length. The *atp*I, *atp*H, *atp*H_i and *atp*A transcripts begin three bases upstream of a "natural" (i.e., found in vivo) 5′ end. The *atp*I transcript encompassed nucleotides from –155 through +15, where the first base of the open reading frame is +1. The *atp*H transcript encompassed nucleotides –53 to +73, while *atp*H_i went from +122 to +249, *atp*F from –79 to +30 and *atp*A from –40 to +47. The *atp*F transcript begins 309 bases downstream from its natural 5′ end. All five transcripts began with three non-chloroplast-encoded nucleotides because the T7 RNA polymerase initiates transcription within its own promoter at the first G of a G_3 sequence. The binding substrates also have a 5′ tri-phosphate rather than the mono-phosphate present in vivo, because they are primary transcripts of the T7 RNA polymerase.

Chloroplast soluble protein extract. Protein extracts were prepared as described by Gruissem and colleagues with a few minor modifi-

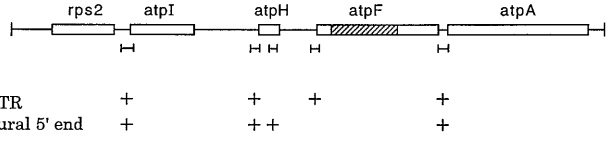


Fig. 1 Organization of the spinach plastid large ATP synthase gene cluster. Transcription begins upstream of *rps*2 and *atp*H and continues through *atp*A. Open reading frames are drawn as *open boxes*. The group-II intron interrupting *atp*F is shown as a *hatched box*. *Small horizontal lines* below the map designate the binding substrates. From left to right, they are *atp*I, *atp*H, *atp*H_i, *atp*F, and *atp*A. The presence of a 5′ UTR or a natural 5′ end within a binding substrate is noted below it

cations (Gruissem et al. 1986). Briefly, soluble extracts were obtained by passing a clarified chloroplast lysate over a DEAE-Sephadex column and precipitating to 60% saturation with ammonium sulfate. The precipitate was resuspended in 1 ml of buffer E (20 mM HEPES pH 7.9, 60 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, and 17% glycerol and passed over a heparin-agarose column (Sigma) (Gruissem et al. 1986). The flow-through from this column was collected and the protein concentration determined by a Coomassie assay (Pierce). Protein concentrations of 1–10 mg/ml were used for this study. One of the first steps in extract preparation is centrifugation at 150 000g. This is sufficient to remove all ribosomes and polysomes from the extract. However, individual ribosomal protein subunits could still be present.

Gel mobility shift assays. Gel mobility shift assays were essentially as described by Hsu-Ching and Stern (1991). Briefly, 10–30 μg of chloroplast extract was incubated in binding buffer (10 mM $MgCl_2$, 40 mM KCl, 10 mM HEPES pH 7.9, 0.05 mM EDTA, 3 mM DTT in 8.8% glycerol), 10 μg of yeast RNA (Boehringer Mannheim Biochemicals), and 10–30 fmol of radiolabeled RNA, in a total reaction volume of 12 μl. In reactions with competitor RNAs, unlabeled RNA was added to the radiolabeled RNA, prior to the addition of extract, at the molar excess noted in the figure legends. Reactions were incubated for 10 min at 25 °C and loaded onto non-denaturing 6% polyacrylamide gels (79:1 acrylamide:bis acrylamide), followed by electrophoresis at 300 V, 4 °C. Identical results were obtained if 10 μg of *Escherichia coli* tRNAs (Sigma) were used as non-specific competitors instead of total yeast RNA. After drying, gels were exposed to a Phosphorimager screen and analyzed with Molecular Dynamics software.

Proteinase K treatment. Extracts for protease sensitivity experiments were incubated at room temperature (RT) for 30 min with 2 U/μl of proteinase K (Boehringer Mannheim Biochemicals). Mock-treated extracts were incubated at RT without the addition of protease.

