Molecular cloning of the maize gene crp1 reveals similarity between regulators of mitochondrial and chloroplast gene expression

Dianna G.Fisk, Macie B.Walker and Alice Barkan

Institute of Molecular Biology, University of Oregon, Eugene, OR 97403-1229, USA

1Corresponding author
e-mail: abarkan@molbio.uoregon.edu

The maize nuclear gene crp1 is required for the translation of the chloroplast petA and petD mRNAs and for the processing of the petD mRNA from a polycistronic precursor. In order to understand the biochemical role of the crp1 gene product and the interconnections between chloroplast translation and RNA metabolism, the crp1 gene and cDNA were cloned. The predicted crp1 gene product (CRP1) is related to nuclear genes in fungi that play an analogous role in mitochondrial gene expression, suggesting an underlying mechanistic similarity. Analysis of double mutants that lack both chloroplast ribosomes and crp1 function indicated that CRP1 activates a site-specific endoribonuclease independently of any role it plays in translation. Antibodies prepared to recombinant CRP1 were used to demonstrate that CRP1 is localized to the chloroplast stroma and that it is a component of a multisubunit complex. The CRP1 complex is not associated detectably with either chloroplast membranes or chloroplast ribosomes. Models for CRP1 function and its relationship to other activators of organelar translation are discussed.

Keywords: chloroplast/mitochondria/Mutator/RNA processing/translational control

Introduction

The expression of many chloroplast genes is induced during the formation of photosynthetic cell types in multicellular plants (reviewed by Mullet, 1988, 1993; Sugita and Sugiura, 1996). Because chloroplast genomes lack apparent regulatory genes, chloroplast gene expression presumably is modulated by regulatory proteins that are encoded in the nucleus. A number of regulatory genes have been identified by mutations in the nuclear genome that cause defects in chloroplast gene expression (reviewed by Rochaix, 1996; Goldschmidt-Clermont, 1998; Leon et al., 1998). Although the mutant phenotypes demonstrate that such genes function, either directly or indirectly, in gene expression, the biochemical roles of their products are as yet unknown. Other potential regulatory proteins have been identified by virtue of their ability to bind chloroplast nucleic acids in vitro; these have proposed roles in the regulated transcription, translation and stability of chloroplast mRNAs (reviewed by Sugita and Sugiura, 1996). Enzymes that function as endo- and exo-ribonucleases have been purified from chloroplasts (reviewed by Barkan and Stern, 1998) and may play regulatory roles as well.

Previously, we described a nuclear gene in maize, crp1, mutations in which lead to the complete absence of the cytochrome b6f complex (cyt b6f) and a decrease in the abundance of the photosystem I (PSI) complex (Barkan et al., 1994). The loss of cyt b6f results from the failure to translate the petA mRNA (encoding cytochrome f) and from a decrease in the rate of synthesis of the petD gene product (subunit 4 of cyt b6f). The defect in petD mRNA translation is accompanied by a failure to cleave petD-coding RNA from upstream sequences in a polycistronic precursor RNA. The simultaneous loss of petD mRNA processing and translation in crp1 mutants is consistent with the notion that the processing event increases the efficiency with which petD mRNA is translated.

The molecular phenotype of crp1 mutants can be explained in several ways. It is possible that the crp1 gene product (CRP1) activates petD mRNA processing, petD translation and petA translation independently. Alternatively, CRP1 might directly modulate just one step in an interdependent network of molecular interactions. For example, CRP1 might activate the processing of the petD mRNA directly, and this processing event might, in turn, be needed for the efficient translation of the petD and petA mRNAs. Elucidation of the biochemical role of CRP1 will further our understanding of the interactions between the translation and RNA processing machineries that co-exist in the chloroplast stroma.

We now describe genetic data indicating that the failure to process petD mRNA in crp1 mutants does not arise from a defect in the translation of the petD mRNA. We have cloned the crp1 gene and cDNA and found an intriguing similarity between CRP1 and proteins in fungi required for the translation and metabolism of the mitochondrial cox1 mRNA. Fractionation experiments revealed that CRP1 is a component of a multisubunit complex located in the chloroplast stroma and that it is not associated detectably with chloroplast ribosomes.

