An mRNA 3’ processing site targets downstream sequences for rapid degradation in *Chlamydomonas* chloroplasts

by

CONTACT _Con-49342932793_ Amanda Hicks, Robert G. Drager, David C. Higgs‡, and David B. Stern§

*Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, NY 14853

‡Department of Biological Sciences, University of Wisconsin-Parkside, Kenosha, WI 53141

§To whom reprint requests should be addressed. e-mail ds28@cornell.edu.

Running title: Rapid RNA degradation in *Chlamydomonas*
SUMMARY

In *Chlamydomonas* chloroplasts, *atpB* pre-mRNA matures through a two-step process. Initially, endonuclease cleavage occurs 15 nt downstream of the mature 3’ end, which itself lies at the end of a stem-loop-forming inverted repeat (IR) sequence. This intermediate product is then trimmed by a 3’-5’ exonuclease activity. Although the initial endonucleolytic cleavage by definition generates two products, the downstream product of *atpB* pre-mRNA endonucleolytic processing cannot be detected, even transiently. This product thus appears to be highly unstable, and it can be hypothesized that specific mechanisms exist to prevent its accumulation. In experiments described here, the *atpB* 3’ maturation site was placed upstream of reporter genes *in vivo*. Constructs containing both the IR and endonuclease cleavage site (ECS) did not accumulate the reporter gene mRNA, whereas constructs containing only the IR did accumulate the reporter mRNA. The ECS alone gave an intermediate result, suggesting that the IR and ECS act synergistically. Additional secondary structures were used to test whether 5’-3’ and/or 3’-5’ exonuclease activities mediated degradation. Because these structures did not prevent degradation, rapid endonucleolytic cleavages most likely trigger RNA destruction after ECS cleavage. On the other hand, fragments resulting from cleavage within the endogenous *atpB* mRNA could occasionally be detected as antisense transcripts of the adjacent reporter genes. Since endonuclease cleavages are also involved in the 5’ maturation of chloroplast mRNAs, where only the downstream cleavage product accumulates, it appears that chloroplast endoribonuclease activities have evolved mechanisms to selectively stabilize different ECS products.
INTRODUCTION

Maturation of mRNA can involve multiple steps in both prokaryotic and eukaryotic systems, and results in functional transcripts with a stability and subcellular localization appropriate to their functions. RNA sequence and secondary structure, along with ribonucleases and a variety of accessory factors, are used to achieve these goals. Built into this process is the necessity to recognize nonfunctional RNAs and eliminate them; destruction of nonsense codon containing mRNAs is a good example of the complexity of such surveillance mechanisms (reviewed in 1).

Our laboratory has focused on 5’ end and 3’ end maturation of chloroplast mRNAs, using both vascular plants and the unicellular green alga *Chlamydomonas reinhardtii* as models. In this respect, chloroplast transcripts have mostly prokaryotic features such as the lack of a trimethylguanosine 5’ cap, a 3’ stem-loop-forming inverted repeat (IR) structure, and they are destabilized by polyadenylation (reviewed in 2,3). Processing of chloroplast mRNAs, with rare exceptions, depends on nucleus-encoded proteins, several of which have been identified genetically through screens for plants or *Chlamydomonas* strains unable to carry out photosynthesis. The phenotypes suggest that defects in intercistronic processing of polycistronic transcripts (4,5), or in correct 5’ end maturation (6-8), can cause RNA instability and/or translational defects. Also, we have previously shown that correctly 3’ end processed mRNA is translated preferentially in *Chlamydomonas* chloroplasts (9). Therefore, 5’ and 3’ end maturation are essential steps in chloroplast gene expression.

Biogenesis of the *Chlamydomonas* chloroplast *atpB* mRNA is particularly well characterized. The *atpB* gene contains a somewhat unusual promoter, with essential elements
included in the 5’ UTR (10). Following transcription, two 5’ ends can be found in the mature mRNA (11), a situation also found for many other chloroplast transcripts and presumed or proven to result from a primary transcript undergoing partial endonucleolytic cleavage. The region spanning from 10-89 nt downstream of the \textit{atpB} stop codon contains an IR sequence predicted to form an AU-rich stem-loop structure, and the mature 3’ end is found 3-4 nt downstream of the IR (12,13). Deletions which destabilize this structure cause RNA instability (12), however the sequence can be functionally replaced by an IR from spinach chloroplasts (13), or by a polyguanosine tract, which forms a tertiary structure impervious to either 5’–3’ or 3’–5’ exonucleases (8,14).

