Chloroplast mRNA maturation is catalyzed by nucleaus-encoded processing enzymes. We previously described a recessive nuclear mutation (*crp3*) that affects 3'-end formation of several chloroplast mRNAs in *Chlamydomonas reinhardtii* (Levy, H., Kindle, K. L., and Stern, D. B. (1997) *Plant Cell* 9, 825–836). In the *crp3* background, *atpB* mRNA lacking a 3'-inverted repeat normally required for stability accumulates as a discrete transcript. The mutation also affects the *atpA* gene cluster; polycistronic mRNAs with *psbI* or *cemA* 3'-ends accumulate to a lower level in the *crp3* background. Here, we demonstrate that the *crp3* mutation also alters 3'-end formation of *psbI* mRNA and *cemA*-containing mRNAs. A novel 3'-end is formed in polycistronic *psbI* transcripts, and this is the only terminus observed when the *psbI* 3'-untranslated region is fused to an *aadA* reporter gene. Accumulation of mRNAs with 3'-ends between *cemA* and *atpB*, which is immediately downstream, was reduced. However, this sequence was not recognized as a 3'-end formation element in chimeric genes. The *crp3* mutation was able to confer stability to three different *atpB* 3'-stem-loop-disrupting mutations that lack sequence similarity, but are located at a similar distance from the translation termination codon. We propose that the wild-type CRP3 gene product is part of the general 3' → 5' processing machinery.

Gene expression in chloroplasts depends on nuclear gene products, which mediate transcription and post-transcriptional processes (reviewed in Refs. 1 and 2), including mRNA processing, splicing, RNA stabilization, and translation initiation. Many nuclear factors have been identified by genetic screens, e.g. in maize and Arabidopsis by high chlorophyll fluorescence or leaf pigmentation patterns (3–7) and in the unicellular green alga *Chlamydomonas reinhardtii* by high chlorophyll fluorescence or a failure to grow phototautrophically (reviewed in Ref. 1). These mutations are recessive and can affect one or several chloroplast transcripts.

Progress in defining the precise targets of these nuclear factors has been limited to *Chlamydomonas*, where chloroplast transformation and the use of chimeric reporter genes are routine. Nuclear factors required for *psbD* and *petD* mRNA stability, for example, have been shown to interact with the 5'-untranslated region (UTR), protecting the mRNA from 5' → 3' exonucleolytic degradation (8–10). In addition, point and deletion mutagenesis has been used to define potential sites of interaction between nuclear translation factors and the 5'-UTRs of *petD* (11), *psbD* (10), and *psbA* (12). These studies suggest a complex interplay between nuclear proteins and multiple cis-elements in chloroplast mRNAs.

In contrast to the 5'-UTR, whose functions in mRNA stability and translation are relatively well described, the role of the 3'-UTR in chloroplast gene expression is enigmatic. Although stem-loop-forming inverted repeat structures are commonly found in the 3'-UTR and stabilize RNAs in vitro and in vivo (13–15), the inverted repeats can be replaced in vivo by a polyguanosine sequence, which also forms a strong secondary structure (16). This suggests that at least for *atpB* mRNA, specific sequences are not required for formation of a stable 3'-end. Furthermore, the 3'-UTRs of various chloroplast genes are interchangeable both in tobacco (17) and in *Chlamydomonas* (18, 19), suggesting that gene-specific regulation is not accomplished through elements in this region. Nonetheless, the stabilizing functions of 3'-UTRs are often orientation-dependent (18, 19), and recent data from *Chlamydomonas* suggest that 3'-end formation may stimulate translation initiation in vivo (20).