Results

RNA binding analysis

RNAs corresponding to the 5′ untranslated region (5′ UTR) of all four ATP synthase genes from the cluster (*atp*I, *atp*H, *atp*F, and *atp*A), as well as one RNA that begins within a coding region (designated *atp*H_i), were incubated individually in chloroplast extracts and examined by mobility shift analysis. Twenty-two 5′ ends have been identified in transcripts from the large ATP synthase gene cluster (Stollar et al. 1994). Three of the four 5′ UTR-containing transcripts examined in the binding assays, *atp*I, *atp*H and *atp*A, have 5′ ends within three bases of in vivo 5′ ends (see Materials and methods). In order to distinguish between the binding of 5′ end-specific polypeptides and those that recognize other sequences in the 5′ UTRs, an RNA that contained a coding sequence but also corresponded to an in vivo 5′ end, *atp*H_i, was also examined. The results of these binding assays are shown in Fig. 2. Variations in intensity of the various binding substrates in Fig. 2 are dependent upon their specific activity and exposure range. Each binding reaction has 10 fmol of substrate.

RNAs corresponding to *atp*I, *atp*H and *atp*A 5′ UTRs are bound by chloroplast polypeptides. In contrast, neither the *atp*F 5′ UTR nor the interior fragment from the *atp*H ORF, *atp*H_i, bound to polypeptides in the extract. The three RNAs that were bound in the extract had two features in common, 5′ UTRs and "natural" (i.e., similar to in vivo) 5′ ends. RNAs that had only a 5′ UTR (*atp*F) or a natural 5′ end but not a 5′ UTR (*atp*H_i) were not bound in the extract.

To determine whether the natural 5′ end was actually necessary for binding to these transcripts, RNAs beginning 20 nucleotides upstream of or downstream from the natural *atp*I 5′ end were assayed for their ability to bind polypeptides in the chloroplast extract. Both RNAs bound to polypeptides in the extract with a similar affinity to the original *atp*I transcript. In addition, both RNAs competed well against binding of the original *atp*I binding substrate, implying that the same polypeptides bound all three RNAs (Both and Hollingsworth, unpublished). Therefore a 5′ end corresponding closely to one found in vivo is not necessary for binding to polypeptides in this chloroplast extract.

There were multiple binding complexes observed for each of the RNAs (Fig. 2). The *atp*I binding assays revealed two complexes, with the faster mobility complex