Both \textit{in vitro} and \textit{in vivo} approaches have shown that chloroplast IR sequences do not efficiently terminate transcription, and those tested include \textit{Chlamydomonas} \textit{atpB} (13,15). This implies that post-transcriptional 3’ end maturation is required. We have previously shown that \textit{atpB} mRNA undergoes a two-step maturation process (13). In the first step, endonuclease cleavage occurs at 3 consecutive positions 8-10 nt downstream of the mature 3’ ends (Fig. 1A). This cleavage is rapid, and \textit{in vitro} can be detected within seconds of adding an artificial transcript to a \textit{Chlamydomonas} chloroplast stromal extract. The second step is 3’–5’ exonucleolytic trimming, a slower step which requires about 15 min \textit{in vitro}. Surprisingly, however, the distal product of endonuclease cleavage could not be detected \textit{in vitro}, even when the \textit{atpB} endonuclease cleavage site (ECS) was placed between two stem-loops, or when the artificial transcript was labeled at its 3’ end. This suggested that some mechanism rapidly degraded the downstream cleavage product, unlike the unprocessed pre-mRNA or matured \textit{atpB} fragment, both of which were relatively stable in the \textit{in vitro} system. The \textit{atpB} mRNA itself is
quite stable \textit{in vivo}, with a half-life estimated at 2-10 h depending on growth conditions (16). Here, we show that cleavage at the \textit{atpB} ECS potentiates rapid degradation of the downstream fragment \textit{in vivo}, even if it is a coding region or flanked by protective RNA structures. Our data suggest the activity is an endonuclease which acts as part of a targeted RNA recycling mechanism.

**EXPERIMENTAL PROCEDURES**

**Culture conditions and RNA isolation.** The strains used in this study were wild-type P17 (12), which is derived from CC373 (17), the \textit{atpB} deletion mutant used as a transformation recipient. Cells were grown in TAP medium (18) under constant fluorescent light.

**Gene constructions and transformations.** The \textit{uidA} constructs shown in Figure 2 were based on pDG2 (19). This plasmid contains the \textit{petD} promoter and 5’ UTR translationally fused to the \textit{uidA} coding region and \textit{rbcL} 3’ UTR, and the chimeric gene is inserted into the large inverted repeat of the chloroplast genome downstream of the \textit{atpB} gene (11). We previously engineered a BglII site into the \textit{petD} 5’ UTR at position +25 relative to the mature RNA 5’ end, which lengthens the transcript by 6 nt and is present in pDG2 (19). The BglII site was used as an insertion point for the various \textit{atpB} fragments to make the \textit{petD-uidA} and \textit{petD-aadA} (DG and DA) constructs. The 242 bp ECS fragment, which contains the distal half of the \textit{atpB} 3’ stem loop, the ECS and downstream sequences, was amplified from the WT \textit{atpB} gene using primers NFIR.3 (loop of IR) and DBS5 (see primer table). This PCR product was cloned into the ddT-tailed EcoRV site of pBluescript II SK+, and released with BglII (in primer NFIR.3) and XbaI (in primer DBS5). This BglII-XbaI fragment was ligated into the unique BglII site (+25) of
pDG2 after blunting XbaI and the 3’ half of petD BglII, generating the ECS-DG plasmid. +IRECS and –IRECS: The WT atpB 3’ UTR was amplified with primers DBS4 and DBS5, yielding the same 341 bp fragment previously used for in vitro mRNA maturation studies (13). The insert was cloned and then released with XhoI and XbaI, blunted, and inserted in the blunted BglII site of the petD 5’ UTR. Both orientations were obtained to make plasmids +IRECS-DG and -IRECS-DG. IRECSIR: This 679 bp insert was amplified from the plasmid containing tandem Chlamydomonas atpB and spinach petD 3 UTRs (850; 13) with primers DBS4 and 8901. The fragment was inserted into the pDG2 BglII site to generate the IRECSIR-DG plasmid. Finally, the 151 bp IR fragment was made from the source plasmid atpB21, a derivative of the WT atpB gene in which 6 nt of the distal stem of the atpB 3 stem-loop has been deleted, as well as downstream sequences (12). At the site of the deletion a BglII site was inserted. The atpB21 Chlamydomonas strain accumulates a discrete atpB transcript of the wild-type size, indicating that the short deletion does not significantly compromise RNA stability (13). The IR insert was amplified from atpB21 with primers DBS4 (XhoI) and 8906 (HindIII), inserted into pBluescript, excised with XhoI and BglII (the 21 3 deletion endpoint), and inserted into the petD 5 UTR BglII site after blunting XhoI and 5’ half of petD BglII, creating plasmid IR-DG. The pDAAD (20) based constructs shown in Figure 3 were made by releasing the petD promoter and 5’ UTR fragments from ECS-DG and +IRECS-DG with XhoI (upstream of promoter) and SmaI (3’ to the translation start) and using them to replace the equivalent XhoI-SmaI fragments of pDAAD, generating plasmids ECS-DA and +IRECS-DA.