To address the function of the 3'-UTR in more depth, we have taken a molecular genetic approach. Previously, a series of 3'-deletions were engineered downstream of the *atpB* gene. *Chlamydomonas* strains with UTRs that lacked the potential to form a stable 3'-stem-loop structure grew slowly under phototautrophic conditions, were sensitive to high intensity light, accumulated a reduced amount of *atpB* mRNA that was heterogeneous in size, and accumulated a similarly reduced level of the ATPase β-subunit, the product of the *atpB* gene (15). Two types of phenotypic revertants were isolated from a prototypical strain of this series, *atpBΔ26*, by virtue of their ability to grow rapidly on minimal medium and their tolerance of high light. One class resulted from a dramatic amplification of the mutant *atpB* gene in the chloroplast, so that heterogeneous and unstable RNA accumulated to a high level and thus increased ATPase accumulation (21). A second type resulted from a mutation in a nuclear gene, which we have termed *crp3* (chloroplast RNA processing). This recessive mutation allows a discrete transcript to accumulate from the 3'-deleted copy of *atpB*, which results in increased accumulation of the ATPase β-subunit due to enhanced transcript stability and more efficient translation (20, 22). Interestingly, the *crp3* mutation also caused changes in other chloroplast transcripts, including accumulation of putative processing intermediates and altered

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**Target and Specificity of a Nuclear Gene Product That Participates in mRNA 3'-End Formation in *Chlamydomonas* Chloroplasts**

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1 The abbreviations used are: UTR, untranslated region; PCR, polymerase chain reaction.
stability of transcripts from the atpA gene cluster (22). Thus, CRP3 appears to encode a factor involved in the maturation of several chloroplast transcripts. In this report, we examine the specificity of suppression of RNA instability by crp3 and show that it has a target in the 3'-UTR. A possible role for CRP3 in wild-type cells is discussed.

**EXPERIMENTAL PROCEDURES**

**Strains, Culture Conditions, and Genetic Analysis**—The strains used in this study are shown in Table I. Cells were grown in TAP medium (23) under constant fluorescent lighting. Genetic crosses were performed using standard techniques (23).

**Plasmids and Probes**—We have previously described the structure and expression of the atpA gene cluster, which contains the atpA, psbI, cemA, and atpH genes (24). Plasmid pCEMA, which contains most of the cema (ycf10) coding region, was generated by inserting a 1-kilobase EcoRI-HindIII fragment of the chloroplast EcoRI fragment 22 into pBluescript SK+ (Stratagene). This plasmid served as a template for amplifying the entire ORF by PCR using T7 and T3 primers, and the PCR fragment was used as a probe in RNA filter hybridizations. The psbI coding region probe was inserted into pBluescript SK+ as a 296-base pair PCR-amplified fragment using primers psbI1 and psbI3, covering from −35 relative to the translation initiation codon to +93 relative to the termination codon. The psbI-cemA intergenic region was amplified with primers psbI2 and psbI4, covering from −31 to +405 relative to the psbI termination codon. The cema-atpH intergenic region was amplified with primers cemA1 and cemA2, covering from −44 to +207 relative to the cemA termination codon. Primers psbI2 and cemA1 had added SphI sites, and primers psbI4 and cemA2 had added XbaI sites. The psbI2-psbI4 and cemA1-cemA2 PCR products were inserted into pBluescript SK+, and the orientation of the inserts was determined by XbaI digestion. Since pBluescript has one XbaI site in its polylinker, in one of the orientations, the PCR fragment could be excised as an XbaI fragment. This fragment was cloned into XbaI-digested pDAAD, which contains the adaA selectable marker downstream of the atpB gene (25). The orientation of the insertions was determined by digesting with SphI, as one site was present in the insert, and a second site was present in pDAAD between the adaA coding region and the rbcL 3'-UTR. For 3'-ends mapping of atpB324 and atpB247 RNAs, the atpB 3'-regions from each strain were PCR-amplified using primers 9001 and 9001(15) and inserted into the EcoRV site of T-tailed pBluescript SK (26).

**Chloroplast Transformation**—Chlamydomonas chloroplast transformation was carried out as described previously (15). Transformants were selected by their ability to grow on TAP plates containing 100 μg/ml spectinomycin. Transformants were colony-purified, and the expected insertions were confirmed by DNA filter hybridizations and appropriate PCR amplifications (data not shown).