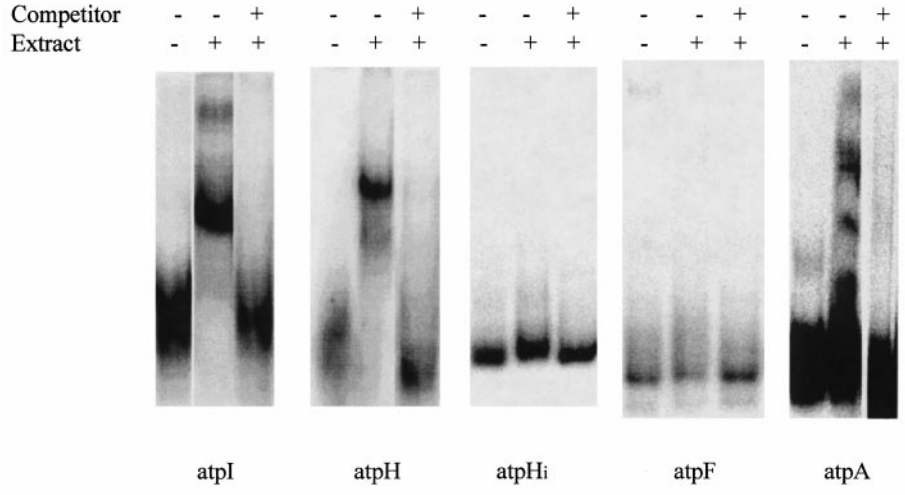
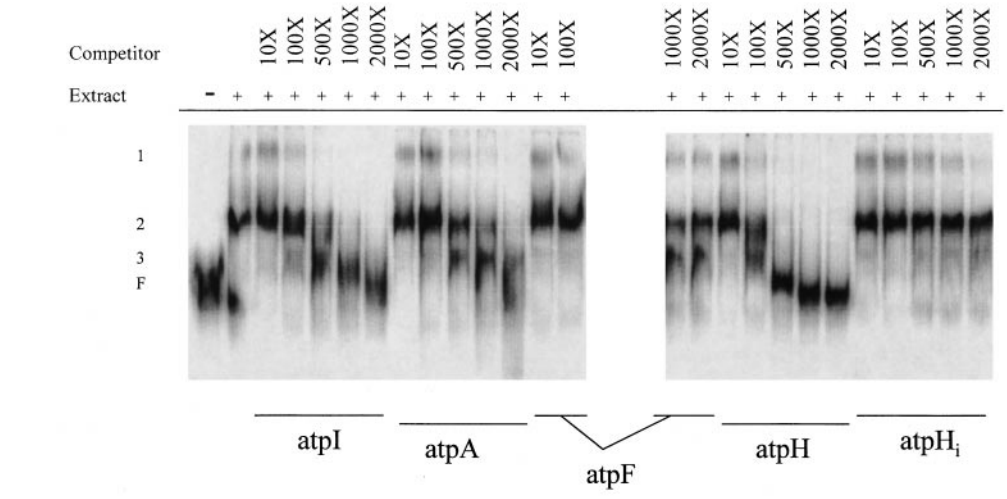
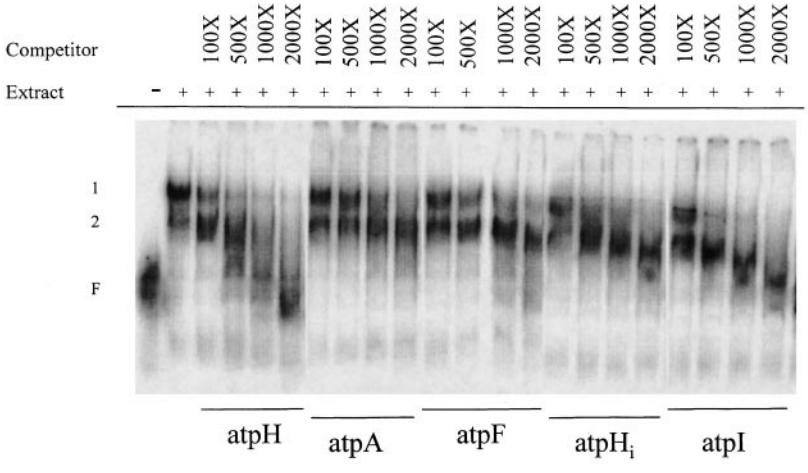


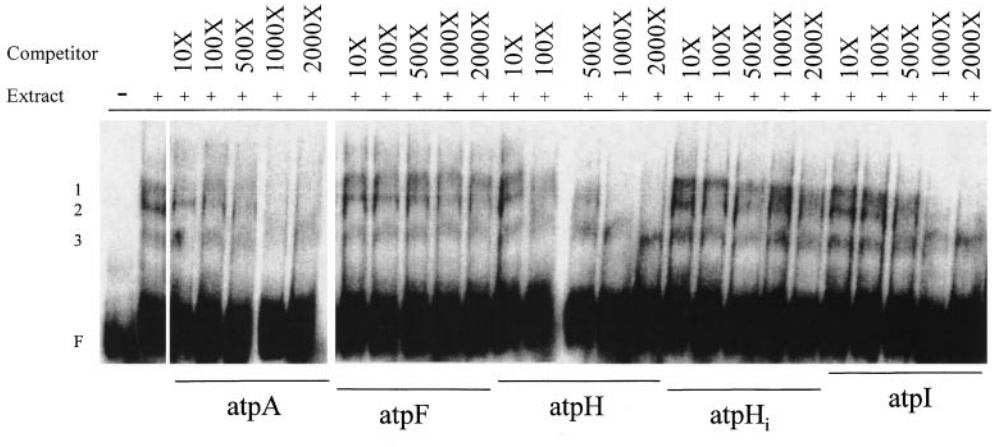
Fig. 2 Mobility shift assays of ATP synthase transcripts in a spinach chloroplast extract. Radiolabeled RNA (10 fmol) was incubated in a soluble chloroplast extract in the presence of a 10 000-fold excess of unlabeled total yeast RNA. Specific unlabeled competitor (1000-fold excess) was added as indicated. The specific competitor has precisely the same sequence as the radiolabeled binding substrate