Clones used for experiments in Figure 4: WT and CC373 were referenced above. Strain 8 has been described previously (12), and has a complete atpB transcription unit and a 2 kb
deletion beginning 87 bp downstream of the IR. At the site of this deletion a BglII site was inserted, and this was used to create the 8pG strain. The plasmid was digested at its unique BglII site, and annealed oligonucleotides carrying a G18 motif, a flanking EcoRI RFLP to determine orientation, and sticky ends compatible with BglII (14) were ligated into it. For IRECSpG, a 

atpB 3 UTR-pG containing fragment was amplified from plasmid atpB8pG using primers DBS4 and 8901 and inserted into a ddT-tailed EcoRV site of pBluescript II SK+. This fragment was released with XhoI (in primer DBS4) and BglII (in primer 8901) and inserted into the BglII site of pDG2, after blunting XhoI and the 3' petD BglII site, creating plasmid IRECSpGDG.

**RNA isolation, filter hybridizations and primer extension.** RNA was isolated from 10 ml of cells as previously described (21). For RNA filter hybridizations, 10 µg of total RNA was fractionated in 1.2% agarose, 6% formaldehyde gels, transferred to Hybond-N (Amersham) and cross-linked by UV irradiation. Double-stranded DNA probes were labeled by random priming (22), and filters were prehybridized and hybridized according to Church and Gilbert (23). Double-stranded probes were the following: for uidA, an internal EcoRV fragment; for petD, the complete 362 bp 5’ UTR was amplified using primers WS13 and WS11; for aadA the coding region was excised from patpX-AAD (24); and for atpB primers DBS1 and atpBstart were used to amplify the 5’ UTR, since this region is not deleted in CC373.

For strand-specific RNA probes, filter hybridizations were performed using the Zeta Probe protocol from Bio-Rad (www.bio-rad.com) with the following modifications: prehybridization and hybridization were at 65°C for a minimum of 3 hours and 8 hours, respectively. All probes were made by linearizing DNA templates and transcribing with T3 or T7 RNA polymerase in the
presence of $\alpha^{32}$P-UTP (25). The following probes were used: For *uidA* the template was pBGEV (26). Antisense transcripts were made with T7 following HindIII digestion, and sense transcripts with T3 following EcoRI digestion. For *aadA* the template was amplified from *patpXAAD* with primers lar005 (beginning with a T7 promoter; sense transcripts) and lar006 (beginning with a T3 promoters; antisense transcripts). For probes spanning the region downstream of the *atpB* 3’ UTR, primers NFIR.3 and 8901 (minus added restriction sites) were modified to be preceded with T7 and T3 promoters, respectively. T3 transcription produced an antisense transcript, and T7 transcription a sense transcript. All RNA hybridizations were analyzed using the Storm system (Molecular Dynamics Inc., Sunnyvale, CA).

For primer extension, 2 ng primer DBS4anti (P3 in Figure 2C) were labeled with $\gamma^{32}$P-ATP and polynucleotide kinase, and mixed with 20 µg *Chlamydomonas* RNA in a final volume of 8 µL. The reaction also contained 0.7 µL 10 mM dNTPs and 0.7 µL 10X reaction buffer (10X buffer is 100 mM Tris-HCl pH 8.5; 60 mM MgCl$_2$; 500 mM KCl; 10 mM DTT). The reaction was incubated for 5 min at 75°C and 5 min at 50°C, 4.5 u AMV reverse transcriptase (Promega) were added, and incubation was continued for 15 min at 50°C. 5 µL sequencing dye was then added and samples were analyzed by denaturing polyacrylamide gel electrophoresis.

**RESULTS**

The IR and ECS synergistically activate downstream RNA decay. Degradation of downstream sequences is associated with ECS cleavage (Fig. 1A), and may be associated with multiple mechanisms (Fig. 1B). Mutagenesis of the ECS showed, however, that cleavage
occurred in vivo even after multiple mutations were introduced (27). This suggested that the ECS alone did not determine the processing site. To determine which RNA element(s) might be required for ECS downstream product destabilization, a series of reporter gene constructs were assembled and introduced into Chlamydomonas chloroplasts by biolistic transformation, as shown in Figure 2A. These strains contained a chimeric petD promoter/5 UTR-uidA (β-glucuronidase) coding region-rbcL 3 UTR reporter gene, which was located downstream of and in opposite orientation to the endogenous atpB gene. This insertion site has been used for numerous GUS gene fusions in the past (10,11,19), and does not affect expression of atpB, which is used as the selectable marker for transformation of the nonphotosynthetic recipient strain CC373 (see Experimental Procedures).