**Isolation of Nucleic Acids, Filter Hybridizations, and Ribonuclease Protection**—For whole-cell nucleic acid preparations, cells were grown in TAP medium, and RNA was isolated as described previously (22). RNA was size-fractionated on 6% formaldehyde and 1.1% agarose gels, transferred to nylon membranes, and hybridized with 32P-labeled probes as described previously (22). The 3'-ends of atpB psbI, and cema4 transcripts were mapped using the plasmids described above and RNase protection as described previously (27). Gel imaging and quantification were performed using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

**RESULTS**

**A aberrant Processing of the psbI 3'-UTR in the crp3 Background**—The Chlamydomonas atpA gene cluster contains four genes, which are transcribed into eight known mono- and polycistronic mRNAs, as shown in Fig. 1 (diagram) and described previously (24, 28, 29). The crp3 mutation was previously shown to affect the accumulation of mRNAs that terminate downstream of the psbI and cemA (ycf10) coding regions; the amount of psbI-terminated RNAs was moderately reduced, whereas the amount of cema-terminated RNAs was strongly reduced (22). To examine the effect of the crp3 mutation on these RNAs in detail, we performed filter hybridizations using total RNA extracted from wild-type, crp3, or atpA33 cells (24); in atpA33, the psbI promoter has been deleted.

Fig. 1A shows the results of probing with the psbI coding region. In wild-type cells, six RNAs were detected of which the most abundant was the monocistronic psbI message, transcript 7. In crp3 cells, it was expected that RNAs 2, 3, 6, and 7 might be affected since their 3'-ends are downstream of psbI or cemA. Indeed, RNA 2 was virtually undetectable; however, only a small change, if any, was seen in the accumulation of the bands corresponding to RNAs 3 and 6, which are similar in size. Most remarkably, a new species was observed that migrated slightly slower than RNA 7; this is marked as RNA 7* in Fig. 1A. This suggested that monocistronic psbI mRNA might be aberrantly processed in crp3 cells. This is supported by results from atpA33, in which the psbI-proximal promoter has been deleted: neither RNA 7 nor the new transcript was observed. However, RNA 3 accumulated to a high level (it is shorter due to the atp33 deletion). This suggests that crp3 selectively affects transcripts terminating downstream of psbI.

The difference in size between the wild-type and novel monocistronic psbI mRNAs could result from differences at the 5'- or 3'-ends. The 5'-end of psbI mRNA was mapped by primer extension, using total RNA from wild-type and crp3 cells, as described previously (24), and no differences were observed between wild-type and crp3 cells (data not shown).

The 3'-end of psbI mRNA was mapped by RNase protection, using a uniformly labeled antisense RNA spanning the intergenic region between psbI and cemA as shown in Fig. 1B, two major protected bands were obtained using wild-type RNA. The longer fragment (readthrough) represents protection by RNAs 1, 2, 5, and 6, whereas the shorter fragment (ut) represents protection by RNAs 3 and 7. By comparison with a DNA sequencing ladder, the psbI 3'-end was mapped to 83 ± 3 nucleotides downstream of the psbI termination codon (data not shown). In crp3, an ~50% reduction in the level of transcripts with the wild-type psbI 3'-end was observed (wt- crp3). In addition, a longer protected product that represents a novel psbI 3'-end was mapped. This product is ~100 nucleotides longer than the wild-type product, represents ~50% of the psbI 3'-ends, and corresponds to RNA 7* in Fig. 1A and possibly to a slightly longer RNA 3.

In an attempt to distinguish between the 3'-ends of RNAs 3* and 7*, we took advantage of the fact that in strain atpAΔ3, RNA 3 accumulates, but RNA 7 does not (Fig. 1A). The 3'-ends of psbI-containing transcripts were again mapped by RNase
Fig. 1. Analysis of psbI 3′-end formation. A, total RNA from the indicated strains was subjected to gel electrophoresis and filter hybridization as described under “Experimental Procedures,” using as probes psbI and psbA coding regions, the latter as a loading control. RNA species are identified by numbers as indicated in the diagram. RNAs denoted by asterisks (3*, 6*, and 7*) are unique to the crp3 background and are discussed under “Results.” The diagram is adapted from Draper et al. (24), and indicates the extent of the psbI promoter deletion in strain atpAΔ3. Bent arrows represent promoters, and RNA sizes are given in kilobases. B, RNase protection was performed on the psbI-cemA intergenic region using the uniformly labeled antisense RNA probe marked by a heavy line in the diagram and total RNA from the strains indicated at the top of the gel. Lane probe is probe alone; lane tRNA is a reaction containing 10 μg of yeast tRNA instead of Chlamydomonas RNA. The protected bands marked readthrough, novel, and wt are discussed under “Results.” They result from protection of the RNAs listed in parentheses. The sizes of the protected bands are 424 nucleotides (readthrough), ~180 nucleotides (novel); and ~80 nucleotides (wt). The mapped wild-type (wt) and novel 3′ termini are indicated by vertical arrows between psbI and cemA. bp, base pairs.