a **Binding substrate: atpI**



b **Binding substrate: atpH**



c **Binding substrate: atpA**

Fig. 3a–c Competition assays. Ten femtomol of each UTR was incubated in the presence (+) or absence (–) of chloroplast extracts with a varying-fold excess (as noted) of unlabeled transcripts. The mixtures were subjected to gel mobility shift analysis. The identity of the competitor RNA is given below the lanes. The identity of the radiolabeled probes is: **a** *atp*I UTR; **b** *atp*H UTR; **c** *atp*A UTR. Each complex is identified by a *number* to the left of the image. Numbers were assigned in increasing order such that the slowest migrating complex is designated "*1*." *F* denotes the migration of the unbound RNA

distinctly more intense than the slower. The *atp*H transcript was also involved in two complexes, but of relatively similar (within an order of magnitude) intensities. The *atp*A transcripts formed three complexes, each within an order of magnitude of the same intensity as the others.

The multiple complexes observed to form on a given transcript may be unrelated and formed from entirely different proteins. Alternatively, the faster complexes could be subcomplexes of the slower. To test the latter possibility, titration experiments were performed, varying the concentration of either protein or RNA. First, varying quantities of extract were subjected to the binding assay in the presence of limiting (i.e., substoichiometric) RNA concentrations, to determine whether disappearance of one complex could be correlated with a proportionally greater signal from another complex. Increasing concentrations of binding extract (up to four-fold more than in the standard assay) did not reproducibly alter the relative ratios of the complexes (data not shown). We also discovered that complex formation under these conditions was an all-or-none phenomenon. When protein concentration was lowered to the point that one complex did not form, none of the complexes formed, implying that the protein(s) necessary for initiating complex formation are in limited quantities in the extract (data not shown). In contrast to the protein titration, RNA competition experiments (reported below) do reveal proportional increases in faster-migrating complexes in parallel with decreased signals from slower complexes. Thus it appears that the multiple binding complexes do have some polypeptides in common.

To determine whether the mobility shifts were protein-dependent, extracts were digested with proteinase K prior to binding analysis. This treatment destroyed the ability of the extracts to produce an RNA mobility shift (data not shown).

Competition experiments

Competition experiments were performed to determine the specificity of the polypeptide(s) binding the 5′ UTRs. In these experiments, complex formation with *atp*I, *atp*H, or *atp*A 5′ UTRs was challenged with increasing amounts of unlabeled competitor. Each set of competitions was performed with a single extract preparation. Although the amount of RNA bound per μg of protein varies in different extract preparations, the efficiency of one competitor as compared to the others was consistent between all the extract preparations.

Results of the competition assays against *atp*I, *atp*H, and *atp*A binding substrates are shown in Figs. 3A, B, and C, respectively. A summary of the competition results is given in Table 1.

atpI

All transcripts containing 5′ UTRs were able to compete, with varying degrees of efficiency, against *atp*I (Fig. 3a).