The reference strain, DG2, has the structure shown at the top of Figure 2A. Five additional strains were created which have insertions at the +25 position relative to the mature uidA mRNA 5’ end. We have previously shown that deletions up to +25 do not affect transcription rates (19), and that small mutations or a polyguanosine insertion within the +1-25 region do not decrease petD RNA levels (21,28). In addition, the sequences around this position are not important for petD mRNA 5’ end maturation (28), although any insertion into this site translationally inactivates the message, for unknown reasons. For this reason, analyses were restricted to RNA accumulation. The +25 insertions are shown symbolically in Figure 2A and include the following sequences: ECS, a 242 bp fragment which lacks one stem of the atpB 3’ IR, and includes the ECS; +IRECS, a 341 bp fragment which includes the full 3’ IR and ECS in the sense orientation relative to uidA; -IRECS, which includes the 3’ IR and ECS in the antisense orientation relative to uidA; IRECSIR, a 679 bp fragment which contains the atpB 3’ IR and ECS, with a second IR,
derived from the spinach chloroplast petD gene, immediately downstream; and IR, a 151 bp fragment which has the nearly complete atpB 3’ IR but lacks the ECS.

Homoplasmic transformants were obtained and uidA mRNA was examined with strand-specific probes, with petD mRNA as a loading control. Figure 2B (top panel) shows results with a probe which detects sense uidA mRNA. As expected, no hybridization occurs with RNA from wild-type cells or CC373, the transformation recipient. On the other hand, a transcript of 2.5 kb accumulates in DG2; this mRNA has a 5’ end at position +1 in the petD 5’ UTR (19) and terminates in the rbcL 3’ UTR at a stem-loop structure (29). Among the five newly-created strains, 3 accumulated a sense uidA transcript. Of these, the lowest accumulation, relative to petD, was in ECS, with higher amounts in –IRECS and IR. The sizes of the chimeric uidA transcripts in these strains are slightly larger than in DG2, commensurate with the sizes of insertions at +25. We thus infer that these transcripts have been correctly 5’ end processed. On the other hand, neither +IRECS nor IRECSIR accumulated sense uidA mRNA. These are also the only two strains that have both the atpB IR and ECS at +25. Since the IR is not responsible for transcription termination – for example it is present in strain IR (see also 13,15), it can be concluded that the IR-ECS combination prevents accumulation of uidA mRNA through RNA degradation. We would explain accumulation in strain ECS by inefficient cleavage in the absence of the IR: uncleaved RNA accumulates, whereas RNAs that are cleaved undergo downstream degradation.

The bottom panel of Figure 2B shows results with a probe designed to detect antisense uidA mRNA. Surprisingly, hybridization was detected in four strains, most prominently in +IRECS and most weakly in ECS (the band marked with an asterisk is an rRNA hybridization artifact).
Based on their sizes, these RNAs had 5’ ends in or near the intergenic region between atpB and the 3’ end of the uidA cassette, and 3’ ends at the IR sequences inserted at the petD +25 position (see Fig. 6 and Discussion). We have already shown that the atpB 3’ IR can stabilize RNAs when it is in the antisense orientation (20). To map the 5’ ends, we selected +IRECS and IRECSIR and used primer extension, with RNA from untransformed cells as a negative control. Three primers were tested, as shown at the top of Figure 2C (P1, P2, P3). Using P1, no transformant-specific products were detected, however using P2, high molecular weight products were seen for +IRECS and IRECSIR, but not for the WT control (data not shown). To map the end precisely, we used the 26 nt primer P3, which anneals upstream of the atpB stop codon. 5’ ends mapping 6 nt upstream of the primer were found in both transformants, whereas this product was not seen in the control. The additional bands probably result from abortive extension of the abundant endogenous atpB transcript, which extends 1.9 kb upstream and accumulates in all strains. The mapped 5’ ends in +IRECS and IRECSIR lie 72 nt upstream of the atpB stop codon, and may represent a normal cleavage site during atpB mRNA degradation. We hypothesize that they accumulate in these transformants due to their stabilization at the 3’ end by IR structures, and for unknown reasons, perhaps structural, are not subject to cleavage at the ECS. Note that the IRECSIR antisense transcript is slightly longer (Fig. 2B), in agreement with the fact that it has a petD 5’ UTR insert 338 bp longer than that of +IRECS, which would be included at the 3’ end of the antisense transcript.

In wild-type cells no antisense transcript accumulated, based on primer extension. Assuming the same atpB-internal cleavage occurs in wild-type cells, a short transcript would be generated also flanked by a 3’ IR. However, this hypothetical transcript would only be 168 nt
and might be unstable and/or subject to loss during RNA isolation. The lack of an antisense transcript in strain IR, on the other hand, was unexpected given its accumulation in the other IR-containing strains. We note that the +25 insertion in IR is not the entire stem-loop, as it lacks the last 6 of 20 nt in the second stem (12). Given its AT-richness, the antisense IR sequence may form too weak of a secondary structure to stabilize the uidA antisense transcript.