Results

The defect in petD RNA processing in crp1 mutants is not a consequence of a defect in translation

The synthesis of the chloroplast petD gene product is reduced dramatically in crp1 mutants. This is accompanied by the failure to cleave a monocistronic petD mRNA from polycistronic precursors that include upstream sequences (Barkan et al., 1994). We considered the possibility that the defect in petD mRNA processing in crp1 mutants was a secondary effect of a more direct block in petD transla-
tion. This seemed plausible because the processing site is only 140 nucleotides from the start codon and there is evidence that bound ribosomes can influence RNA processing (Braun et al., 1998; Semrad and Schroeder, 1998). For example, it is possible that CRP1 is required for ribosomes to bind petD RNA and that a bound ribosome is required to generate an RNA structure recognized by processing enzymes. In this case, crp1 mutants would fail to process the RNA because the requisite ribosome binding event did not occur. Alternatively, if crp1 mutations prevent ribosomes from clearing the petD start codon, the trapped ribosome subsequently could block processing by influencing RNA structure or masking sequences needed for processing.

We tested the first possibility by analyzing petD RNA processing in two maize mutants lacking detectable plastid ribosomes (wl and iqaip). petD RNA processing was normal in both mutants, indicating that an initiating ribosome is not required for processing (Figure 1). To address the second possibility, w1/crp1 double mutants were constructed. The absence of processed petD mRNA in w1/crp1 mutants (Figure 1) indicates that petD RNA processing is dependent upon the wild-type allele of crp1 even in the absence of plastid ribosomes. Therefore, the failure to process petD RNA in crp1 mutants is not caused by the aberrant stalling of ribosomes. Taken together, these results indicate that ribosomes play no role in the processing of the petD mRNA and that crp1 influences petD RNA processing independently of any effect it might have on petD translation.

**Molecular cloning of the crp1 gene**

The reference allele of crp1 (crp1-m1::MuDR, hereafter referred to as crp1-m1) arose in a maize line harboring active Mutator (Mu) transposons and exhibited somatic instability, suggesting that it was caused by the insertion of a Mu transposon. To identify a Mu insertion genetically linked to crp1, DNA from mutant seedlings derived from diverse branches of the crp1 pedigree was analyzed by Southern hybridization, using probes corresponding to each member of the Mu family (Chandler and Hardeman, 1992). A MuDR probe detected a 3.7 kb HindIII fragment that was present in all mutant seedlings but absent in closely related homozygous wild-type plants (data not shown). This insertion was very tightly linked to crp1 (<2 map units).

The 3.7 kb HindIII fragment containing MuDR sequence was cloned from a size-enriched genomic library of crp1-m1 mutant DNA (clone A in Figure 2A). As expected, one end of clone A mapped to a HindIII site within MuDR. The 1033 bp of genomic DNA flanking the MuDR insertion in clone A was subcloned (clone B in Figure 2A). To prove that this sequence was from the crp1 gene, we took advantage of hcf111, an independently derived crp1 allele that also arose in a Mu-active maize line (Cook and Miles, 1989; Barkan et al., 1994). Genomic Southern blots probed with Mu1 revealed a 4.8 kb BamHI fragment that was genetically linked to hcf111 and that co-migrated with a band detected by clone B (data not shown). These findings suggested that a Mu1 element in hcf111 mutants lies within sequences corresponding to clone B. To explore that possibility, PCR was used to probe the structure of hcf111 DNA in this region. Primers 2 and 9, whose sequences flank the cloned insertion site, amplified the predicted 498 bp fragment from wild-type DNA (Figure 2B). As predicted, this primer pair failed to amplify DNA in homozygous crp1-m1 mutants because intact Mu elements prevent amplification by flanking primers (Voelker et al., 1997). This same primer pair failed to
allele due to the presence of a 498 bp fragment of wild-type DNA and did not amplify either mutant Arrows represent primer-binding sites. Primers 2 and 9 amplified a 353 bp fragment from crp1-m1 and a 116 bp fragment from hcf111 insertion sites. The box identifies the 9 bp target site that was duplicated upon insertion of the transposable element (Chandler and Hardeman, 1992). Nucleotide positions are numbered with respect to the initiation codon.

amplify DNA from homozygous hcf111 plants, supporting the idea that hcf111 mutants also have a Mu element mapping between those primer-binding sites. To map the Mu insertion site in hcf111, primer 2 and primer 9 were each used in conjunction with the Mu primer, whose sequence corresponds to the ends of the terminal inverted repeats of all Mu transposons. As expected, both of these primer pairs failed to amplify DNA from homozygous wild-type plants. With homozygous crp1-m1 DNA as template, the primer 2–Mu and primer 9–Mu primer pairs amplified the predicted 262 and 353 bp fragments, respectively (Figure 2B). With homozygous hcf111 DNA as template, these same primer pairs amplified fragments of 116 and 499 bp, confirming the presence of a Mu element in this region of the hcf111 genome. DNA sequence analysis of the PCR products revealed sequence elements specific to the Mu1 member of the Mutator family. These results demonstrated that a Mu1 insertion in hcf111 mutants maps 146 bp away from the MuDR insertion in crp1-m1 mutants. The nucleotide sequence of each insertion site is shown in Figure 2C.