Table 1 Summary of competition analysis: The identity of the bound complexes being challenged is noted in the leftmost column. Competitor RNAs are designated on the top row. "+" designates complete competition with approximately the same-fold excess as the self-competition. "(+)" designates partial competition at a fold-excess that gave complete self-competition. "–" designates no competition

Complex	Competitor				
	*atp*I	*atp*H	*atp*H_i	*atp*F	*atp*A
*atp*I-1	+	+	(+)	(+)	+
*atp*I-2	+	+	–	(+)	+
*atp*H-1	+	+	(+)	(+)	(+)
*atp*H-2	+	+	–	–	–
*atp*A-1	+	+	–	–	+
*atp*A-2	+	+	–	–	+
*atp*A-3	–	–	–	–	+

A new complex, of faster mobility than the original two, was detected during the competition experiments and designated "3" (Fig. 3a). Complex 3 was clearly visible in competition against all of the competitors that contained a 5′ UTR sequence (*atp*I, *atp*H, *atp*F, and *atp*A). However, the kinetics of the competitions differed with the various competitors. Only *atp*H was able to compete against *atp*I, binding as efficiently as *atp*I itself. *atp*A was the next most efficient competitor against *atp*I, competing complexes 1 and 2 to complex 3. Competitions of complex 3 to free RNA by *atp*A occurred only at the highest fold-excess assayed. The *atp*F transcript was a less-efficient competitor than the others, able to only partially compete against the formation of complexes 1 and 2. The only competitor that did not contain a 5′ UTR sequence, *atp*H_i, competed poorly, and then only at the highest-fold excess, against binding of *atp*I.

atpH

Competition against *atp*H was most efficient with *atp*I, which successfully competed against both of the *atp*H complexes (Fig. 3b). We were surprised to discover that none of the other three transcripts competed efficiently against the binding of *atp*H. They were able to compete for the binding of factors involved only in the slower migrating complex (designated 1 in Fig. 3b), and then only at the highest (2000-fold) excess. As with the competition experiments against *atp*I, it appeared that some new complexes of faster mobility could be detected in these competition experiments. However, there was no one distinct complex formed. Instead, a series of subcomplexes of increasing mobility were detected.

atpA

In contrast to the *atp*I and *atp*H competition experiments, the only efficient and complete competitor against *atp*A-

binding was itself. *atp*H and *atp*I transcripts competed well against factors involved in the two slowest mobility *atp*A complexes (designated 1 and 2 in Fig. 3C), but not at all against complex 3. Neither *atp*F nor *atp*H_i transcripts competed for any of the factors involved in the three *atp*A complexes.

It was interesting that even though the *atp*A transcript was not bound as efficiently in the extract as *atp*I and *atp*H, *atp*A complex 3 could not be competed by *atp*I or *atp*H. Given the number of kinetic and thermodynamic factors involved in a typical protein:RNA interaction, there are many possible explanations for the less-efficient binding of *atp*A as compared to the other two binding substrates. However, the point of these competition assays was to determine whether any of the ATP synthase RNA:protein complexes have any polypeptides in common. Regardless of the relative affinities and specificities of these polypeptides, we conclude that many, but not all, of these polypeptides are shared by the various complexes.

Competition of psbA against atp*I binding*

To test how specific the binding activity is for ATP synthase transcripts, competition experiments were performed using a transcript corresponding to the 5′ UTR of spinach chloroplast *psb*A as a competitor against the binding of *atp*I. The *psb*A gene encodes the D1 protein of photosystem II in thylakoid membranes (Hasler et al. 1997; Kim and Mullet 1994). Only partial competition against the slowest *atp*I complex was observed, even at 2000-fold excess over the *atp*I binding substrate (Fig. 4).