Modified Processing or Stability of the cemA 3′-End—The cemA transcript does not accumulate in a monocistronic form, but only in polycistronic mRNAs transcribed from the atpA and psbI promoters (Fig. 1). Fig. 2 (diagram and A) highlights the four cemA-containing transcripts, which in wild-type cells are atpA-psbI-cemA-athP (RNA 1), atpA-psbI-cemA (RNA 2), psbI-cemA-athP (RNA 5), and psbI-cemA (RNA 6). In the crp3 background, a substantial decrease in the levels of RNAs 2 and 6 was observed; these are the two transcripts that terminate between cemA and atpH. When gels were run longer to increase resolution (see Fig. 4A), it was clear that the reduction in RNA 6 abundance was accompanied by an accumulation of a slightly larger species (RNA 6*) that, based on its estimated size, had its 3′-end within the atpH coding region. Furthermore, the reduction in RNA 6 was accompanied by an increase in RNA 5, consistent with a precursor-product relationship.

To discern whether 3′-end maturation downstream of cemA is modified in crp3 and to detect any residual RNA 2, RNase protection was performed using the uniformly labeled antisense RNA probe corresponding to the cemA-athP intergenic region, as shown in Fig. 2 (diagram). Fig. 2B shows the results of this experiment. Two major species were protected by RNA from wild-type cells, the shorter one representing the 3′-ends of RNAs 2 and 6 and the longer one representing RNAs 1 and 5,
which contain atpH (readthrough). In the mutant background (crp3), the cemA 3'-end accumulated to a lower level, which was expected since RNA 6 was clearly reduced in abundance. To examine RNA 2, 3'-ends between cemA and atpH were mapped in strain atpAΔ3-crp3, which fails to accumulate RNAs 5 and 6 due to a deletion of the psbI promoter. In atpAΔ3 cells, the amount of probe protected by cemA 3'-ends was reduced relative to wild-type cells. This is consistent with our previous observation that whereas RNA 2 increases in abundance in atpAΔ3 cells relative to wild-type cells (Fig. 1A), this increase is not in proportion to the decline in RNA 6 (see Fig. 6 in Ref. 24).

In atpAΔ3-crp3 cells, a further reduction in probe protected by cemA 3'-ends compared with the wild-type sibling (atpAΔ5) was observed; however, the protected product was clearly present. This suggests that although not readily detectable on RNA filter blots, RNA 2 does accumulate to some extent in the crp3 background. However, unlike psbl, no heterogeneity in the size of the protected band was seen, suggesting that any processing that takes place between cemA and psbl occurs at the same site as in wild-type cells.

It should be noted that the size of the probe protected by RNA 8 would be only 31 nucleotides and therefore not visible in this experiment. Furthermore, the novel RNA 6*, which apparently has a 3'-end within the atpH coding region, would fully protect the probe and contribute slightly to the band labeled readthrough.