Clone B detected an mRNA in leaf tissue (data not shown) and was used to obtain a partial cDNA clone from a seedling leaf cDNA library. This cDNA hybridized to a 2.7 kb RNA in wild-type leaves that was absent from both hcf111 and crp1-m1 mutant leaves (Figure 3). That two independent mutant alleles had Mu insertions mapping very near to one another, and that both insertions disrupted the expression of this transcript, provides very strong evidence that this transcript encodes the crp1 gene product. crp1-m1 is likely to be a null allele because it does not accumulate detectable crp1 mRNA (Figure 3) and because the phenotype of plants that are hemizygous for crp1-m1 (crp1-m1/0) is not detectably more severe than that of homozygous (crp1-1/crp1-1) mutants (data not shown).

**CRP1 is related to fungal proteins that mediate post-transcriptional steps in mitochondrial gene expression**

The crp1 cDNA obtained from the cDNA library was several hundred nucleotides shorter than the 2.7 kb mRNA detected by RNA gel blot hybridization. The remaining cDNA sequence was obtained by 5' RACE. The full-length cDNA sequence contains a continuous open reading frame of 668 codons. The N-terminus of the predicted gene product exhibits features of chloroplast transit peptides (Cline and Henry, 1996): 12 uncharged amino acids are followed by a 50 amino acid segment that is rich in hydroxylated amino acids (32%) and that has an overall basic charge (Figure 4A). The sites of the Mu insertions in crp1-m1 and hcf111 are shown in Figures 2A and 4A. Both insertions disrupt sequences encoding amino acids near the N-terminus of the predicted protein. CRP1 lacks
Fig. 4. Partial amino acid sequence of the deduced *crp1* gene product and its relationship to fungal proteins that control mitochondrial gene expression. (A) Amino acid sequence of the N-terminal 100 amino acids of the deduced *crp1* gene product. The sites of the *Mu* insertions in the mutant alleles are illustrated. The complete cDNA sequence has been entered in DDBJ/EMBL/GenBank (accession No. AF073522). (B) Multiple sequence alignment of a 300 amino acid region of CRP1, Pet309p and Cya5p. The alignment was calculated using ClustalW 1.7 (Thompson et al., 1994) and Boxshade (Bioinformatics group, ISREC, Lausanne, Switzerland).

strongly predicted transmembrane domains, suggesting that it is a soluble protein.

Database searches with the deduced CRP1 sequence revealed near identity with the product of an expressed sequence tag from rice (accession No. C74592) and extensive similarity with the products of a family of distinct expressed sequence tags (ESTs) in *Arabidopsis* (data not shown). These proteins are all related to a salt-inducible protein in tobacco (accession No. U08285) of unknown function that is not localized to the chloroplast (Chang et al., 1996). Only the rice EST is likely to represent a *crp1* ortholog as the other deduced proteins exhibit <40% similarity to CRP1. Although phenotype analysis implicates CRP1 in an endonucleolytic processing event, its size and sequence indicate that it does not correspond to any previously identified chloroplast ribonuclease (Nickelsen and Link, 1993; Hayes et al., 1996; Yang et al., 1996).

An intriguing finding was that the two most closely related genes detected outside of the plant kingdom were *CYA5* in *Neurospora crassa* (accession No. AF002169) and *PET309* in *Saccharomyces cerevisiae* (accession No. L06072), which both function in mitochondrial gene expression (Manthey and McEwen, 1995; Coffin et al., 1997). *PET309* activates *coxl* translation and is required for the accumulation of *coxl* mRNAs from intron-bearing alleles. *CYA5* also activates *coxl* translation; the possibility that it functions in mRNA metabolism as well has not been thoroughly addressed. CRP1, Pet309p and Cya5p are most closely related in a 300 amino acid region (Figure 4B). Pairwise comparisons revealed 37% similarity between Cya5p and CRP1 and 33% similarity between Pet309p and either CRP1 or Cya5p. The facts that mutations in these three genes result in analogous mutant phenotypes and that their gene products are related suggest that these proteins play similar biochemical roles. However, no information is available concerning the biochemical role of Pet309p or Cya5p.