A second reporter gene confirms results from uidA. To confirm that the results shown in Figure 2 were not due to a peculiarity of the reporter gene chosen, certain constructs were replicated using the aadA coding region instead of uidA. aadA is a commonly used selectable marker gene in Chlamydomonas chloroplasts and has also been used to study RNA cis elements (8,30). Figure 3 shows results from the reference strain, DAAD (petD-aadA) and two derivatives, ECS-DA and +IRECS-DA. These are in every way analogous to the uidA based constructs DG2, ECS-DG and +IRECS-DG, respectively. When a dsDNA probe recognizing the aadA coding region was used, a 1.5 kb transcript accumulated in DAAD, as expected. This is the chimeric petD-aadA-rbcL message. A 1.7 kb species accumulated in ECS-DA, and a 3.4 kb transcript accumulated in +IRECS-DA. To differentiate between sense and antisense transcripts, a strand-specific probe was used which only detected antisense aadA mRNAs (bottom panel). This identified the 3.4 kb species, but not the 1.5 kb and 1.7 kb transcripts. Thus, a sense aadA transcript accumulates to a low level when the ECS alone is inserted at position +25, not at all when both the IR and ECS are present, and to an increased level when neither is present. On the other hand, the antisense message only accumulated when a stem-loop-forming sequence was present at position +25. These results are entirely consistent with the results for uidA constructs (except the lack of a minor antisense transcript in ECS-DA), and indicate that the reporter gene
coding region did not have a significant effect on the outcome.

**Downstream degradation cannot be blocked by impeding a 5’–3’ exonuclease activity.** The mechanism by which ECS cleavage potentiates downstream degradation could involve one or a combination of ribonuclease activities known to exist in *Chlamydomonas* chloroplasts, as shown in Figure 1B. 5’–3’ exonuclease activity (i) is known from studies of mutants in which particular chloroplast transcripts are unstable due to lack of protection of the 5’ UTR by nucleus-encoded factors, and can be blocked by a polyguanosine (pG<sub>18</sub>) sequence (8,31,32). 3’–5’ exonuclease (ii) is known from 3’ processing studies (14), and can also be impeded by pG, or by a stem-loop. Finally, endonuclease activities exist (iii), for example those which mature the 5’ end of *petD* mRNA (33) or cleave at the *atpB* 3’ ECS. Degradation could also involve multiple mechanisms, for example endonuclease cleavages followed by exonucleolytic digestion to mononucleotides.

To test for the involvement of a 5’–3’ exonuclease, we attempted to block downstream degradation by interposing pG immediately downstream of the ECS. This strategy was carried out in two configurations, as shown in Figure 4A. In the first case, we compared the strain *atpB*8 (12), in which a BglII site has been inserted downstream of the *atpB* ECS, to a strain where pG had been inserted into the BglII site (8pG). If pG blocked downstream degradation, a transcript containing sequences downstream of the ECS might accumulate, although there is no particular structure that would serve as a 3’ end. In the second case, we modified strain +IRECS-DG by introducing pG downstream of the ECS. In this situation, should pG block 5’ to 3’ degradation we would anticipate the accumulation of a *uidA* transcript with pG defining its 5’ end and the *rbcL* IR defining its 3’ end.

RNA accumulation for these strains, and appropriate controls, is shown in Figures 4B and
4C. Figure 4B shows results using a probe which identifies sequences downstream of the normal *atpB* mRNA mature 3’ end. The sequences homologous to this probe vary from strain to strain, and are diagrammed in Figure 4A. In the top panel of Figure 4B, a strand-specific probe was used to detect sense RNAs with respect to *atpB*, and a strongly hybridizing 1.5 kb band was visible for strain CC373, the transformation recipient. Because of a large deletion, CC373 lacks the ECS and therefore would not be expected to destabilize downstream sequences. A fortuitous stabilizing sequence must define the 3’ end of that transcript. The lack of signals for WT and DG2 is not surprising, since each of these strains possesses a wild-type *atpB* gene. Similarly, 8, although it has a deletion, still contains the IR and ECS and should act like the wild-type. In 8pG, however, we might have observed accumulation of an RNA species if pG prevented 5’ to 3’ degradation following ECS cleavage, although we did not introduce a downstream IR to stabilize this putative transcript. We conclude that blocking 5’-3’ exonuclease activity, in this context, does not permit accumulation of the ECS downstream cleavage product.