The psbl-cemA intergenic region confers correct or modified 3'-end processing to a chimeric mRNA, but the cemA-atpH region does not. To test whether the psbl-cemA and cemA-atpH intergenic regions contain information sufficient for the recognition of mRNA processing or stability functions altered in crp3, we tested the ability of these regions to serve as RNA-processing sites in the context of a bacterial aadA reporter gene engineered to be expressed in Chlamydomonas chloroplasts (30). Fig. 3 (diagram) shows the configuration of these four constructs, in which the intergenic regions were inserted in the two possible orientations between the aadA coding region and the Chlamydomonas chloroplast rbcL 3'-UTR. The promoter and 5'-UTR were from the Chlamydomonas chloroplast petD gene. The original petD-aadA-rbcL cassette produces transcriptionally competent mRNAs, which confer spectinomycin and streptomycin resistance to transformed cells (25). We rationalized that if the inserted intergenic sequences were sufficient to promote 3'-end formation, a shorter mRNA ending at the processing site would accumulate. Otherwise, a longer mRNA that represents processing at the rbcL 3'-end would accumulate. In either case, the mRNA was expected to be stable and to confer antibiotic resistance. Indeed, spectinomycin-resistant transformants were recovered when this construct was introduced into both the wild-type (CRP3) and crp3 mutant strains, and these transformants contained the desired mRNA configurations.

Typical RNA accumulation patterns from the transformants and control strains are shown in Fig. 3. The middle panel shows results with an atpB probe. As expected, no discrete transcript accumulated in atpBΔ26, the wild-type recipient strain for the transformation (see “Experimental Procedures”), whereas a low amount of a discrete transcript was detected in the other recipient, atpBΔ26-crp3 (the crp3 mutation stabilizes the normally unstable atpB transcript). In all the transformants, atpB mRNA accumulation was wild-type since the atpBΔ26 deletion had been replaced with wild-type sequences during transformation (see diagram in Fig. 3).

Fig. 3 (lower panel) shows results with a cemA probe. This probe provided verification of the nuclear background; lanes with a low level of RNA 2 and a high level of RNA 5 carry the crp3 mutation. Probing with aadA, as shown in the upper panel, revealed the transcripts produced from the chimeric genes. For psbl insertions downstream of the aadA coding region, different results were obtained depending on the orientation of the insertion. In the (+)-orientation and in the wild-type background, a mRNA that corresponds to processing in the psbl 3'-UTR accumulated, indicating that the psbl-cemA intergenic region carries sufficient information to direct maturation of the psbl 3'-end. In the crp3 background, a slightly larger transcript accumulated. Based on its size, this transcript most likely terminates at the novel psbl 3'-end as an RNA 7° (Fig. 1A). However, there was little evidence for accumulation of aadA-psbl transcripts with the wild-type 3'-end. These results indicate that for the psbl 3'-end, the psbl-cemA intergenic region included in these constructs includes sequences sufficient for processing and stability in the crp3 background.

In transformants containing the psbl 3'-UTR in the (-)-orientation, little or no RNA processed at the psbl 3'-end could be detected. Mainly the longer mRNA processed at the rbcL 3'-end accumulated, and the crp3 mutation had no effect. This indicates that the psbl 3'-UTR functions in an orientation-dependent manner, as has been reported previously for other 3'-UTRs in Chlamydomonas chloroplasts (18, 19). The longer transcripts were also seen when the psbl 3'-UTR was in the (+)-orientation, although in a reduced amount. This is consistent with inefficient transcription termination and incomplete processing. Transcription termination at Chlamydomonas
Chlamydomonas Chloroplast mRNA 3' End Formation

![Diagram](image)

**Fig. 4. Suppression of RNA instability in other atpB 3'-UTR deletions by crp3.** To analyze suppression of the atpBΔ24 (A) and atpBΔ27 (B) deletions, total RNA from the indicated strains (a-d are tetrad products from the cross shown above them) was subjected to gel electrophoresis and filter hybridization as described under “Experimental Procedures.” Using an atpB coding region probe, psbA as a loading control, or cemA to determine crp3 genotype. The crp3 genotype is indicated as w (wild-type, no suppression) or m (mutant, suppresses atpBΔ26). RNA species in the cemA panel are indicated by numbers and refer to the diagram. The approximate locations of the 5' termini of the atpB 3'-deletions in each strain are indicated at the bottom center. wt, wild-type.

chloroplast 3'-inverted repeats has previously been reported to be inefficient (25). However, previous examples of chimeric constructs with two adjacent inverted repeats resulted in accumulation of only the shorter transcript, suggesting that the 3'-psbA processing site may be less efficient than those downstream of atpB (27).