When CRP1 was used to search the COGNITOR database, which catalogs ancient conserved domains in fully sequenced genomes (Tatusov et al., 1997), the four best matches of known function were Pet309p, threonyl-tRNA synthetases, RPM2 (encoding the protein component of yeast mitochondrial RNase P) and AddB, an ATP-dependent nuclease. The similarity with yeast threonyl-tRNA synthetase was considerable, with 31% identity and 46% similarity over a 103 amino acid region (data not shown). The other similarities were less extensive but also involved the segment of CRP1 shown in Figure 4B. That all of these proteins interact with nucleic acids supports the notion that CRP1 plays a direct role in the metabolism and translation of chloroplast mRNAs.

**CRP1 is a component of a multisubunit complex in the chloroplast stroma**

Polyclonal antibodies were raised against a recombinant CRP1 fragment encompassing approximately three-quarters of the full gene product (amino acids 86–583). The antibody detected a protein of ~66 kDa in wild-type leaf tissue that was absent in mutant leaf tissue (Figure 5A). This protein is ~5 kDa smaller than the product of *in vitro* translation of the full-length *crp1* cDNA (Figure 5A). Cleaved N-terminal transit peptides in this size range are typical of chloroplast-localized proteins. CRP1 co-purifies with intact chloroplasts in Percoll gradients (Figure 5B) and is enriched in the chloroplast fraction to at least 2624

D.G.Fisk, M.B.Walker and A.Barkan

**Fig. 4.** Partial amino acid sequence of the deduced *crp1* gene product and its relationship to fungal proteins that control mitochondrial gene expression. (A) Amino acid sequence of the N-terminal 100 amino acids of the deduced *crp1* gene product. The sites of the *Mu* insertions in the mutant alleles are illustrated. The complete cDNA sequence has been entered in DDBJ/EMBL/GenBank (accession No. AF073522). (B) Multiple sequence alignment of a 300 amino acid region of CRP1, Pet309p and Cya5p. The alignment was calculated using ClustalW 1.7 (Thompson et al., 1994) and Boxshade (Bioinformatics group, ISREC, Lausanne, Switzerland).
reduced levels of PSI and cytochrome b6f. The CRP1 mutants. The blot was probed with a polyclonal antibody raised to a translation product of the full-length crp1 cDNA (ivt) was generated with the TNT system (Promega) and electrophoresed in parallel with 10 μg of total leaf protein from wild-type, crp1-m1, hcf111 or thal mutants. The blot was probed with a polyclonal antibody raised to a recombinant CRP1 fragment. The thal mutant, like crp1 mutants, has reduced levels of PSI and cytochrome b6f (Voelker and Barkan, 1995), and is shown to illustrate that the loss of CRP1 is not a consequence of the absence of these complexes. The lower panel shows a portion of the same filter after staining with Ponceau S. (B) Intact chloroplasts purified from wild-type seedlings were fractionated by SDS–PAGE in parallel with an equal quantity (10 μg) of total leaf protein from wild-type and crp1-m1 mutant seedlings. Dilutions of the wild-type leaf and chloroplast samples were included to aid in quantification. The blot was probed sequentially with antibodies to CRP1 and to proteins localized in different cellular compartments: the chloroplast protein 28RNP (Schuster and Gruissem, 1991), the mitochondrial protein MDH and the cytosolic protein PEPCase. (C) Pea chloroplasts were incubated with in vitro-translated radiolabeled CRP1 under conditions that promote import. One aliquot was treated with protease after import to remove extra-chloroplastic protein. Intact chloroplasts were re-isolated and fractionated by SDS–PAGE. The first lane contains the in vitro translation product used as the substrate in the import reactions.

CRP1

**Fig. 5.** CRP1 is a chloroplast-localized protein. (A) The in vitro translation product of the full-length crp1 cDNA (ivt) was generated with the TNT system (Promega) and electrophoresed in parallel with 10 μg of total leaf protein from wild-type, crp1-m1, hcf111 or thal mutants. The blot was probed with a polyclonal antibody raised to a recombinant CRP1 fragment. The thal mutant, like crp1 mutants, has reduced levels of PSI and cytochrome b6f (Voelker and Barkan, 1995), and is shown to illustrate that the loss of CRP1 is not a consequence of the absence of these complexes. The lower panel shows a portion of the same filter after staining with Ponceau S. (B) Intact chloroplasts purified from wild-type seedlings were fractionated by SDS–PAGE in parallel with an equal quantity (10 μg) of total leaf protein from wild-type and crp1-m1 mutant seedlings. Dilutions of the wild-type leaf and chloroplast samples were included to aid in quantification. The blot was probed sequentially with antibodies to CRP1 and to proteins localized in different cellular compartments: the chloroplast protein 28RNP (Schuster and Gruissem, 1991), the mitochondrial protein MDH and the cytosolic protein PEPCase. (C) Pea chloroplasts were incubated with in vitro-translated radiolabeled CRP1 under conditions that promote import. One aliquot was treated with protease after import to remove extra-chloroplastic protein. Intact chloroplasts were re-isolated and fractionated by SDS–PAGE. The first lane contains the in vitro translation product used as the substrate in the import reactions.