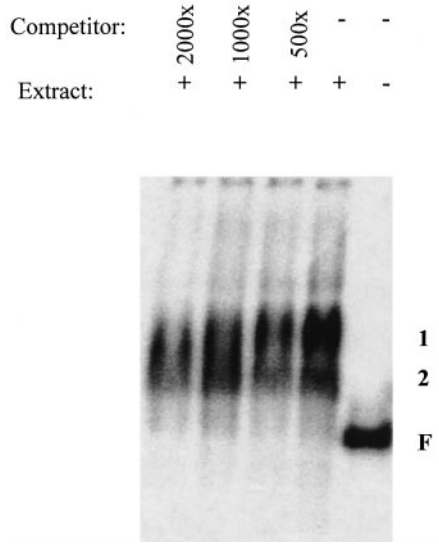


Fig. 4 Competition of the *psb*A 5′ UTR against the binding of the *atp*I 5′ UTR. Ten femtomol of radiolabeled *atp*I 5′ UTR were incubated in the presence (+) or absence (–) of chloroplast extracts and a varying fold-excess of unlabeled *psb*A 5′ UTR. Binding reactions were subjected to gel mobility shift analysis and the results shown here. *F* denotes migration of free RNA and the numbers designate the two *atp*I 5′ UTR-protein complexes

Competition of U_7 against atpI

One of the primary structural features that all of the ATP synthase 5′ UTRs from this gene cluster have in common is an uninterrupted sequence of 7–12 uridines. To determine whether any of the binding polypeptides specifically recognize those U-tracts, binding of the *atp*I 5′ UTR was subjected to competition with U_7 (chemically synthesized by Dr. Philip Gottlieb, SUNY at Buffalo). No competition was observed even at a 2000-fold excess of U_7 (Both and Hollingsworth, unpublished).

Discussion

Translational regulation and stability of chloroplast transcripts can be mediated by information in the 5′ UTRs (Reinbothe et al. 1993; Salvador et al. 1993; Koo and Spremulli 1994; Danon and Mayfield 1994; Nickelsen et al. 1994; Sakamoto et al. 1994; Staub and Maliga 1994; Hauser et al. 1996; Hirose and Sugiura 1996; Klaff et al. 1997; Yohn et al. 1998). The 5′ UTRs of chloroplast transcripts appear to exert their regulatory effects via interactions with RNA binding proteins (Hauser et al. 1996; Hirose and Sugiura 1996; Klaff et al. 1997; Yohn et al. 1998). Many, although not all, of the 5′ UTR-binding proteins studied thus far appear to be specific for individual chloroplast transcripts. Given the limited size of the chloroplast genome (Palmer 1985; Shimada and Sugiura 1991; Gillham 1994), it is likely that these binding polypeptides are nuclear-encoded. In *Chlamydomonas* chloroplasts, nuclear-encoded factors directly interact with specific chloroplast 5′ UTRs to affect translation of the downstream ORFs (Stampacchia et al. 1997; Zerges et al. 1997; Yohn et al. 1998). Thus 5′ UTR/polypeptide interactions play a significant role in nuclear control of chloroplast gene expression.

Although binding as assayed by mobility shift was not detected for *atp*F and *atp*H_i, both of these RNAs can compete, albeit poorly, against the slower mobility complexes formed for *atp*I and *atp*H (Figs. 3A and B, Table 1). Since the highest-fold excess of psbA was also able to compete poorly against the slowest mobility *atp*I complex (Fig. 4), we hypothesize that one or more polypeptides involved in slower-migrating *atp*I and *atp*H complexes may recognize some type of general chloroplast RNA feature. Polypeptides involved in these complexes cannot be completely non-specific, since binding occurs even in the presence of 20,000-fold excess of yeast RNA or *E. coli* tRNAs. Experiments are currently in progress to more specifically examine the RNA features required for binding.

Competition assays of *psb*A 5′ UTR against the binding of *atp*I 5′ UTR detected partial competition of only the slowest migrating *atp*I complex. In contrast, all four substrates derived from the 5′ UTRs from the gene cluster competed against both *atp*I complexes to some degree. Therefore, we hypothesize that some of the polypeptides involved in binding *atp*I are ATP-synthase-specific.