For strains containing the chimeric constructs with the cema-atpH intergenic region downstream of aadA, results were identical regardless of the orientation or nuclear background. In each case, only the longer mRNA, processed at the rbcL 3'-end, could be detected. Therefore, the cemA-atpH intergenic region is not sufficient for correct 3'-end maturation in this context. Possibly, additional sequences within the atpH coding region are required to confer processing activity. Indirect evidence for this is that the new RNA 60, unique to crp3 and discussed above, may terminate within the atpH coding region, suggesting that a determinant recognized by crp3 resides in this region.

The crp3 Mutation Can Suppress Other 3'-UTR Deletions in the atpB Gene—Various deletions in the atpB 3'-UTR that eliminate part or all of the potential stem-loop structure cause mRNA heterogeneity and instability (15, 21). The crp3 mutation was isolated as a suppressor of one of these deletions, atpBΔ26. To address the question of whether crp3 specifically suppresses the atpBΔ26 deletion, e.g., by a sequence-specific mechanism, we crossed atpBΔ26-crp3-mt- to two mt- strains (atpBΔ24 and atpBΔ27) that have different deletion end points in the atpB 3'-UTR. Like atpBΔ26, the deletions remove part of the stem-loop structure; however, the sequence context surrounding the deletion is different. The 5'-deletion end points are shown schematically in Fig. 4 (diagram). Because the chloroplast genome is inherited from the mt- parent, all four tetrad progeny would inherit the atpBΔ24 or atpBΔ27 deletion, whereas the mutant and wild-type alleles of CRP3 would segregate 2:2. PCR was used to verify that chloroplast DNA was indeed inherited from the mt- strain, and at least six complete tetrads were obtained from each cross.

Fig. 4A shows results from a representative tetrad of the cross atpBΔ27 × atpBΔ26-crp3. A psbA probe (middle panel) served as a loading control, and a cemA probe (lower panel) identified the progeny carrying the crp3 mutation (a and b in this tetrad). An atpB probe (upper panel) showed a clear 2:2 segregation for the accumulation of a discrete atpB transcript, which was present only in the crp3 mutant progeny. Therefore, crp3 suppresses the partial absence of a 3'-stem-loop in the atpBΔ27 deletion. The intensity of the atpB transcript in atpBΔ27-crp3 suggests that suppression is even more effective than with atpBΔ26, perhaps because atpBΔ27 retains part of the stabilizing secondary structure.

Fig. 4B shows results obtained in a parallel experiment with atpBΔ24. It is clear from the parental strain that atpBΔ24 accumulates a low amount of a wild-type sized atpB transcript (see also Ref. 21; Fig. 4B), although the deletion into the 3'-stem-loop is larger than the one in atpBΔ27. Because these strains were generated by bi-directional Bal31 deletion, we explain this difference by a fortuitous combination of flanking sequences. Accumulating atpB transcripts of representative tetrad progeny from the cross atpBΔ24 × atpBΔ26-crp3 show a 2:2 segregation for an abundant, discrete transcript. As in the case of atpBΔ27, this phenotype is linked to crp3 as shown by the pattern of cemA hybridization (multiple complete tetrads were analyzed for each cross). These results clearly show that in the case of the atpB 3'-UTR, crp3 can suppress different deletion mutations and thus does not recognize a sequence specific to atpBΔ26.

The atpB mRNAs Have Different 3' Termini in the atpBΔ24, atpBΔ26, and atpBΔ27 Strains—The atpB transcripts in atpBΔ24-crp3, atpBΔ26-crp3, and atpBΔ27-crp3 all had similar mobilities on RNA gel blots. Since each had a different deletion in the atpB 3'-UTR, this suggested that 3'-end formation was occurring at different sequences, rather than at a common cryptic 3'-end maturation site downstream of all of the 3'-deletion end points. To investigate this, the 3'-ends of atpB mRNAs in each of these strains were mapped by RNase protection. The sizes of protected fragments were determined using a DNA sequencing ladder, allowing us to estimate 3'-end locations with an error of approximately ±5 nucleotides.