The middle panel in Figure 4B shows transcripts accumulating for the antisense strand with respect to *atpB*, downstream of the normal 3’ end. Here, we observed a 4.4 kb species in the strain IRESCpGDG. This transcript can be compared to the –IRECS antisense *uidA* mRNA seen in Figure 2B, because the probe has homology to sequences inserted into the +25 position of the *uidA* reporter gene, as well as to the 5’ end of this antisense transcript (the same 4.4 kb species is seen in an overexposure of Fig. 4A; data not shown). Finally, an RNA blot for the same strains was probed with a 5’ UTR sequence from *atpB* (lower panel of Fig. 4B). Since each strain except CC373 has an intact *atpB* gene, a wild-type sized 1.9 kb species accumulated in all cases. This demonstrated that *atpB* transcription was proceeding normally. In CC373 a 1.5 kb mRNA was
detected, as expected given the result from the strand-specific probe (top panel).

The same strains were also examined for *uidA* mRNAs, as shown in Figure 4C, although the only relevant ones were IRESCpGDG and DG2, as the others do not contain a *uidA* gene. With a sense probe, *uidA* mRNA was detected only in DG2. If the pG sequence in IRESCpGDG had protected the downstream sequences from degradation, we would have also expected to see a *uidA* transcript of approximately the same size. Since it did not accumulate, the data corroborate results with the *atpB* downstream probe (Fig. 4B) and taken together, indicate that a 5’Ô3’ exonuclease activity alone is not responsible for degradation of sequences downstream of the *atpB* ECS. An antisense *uidA* transcript was seen for IRESCpGDG; this was also identified with the *atpB* downstream probe and was discussed above.

**DISCUSSION**

In the absence of efficient and site-specific transcription termination, RNA 3’ termini must be formed through post-transcriptional processing. Here, we have focused on 3’ end maturation of the *Chlamydomonas* chloroplast *atpB* mRNA, which occurs through a two-step process. The first step is not unlike that of typical eukaryotic mRNAs, i.e. a site-specific endonuclease cleavage directed by upstream sequence elements. We had previously found that the downstream moiety of this endonuclease cleavage could not be detected *in vivo*, which is not surprising given its lack of functionality. However, it was somewhat surprising that in an *in vitro* system where even relatively unstructured RNAs such as those lacking known stem-loops are only slowly degraded, the downstream cleavage product could not be detected. The experiments performed here strongly argue that one or more rapid endonucleolytic cleavages occur downstream, but not
upstream of the \textit{atpB} ECS, and that this triggers complete degradation of the molecule. The RNA accumulation results are summarized in Figure 5. We suggest that this mechanism may be commonly used in chloroplasts, and perhaps in other systems, as an efficient mode of disposing of nonfunctional transcripts.

Our initial assays were designed to elucidate which elements of the \textit{atpB} pre-mRNA provided information for the degradative activity. We found that having the ECS was an absolute requirement, raising the possibility that the degradative activity might be physically associated with the processing enzyme, and strongly suggesting that cleavage was invariably required for subsequent degradation. This indicated that there was nothing inherently unstable about the downstream sequences, which are indeed derived from genes normally expressed in chloroplasts, or well studied reporter genes. We found that the IR-ECS combination yielded no detectable downstream transcript due to rapid degradation. Since the IR alone did not demonstrably affect reporter gene transcript accumulation, we believe that these two elements act synergistically, probably by making ECS cleavage more efficient. That would explain why in ECS strains, some \textit{uidA} or \textit{aadA} mRNA accumulated; in these cases (ECS-DG and ECS-DA), the accumulating transcript was of an appropriate length to contain the uncleaved ECS within the \textit{petD} 5' UTR.

We have previously shown that mutation of the ECS sequence alone is insufficient to alter \textit{atpB} 3' end formation \textit{in vivo} (27), and the IR itself is thus a candidate for a cleavage determinant.

We next attempted to test whether a 5'\textendash3' exonuclease activity was responsible for initiating downstream degradation, by inserting pG downstream of the ECS in two different contexts. pG resists 5'\textendash3' exonuclease attack both in the cytosol, as demonstrated in yeast (34), and both 5'\textendash3' and 3'\textendash5' exonucleases in \textit{Chlamydomonas} chloroplasts (8,14). pG is routinely
used for trapping degradation intermediates in yeast, and we have used it in *Chlamydomonas* (21). Our results here showed that pG could not protect the downstream *uidA* transcript from degradation (IRECSpG-DG). Since this transcript is protected at its 3’ end by the *rbcL* 3’ IR, the degradation cannot be due solely to any combination of exonuclease activities. In a parallel experiment, the pG motif was inserted downstream of the *atpB* ECS and also in this case, no discrete transcript was detected. However, in the latter case no 3’ end stability motif was placed downstream, and an accumulating transcript might be heterodisperse, much as when the *atpB* 3’ IR itself is deleted (12).