One of the proteins that specifically bind the 5′ UTR of *psb*A in *Chlamydomonas* chloroplasts is a polyA-binding protein (Yohn et al. 1998). In addition, Hirose and Sugiura have shown that an AU-rich region is necessary for translation of a *psb*A-*lacZ* fusion in tobacco chloroplast extracts (Hirose and Sugiura 1996). Although there is no conserved polyA sequence in these ATP synthase 5′ UTRs, they all contain AU-rich regions marked by uninterrupted U repeats of 7–12 nucleotides. To investigate whether these repeats were specifically bound by proteins in the complexes, a polyU oligonucleotide seven bases in length was used in competition against *atp*I-protein complex formation. No competition was observed (Both and Hollingsworth, unpublished). We conclude that the binding proteins involved in the *atp*I complexes have no specificity for polyU sequences by themselves.

Multiple complexes were formed with each 5′ UTR bound by polypeptides in the extract (Fig. 2). In the competition experiments, disappearance of a slower migrating complex was paralleled by an increase in the intensity of faster migrating complexes. This supports the idea that the slower complexes may be derived from the faster. Unfortunately, these polypeptides do not UV cross-link to radiolabeled portions of the RNA under standard conditions (Pinol-Roma et al. 1989; Klaff et al. 1997). Experiments are planned to continue the cross-linking analysis with photo-reactive RNAs to address the question of the size and number of binding polypeptides.

RNA-protein interactions at 5′ UTRs have been shown to affect gene expression at any of several levels, including enhancement or inhibition of translation and control of RNA stability (Klausner et al. 1993; Hauser et al. 1996; Hirose and Sugiura; 1996; Klaff et al. 1997; Stampacchia et al. 1997; Zerges et al. 1997; Yohn et al. 1998). Although we do not know the effect of the interactions reported here, we hypothesize that the multiple classes of binding activities detected in the competition experiments may regulate different aspects of ATP synthase expression, such as the stoichiometry of the subunits and/or the abundance of the ATP synthase holo-complex. Some of the complexes have varying affinities for different ATP synthase 5′ UTRs (Table 1). Depending upon the actual effect of the binding proteins, this variation in affinity could serve as a mechanism to regulate the stoichiometry of individual subunits produced from the cluster by interacting more or less strongly with specific transcripts. This could be a key mechanism in transducing the 2 : 11 : 3 : 1 ORF ratio into the final polypeptide stoichiometry, 1 : 6–12 : 1 : 3. Competition experiments also revealed what appears to be ATP synthase 5′ UTR-specific binding. Except for competition against *atp*A by *atp*F, every 5′ UTR-containing transcript from this gene cluster was able to compete against the binding of all the others to some degree. The shared binding polypeptides could provide a mechanism for global regulation, where the abundance of the entire gene cluster would be up- or down-regulated in response to environmental cues (Strotmann and Bickel-Sandkotter 1984).

There may also be polypeptides that specifically bind to transcripts encoding one of the two ATP synthase subcomplexes, CF_0 and CF_1. *atp*I, *atp*H, and *atp*F encode CF_0 subunits, while *atp*A encodes one of the CF_1 subunits. Although *atp*I and *atp*H 5′ UTRs can compete efficiently against each other for binding chloroplast polypeptides, neither can completely compete against *atp*A complex 3. The converse is also true, the *atp*A 5′ UTR does not efficiently compete against complexes formed with either of the CF_0 transcripts. These data, plus the observation that the *atp*F 5′ UTR competes to some degree against *atp*I and *atp*H, but not *atp*A, lends support to the idea that there may be a level of subcomplex-specific regulation for ATP synthase transcripts. Such coordination of expression has been shown at the translational level for *atp*A and *atp*B (which both encode CF_1 subunits) in *Chlamydomonas*, mediated through the action of three nuclear gene products (Drapier et al. 1992).