The results in Figs. 5 (A and B) show that two and one major atpB 3'-termini were found in atpBΔ24-crp3 and atpBΔ27-crp3, respectively. These ends were also detected in the wild-type (CRP3) background, although at a much lower level; we previously reported similar results for atpBΔ26 (22). Thus, the recessive crp3 mutation appears to selectively stabilize minor RNA species also present in the unsuppressed (CRP3) siblings. In addition to the major protected species, numerous minor bands including the fully protected probe (minus vector sequences) were detected. These bands presumably correspond to species that are longer or shorter than the major discrete transcripts and can be seen in the filter hybridizations shown in Fig. 4. The existence of two major protected species for atpBΔ24-crp3 (Fig. 5B) suggests that two discrete transcripts should be seen on filter blots such as the one in Fig. 4B.

However, the size difference between the two is only ~85 nucleotides, and they were not resolved on that gel.

The mapped 3'-ends of wild-type atpB mRNA and those of the deletion mutants in the crp3 background are presented in Fig. 5C. The 3'-ends in all of the deletion mutants are clustered in an ~195-nucleotide region >2 kilobases downstream of the atpB translation termination codon in wild-type cells. However, because of the extents of the deletions, the most proximal 3'-ends in atpBΔ24, atpBΔ26, and atpBΔ27 are ~70, 100, and
The crp3 Mutation Is Non-allelic to Another Mutation That Affects Accumulation of mRNAs from the atpA Gene Cluster—Another Chlamydomonas nuclear mutant has been isolated, in which the accumulation of transcripts from the atpA gene is altered (31). In strains carrying ncc1, the accumulation of monocistronic atpA mRNA (RNA 4) is strongly reduced, although there is no net effect on the level of the atpA gene product, the α-subunit of the ATP synthase. Thus, like crp3, ncc1 does not cause a non-photosynthetic phenotype. Although ncc1 and crp3 affect different RNAs of the atpA gene cluster, the two mutations could represent alleles at the same locus or interact in some way. To test this possibility, ncc1-mt− was crossed with atpBΔ26-crp3-mt−. Tetrad progeny of this cross were scored by RNA filter hybridizations using atpA and atpB probes, as shown in Fig. 6. A reduction in RNA 4 relative to RNA 3 was taken as evidence that the mutant allele of ncc1 was present, whereas a reduction in RNAs 2 and 3 and a discrete atpB transcript indicated that the strain carried crp3 (all progeny carry the atpB 3′-UTR deletion, atpBΔ26, contributed by the mt− parent).

The simplest interpretation of the data is that crp3 and ncc1 represent independently segregating loci and that the two phenotypes are additive. The majority of the 10 complete tetrads obtained appeared to be tetratypes, such as the one shown in Fig. 6. Tetrad progeny a and d are phenotypically similar to the ncc1 and crp3 parents, respectively. Product c appears to have wild-type alleles at both loci and resembles atpBΔ26. Product b is most likely the double mutant, which exhibits reduced accumulation of RNAs 2–4, but accumulates a discrete atpB transcript, although RNA 2 is not well resolved in this experiment. These results indicate that at least two nuclear gene products participate in the maturation and/or stability of mRNAs transcribed from the atpA gene cluster.
Specificity of the crp3 Mutation—We have reported here a detailed analysis of a Chlamydomonas nuclear mutation that affects the maturation and/or stability of multiple chloroplast mRNAs. Although crp3 is a recessive and therefore probably a loss-of-function mutation, its primary phenotype is the appearance of new RNAs in the chloroplast. These include a discrete atpB transcript lacking a 3′-stem-loop, processing intermediates between the 3′-end of petD and trnR (22), as well as new 3′-ends for psbI- and cemA-containing mRNAs and the stabilization of additional atpB 3′-UTR deletions, as reported here. All of these new RNAs may be produced transiently in wild-type cells; thus, CRP3 might encode or activate a ribonuclease that is responsible for their degradation. Alternatively, CRP3 could encode an RNA-binding protein that is essential for correct 3′-processing and/or that regulates RNA stability; candidates for such factors have arisen from in vitro biochemical studies using chloroplast protein extracts (32–36). In two cases, these chloroplast RNA-binding proteins also have been shown to have ribonuclease activity (32, 37), which is yet another possibility for CRP3.