Given that our data suggest that an endoribonuclease initiates, and perhaps propagates downstream degradation following ECS cleavage, we speculate that a chloroplast relative of *E. coli* RNAse E may be responsible. While RNAse E is an endonuclease, it is strongly stimulated by 5’ monophosphate ends (35), giving it an inherent preference for processing products rather than triphosphorylated primary transcripts. Two other features of RNAse E are consistent with the activity we have observed here. First, concomitantly with 5’ cleavage stimulation by monophosphate groups, RNAse E is responsible in part for cleaving 3’ poly(A) tails (36). We have shown such tails to destabilize *Chlamydomonas atpB* mRNA both in vitro (37) and in vivo (38). Thus, an RNAse E-like activity could cause rapid degradation by attacking both ends. Second, the possibility was raised above that rapid degradation might be associated with processing at the ECS. In this respect, we note that RNAse E is found in a multicomponent degradosome in *E. coli*, which includes other RNA processing activities (39). Whether such a complex is found in *Chlamydomonas* chloroplasts is unknown. In any case, based on sequences of algal chloroplast-encoded RNAse E homologues and nuclear versions found in plants, the
chloroplast appears to contain a shorter protein known as RNAse G or CafA (reviewed in 40). CafA has the same cleavage activity as RNAse E (41), but lacks the domain required for degradosome assembly. Since RNAse E/G is not chloroplast-encoded in *Chlamydomonas*, and no nuclear EST has been reported as of this writing, our model awaits further information.

The data here highlight a vectorial RNA decay pathway that is surprisingly rapid and impervious to secondary structures. It also raises the larger question of how the chloroplast, or other systems, differentiate between the two products of endoribonuclease cleavage. In the case where the upstream segment is unstable, the lack of a stem-loop structure has long been implicated (42). On the other hand, what differentiates downstream products? In the case of the chloroplast, it is well-established that mature mRNA 5’ ends are protected by protein complexes (6,7). While it has been previously demonstrated that one role of the complex is to protect the RNA from 5’–3’ exonuclease (8), perhaps another role is to impede the net 5’–3’ activity described here. One possible mechanism would be that a currently unknown member of the complex acts like *E. coli* CspE, an RNA-binding protein which can impede RNAse E activity by physical interaction (43).

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**REFERENCES**


FIGURE LEGENDS

FIGURE1. A). Previously described steps in atpB mRNA 3’ end maturation. Transcription of a pre-mRNA of unknown and perhaps variable length is followed by precise endonucleolytic cleavage and exonucleolytic trimming, to yield the accumulating transcript. The downstream product of endonuclease cleavage fails to accumulate in vivo or in vitro, and its manner of degradation is the subject of this manuscript. B). Hypothetical pathways for RNA degradation following ECS cleavage. i) Vectorial degradation by a 5’Ù3’ exonuclease activity; ii) rapid degradation by a 3’Ù5’ exonuclease activity; iii) net 5’Ù3’ degradation by a processive endonuclease; iv) any combination of activities is possible.

FIGURE2. Sequence requirements for ECS-originated downstream product degradation. A). Schematic of the chimeric uidA genes used in this study, drawn to the scale shown (except the atpB 3’ IR). The map shows the direction of transcription for the uidA cassettes and the endogenous atpB gene. For the atpB gene, the vertical arrow marks the endonuclease cleavage site (ECS), the approximate 3’ end of that transcript. The atpB 3’ IR is shown inverted to represent its direction of transcription (right to left). Below the map, name and graphic representations of insertions into the petD 5’ UTR are shown, as detailed in Experimental Procedures. The vertical arrow represents the ECS, IR represents the atpB 3’ stem-loop-forming inverted repeat, and DG indicates the petD-GUS (uidA) fusion. For –IRECS-DG, the IR is shown inverted to emphasize its antisense transcription. B). RNA filter hybridization results using strand-specific RNA probes as indicated. The petD transcript was used as a loading control, and was detected with a double-stranded probe. Because the exposures were varied for
clarity, no conclusions should be drawn from this Figure as to the relative accumulations of *uidA* sense transcripts, *uidA* antisense transcripts, and *petD* transcripts. The star at the right of the lower panel indicates an artifactual hybridization to rRNA. CC373 is the transformation recipient. **C)** Primer extension analysis of antisense *uidA* RNAs. The top shows the locations of primers used (P1-P3, not to scale) and the extents of deduced transcripts. The bottom shows gel analysis of P3 primer extension products. At left sizes are shown, which were determined by a labeled 25 bp ladder. At right the migration of primer P3 is shown, and the 5’ end product is marked by an arrow. This qualitative analysis does not reflect the differences in transcript accumulation between +IRECS-DG and IRECSIR-DG (panel **B**).