Given our hypothesis that interactions at the 5′ UTR affect expression of the downstream ORF, the fact that *atp*F-protein complexes are not detected in our assays is intriguing. A trivial explanation for this seemingly conflicting observation is that the *atp*F complex is not stable under our mobility shift assay conditions. Alternatively, regulation of *atp*F expression may be mediated via another mechanism. An obvious candidate for regulation of *atp*F expression is splicing of the group-II intron contained within its ORF (Kim and Hollingsworth 1993). Regulation involving splicing would be likely to require RNA features found downstream from the 5′ UTR. Thus, translational regulation for *atp*F, if it exists, may be mediated at the RNA maturation level, involving sequences other than those at the 5′ UTR.

Roughly 60 nucleotides upstream of the translation start sites of all four ATP synthase ORFs were examined for primary sequence features that they might have in common (Fig. 5). Although the binding substrates extend up to 100 nucleotides upstream of the regions compared Fig. 5, there was no significant similarity detected upstream of the sequences shown. Two sequence similarities were found; a

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atpI AUUGAAUCA. AAAUAAUUUU UUUAAGUUAU AUUUCUGUAA GAGGACAAU. .......AUG

atpH AUUGUAUCAU UAACCAUUUC UUUUUUUUUU UUGUGUGUGU GAGGAACUUU AUC....AUG

atpF AUUGAAAUA. AUACAACGAU UUUUUUGUUU AUCUAUAAGA GGAGAUCAU. .......AUG

atpA AUUUAAUUGU AGGUAUUAUU UUUUUGUUUC AAAAAAAAAA AGAAUUUAAG AAGACUCAUG

     AUUgaAuYR. arRYaaY.uY UUUuu.uUuY au.u.uR.Ra RRRRa..au. .......AUG
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Fig. 5 Sequence comparison of the 5′ untranslated regions of the large ATP synthase gene cluster from spinach chloroplasts: Sequences upstream of the AUG start codon of the four ATP synthase open reading frames from the large ATP synthase gene cluster were aligned using the GCG Pileup program (Genetics computer group 1994). A consensus sequence derived from similarities in the UTRs is given in *bold* below the alignments. *Upper case letters* designate a nucleotide found in all four UTRs. *Lower case letters* designate a nucleotide found in three of the four UTRs. *Y and R* stand for pyrimidine and purine, respectively. Putative ribosome binding sites are *underlined* (Bonham-Smith and Borque 1989)

polypurine stretch near the AUG start codon (which contains the proposed ribosome binding site for *atp*I, *atp*H, and *atp*F) and a polyU stretch 7–12 bases in length 20–30 bases upstream of the AUG. Hirose and Sugiura (1996) have shown that an AU-rich sequence upstream of the AUG of *psb*A is important for translation in tobacco chloroplast extracts. The polyU stretches in the 5′ UTRs of the ATP synthase RNAs are within regions of 14–35 bases that are 93–96% AU-rich, as compared to the 73–82% A/U abundance in the 5′ UTRs of the binding substrates. However, neither U_7 nor the *atp*F 5′ UTR, which contains a 14-base 93% AU-rich sequence, bind or efficiently compete for binding in the extract. Thus, it appears that the AU-rich sequence in ATP synthase 5′ UTRs is not as critical a factor for binding in spinach chloroplast extracts as is the case for *psb*A in tobacco chloroplast extracts.

The polypeptides that interact with transcripts from the large ATP synthase gene cluster are candidates for trans-acting factors that mediate RNA stability and/or translational efficiency. Until recently, relatively little was known about the cis- and trans-acting factors required for efficient translation in chloroplasts (Betts and Spremulli 1994; Gillham et al. 1994; Mayfield et al. 1995; Hirose and Sugiura 1996; Stampacchia et al. 1997; Zerges et al. 1997; Yohn et al. 1998). In particular, there has been much speculation concerning the general applicability of the 5′ UTR/polypeptide interaction in the control of translation and stability in land-plant chloroplasts. Further experiments are in progress to determine the functional significance of these interactions and to define the cis- and trans-acting factors necessary for binding.

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