Two features of crp3 distinguish it from most other nuclear mutations that affect Chlamydomonas chloroplast RNA metabolism. First, we have shown that the target of crp3 is the 3′-UTR, at least for psbI and most likely for atpB and petD as well (22). Other mRNA stability mutations analyzed in Chlamydomonas act on the 5′-UTR (8, 9), although nec1, which we determined is not allelic to crp3, appears to act on the atpA 3′-UTR (31). Although we have examined only a small number of mRNAs in the crp3 background, we have never seen any change in mRNA 5′-termini. A second feature is that crp3 is not gene-specific, unlike other mutations affecting RNA stability in Chlamydomonas (8, 38–41). It is likely that additional nuclear loci with functions and mutant phenotypes similar to crp3 will be found. Since crp3 was isolated as a suppressor of a light-sensitive growth phenotype caused by unstable RNA, this type of screen may prove useful in the future.

In vascular plants, and in particular in maize and Arabidopsis, nuclear mutants have been studied in which multiple chloroplast RNAs are affected. For example, crp1 in maize affects both RNA processing and translation (3), and the crs mutants generally affect splicing of numerous mRNAs (5). In Arabidopsis, several mutants have been isolated that display highly altered RNA accumulation patterns, which are difficult to explain by a single target for the product of the affected nuclear locus (4, 6, 7). These Arabidopsis mutants appear to be even more pleiotropic than crp3, although it is possible that other alleles of crp3 could have more variable phenotypes. The dissimilarity between the type of mutation most often isolated in Chlamydomonas and that most frequently found in vascular plants may simply be a consequence of the type of genetic screens available or the relatively small number of mutants that have been carefully studied to date, or it could reflect fundamental differences in gene expression strategies. It should be noted that nearly all mature Chlamydomonas chloroplast transcripts are monocistrionic, whereas polycistrionic transcripts are common in vascular plants. However, this may simply be a consequence of efficient processing of polycistrionic transcripts in Chlamydomonas, as discussed further below, rather than a difference in chloroplast gene expression mechanisms.

Accumulation of New mRNA 3′ Termini—Loss-of-function mutations in some bacterial ribonuclease genes cause phenotypes that may be related to crp3. For example, when transcripts of the dicistrionic rpoO-pnp operon of Escherichia coli are examined in RNase III or RNase E temperature-sensitive mutants at the nonpermissive temperature, RNAs with new 5′ or 3′ termini accumulate (42). This occurs because the primary transcripts are rapidly processed in the wild-type background. Similar phenomena have been reported for other RNAs, e.g. puf and unc (43, 44). Thus, crp3 may be a partial loss-of-function mutant, in which the accumulation of intermediates is a consequence of inefficient processing or degradation by the mutant ribonuclease. This in turn suggests that processing of primary transcripts may be common in Chlamydomonas chloroplasts and indeed may even be more efficient than in vascular plants, where partially processed RNAs accumulate readily (e.g. Refs. 45–47). For example, the Chlamydomonas petD transcript matures rapidly, and its processing pathway can be discerned only from mutant strains (48, 49). Furthermore, 3′-processing of transcripts such as atpB is efficient both in vivo and in vitro (27).

Definition of crp3 Targets—We attempted to define a molecular target for the crp3 product in two ways. First, we inserted two putative targets into a chimeric reporter gene and tested whether accumulation of the reporter gene transcripts responded to the crp3 genotype. As shown in Fig. 3, a psbI-3′-UTR sequence did respond as expected, whereas a cemA 3′-UTR sequence did not. This implies that in the case of cemA 3′-end formation, RNA structures formed through long-range interactions, rather than a specific primary sequence, may constitute the key elements. A second hint at a crp3 target came from its lack of specificity in suppressing atpB 3′-UTR deletions (Figs. 4 and 5). Since each RNA differed at its 3′-end but contained the same coding region, one interpretation of the ability of crp3 to allow stable mRNA accumulation is that an element within the atpB coding region determines the atpB 3′ terminus. This would also account for the fact that each stable RNA in the crp3 background was of approximately the same length.

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