**FIGURE 3.** RNA accumulation for constructs based on the *aadA* gene. The top line shows the gene configuration in the transformants, with the shaded box on the right indicating the endogenous *atpB* gene, as detailed in Figure 2. Hybridization probes are indicated, either corresponding to the *aadA* coding region or the *petD* coding region as a loading control. No inference should be made as to the relative accumulations of *aadA* vs. *petD* transcripts.

**FIGURE 4.** Tests for involvement of 5’–3’ exonuclease activity in RNA degradation following ECS cleavage. **A**). Diagrams of relevant regions of cpDNA in the strains as indicated, with conventions as described in the legend to Figure 2A. In the top 4 strains, the *atpB* gene has been modified (except the wild-type control) and there is no chimeric reporter gene. CC373, the transformation recipient, has a 2.5 kb deletion beginning near the 5 end of the coding region. 8 has a 2 kb deletion beginning further downstream, and a plasmid containing this version of *atpB*
was used as a template for PCR amplification of the \textit{atpB} 3' UTR hybridization probe described in detail in Experimental Procedures. 8pG is identical to 8 except for the insertion of a G$_{18}$ motif. The gray lines above each strain show the places in which the probe amplified from the 8 plasmid could hybridize. In WT these two regions are separated by 2 kb because it lacks a deletion relative to 8. The lower two strains have a wild-type \textit{atpB} gene but contain reporter genes. IRECSpGDG has an insertion of the \textit{atpB} 3' IR and ECS (vertical arrow) followed by G$_{18}$ (larger arrowhead); DG2 is an unmodified control strain. Probe homology is shown by gray lines; note that in IRECSpGDG the \textit{atpB} probe can hybridize in 3 separate regions including within the modified \textit{petD} 5' UTR. \textit{B}). Filter hybridizations with RNA probes as indicated, and a dsDNA probe for \textit{petD}, the loading control. In the top two panels, the \textit{atpB} probe is the PCR probe shown in panel \textit{A}, in the lowest panel the probe is derived from the \textit{atpB} 5' UTR. \textit{C}). Filter hybridizations with RNA probes as indicated, and a dsDNA probe for \textit{petD}, the loading control.

\textbf{FIGURE 5.} Summary of RNA accumulation results. Unmodified \textit{uidA} and \textit{atpB} genes are shown at the top for reference; this is equivalent to strain DG2. Shown immediately below this diagram are transcripts accumulating in DG2, namely \textit{uidA} and the wild-type \textit{atpB} transcript; the latter accumulates in all strains (except a shorter version in CC373). Note that the \textit{rbcL} 3' UTR forms a stem-loop structure based on a prediction from the sequence (29). The second row of transcripts shows the proposed endonucleolytic wave giving rise to \textit{uidA} mRNA degradation in the 3 strains listed at the right. In the third row, modified \textit{uidA} transcripts “escaping” this
degradation are shown; the actual structure represents –IRECS-DG. At the bottom, antisense *uidA* transcripts accumulate in the strains listed at right; the actual structure represents –IRECS-DG.

**Table of primers:**

- **atpBstart:** GCCCCAAGGCATTCATA
- **DBS1:** CAAAATTCTCCACCAGC
- **DBS4:** CCAGAACAAGCATTCTACTTAGGG
- **DBS4anti (P3 in Fig. 2C):** CCTACTAAGTAGAATGCTTGTTCTGG
- **DBS5:** XbaI-CTGTATCTCGTGGACGAC
- **lar005 (T7 underlined):** TAATACGACTCACTATAGGGGGCTCGTGAAAGCGGTTATCG
- **lar006 (T3 underlined):** AATTAACCCTCACTAAAGGGGTTATTTGCCAAACTACCTTA
- **NFIR.3:** BglII-ATTTGGACACCATTAAGTTG
- **NFIR.5:** BglII-CAGGTAGCCGAAGGG
- **P1 (ah-rbcl3'-atpB):** CCCCTTCCCTTCCGGACGTCC
- **P2 (ah-atpBecs):** GGGAAAGGTGCAACTACGTGGG
- **WS11:** SmaI-ACTGACATATTTATTTATCCGTTAA
- **WS13:** BglII-TTTTTAGCATGAAACATTAGAAATAC
- **8901:** CAAAATAATCTGTCCGG
- **8906:** GACAAGCTTTACATCCC
A

- **atpB**
- **pre-mRNA**
- **endo processing**
- **rapid degradation**
- **exonucleolytic trimming**

B

i) **5' to 3' exo**

ii) **3' to 5' exo**

iii) **processive endonuclease**

iv) **combination (endo + exo; 2 exos)**

Figure 1
Figure 3
An mRNA 3’ processing site targets downstream sequences for rapid degradation in chlamydomonas chloroplasts

Amanda J. Hicks, Robert G. Drager, David C. Higgs and David B. Stern

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