CRP1

**Fig. 6.** CRP1 is localized to the chloroplast stroma. Chloroplasts from wild-type leaves were purified, lysed and fractionated as described by Keegstra and Yousif (1986). The same proportion of each fraction was separated by SDS–PAGE and transferred to nitrocellulose membrane. The blot was probed sequentially with antibodies to CRP1 and to proteins specific to different chloroplast compartments: cpn60 (a stromal marker), AtpB (an extrinsic thylakoid marker), OE33 (a marker for the thylakoid lumen) and IM35 (an inner envelope marker).

**Fig. 6.** CRP1 is localized to the chloroplast stroma. Chloroplasts from wild-type leaves were purified, lysed and fractionated as described by Keegstra and Yousif (1986). The same proportion of each fraction was separated by SDS–PAGE and transferred to nitrocellulose membrane. The blot was probed sequentially with antibodies to CRP1 and to proteins specific to different chloroplast compartments: cpn60 (a stromal marker), AtpB (an extrinsic thylakoid marker), OE33 (a marker for the thylakoid lumen) and IM35 (an inner envelope marker).

Because crp1 mutants exhibit translation defects, it seemed plausible that CRP1 might be associated with either ribosomes or polysomes. To address these possibilities, wild-type and w/l leaf extracts were fractionated in sucrose gradients under conditions that maintain polysome integrity and that optimize the resolution of particles >70S (Figure 7A). Although a significant amount of CRP1 was found in fractions containing 30S, 50S and 70S ribosomes (fractions 4–10), CRP1 derived from w/l and wild-type leaves fractionated similarly, arguing that the sedimentation in these fractions does not result from an association with ribosomes. CRP1 was barely detectable in gradient fractions containing polysomes (fractions 11 and above) and, again, the wild-type and w/l extracts behaved similarly (data not shown). When stroma was fractionated in sucrose gradients under conditions that better resolve ribosomal subunits from material <30S, the vast majority of CRP1 was found in a single peak near the top of the gradient; <5% sedimented with 30S–70S particles in both wild-type and w/l leaves (data not shown). Therefore, no more than a minute proportion of CRP1 is tightly associated with polysomes or ribosomes.

To determine whether native CRP1 is monomeric or multimeric, a CRP1-enriched extract was analyzed by size
exclusion chromatography. CRP1 peaked in a fraction containing proteins ranging from 326 to 362 kDa (Figure 7B). No other peaks were detected (data not shown). Because the CRP1 monomer is ~66 kDa, native CRP1 must be part of a multisubunit complex.

Discussion

Numerous nuclear genes that control the translation, processing and stability of chloroplast mRNAs have been identified in both plants and algae by virtue of mutant phenotypes (reviewed by Goldschmidt-Clermont, 1998; Leon et al., 1998), but the nature of their genes and products has not been reported. A biochemical dissection of the roles of these gene products and their interactions will require the molecular cloning of the corresponding genes. We described the molecular cloning of the maize nuclear gene crp1, which is required for the translation and processing of a small subset of chloroplast mRNAs. Interestingly, CRP1 bears significant homology to the fungal proteins Pet309p and Cya5p, which activate the translation of the mitochondrial cox1 mRNA. These functional and structural homologies suggest that these proteins are likely to share mechanistic similarities, but no biochemical studies of Pet309p and Cya5p have been reported.

Role of CRP1 in mRNA processing and translation

To understand the biochemical role of CRP1, it is necessary to distinguish its direct effects from its pleiotropic effects. It is possible that CRP1 influences petA translation, petD translation and petD RNA processing independently, or that CRP1 directly influences just a subset of these processes. Experiments described here eliminate one possible causal relationship: that the petD mRNA processing defect in crp1 mutants is a consequence of aberrant interactions between ribosomes and the petD mRNA. We found that (i) processing occurs normally in mutants lacking detectable chloroplast ribosomes and (ii) petD mRNA processing requires crp1 function even when plastid ribosomes are absent, indicating that processing in crp1 mutants is not blocked by stalled ribosomes. Therefore, crp1 influences petD RNA processing independently of any effect it might have on petD translation.

We previously proposed that the processing of the petD mRNA might increase its translational efficiency by destabilizing secondary structures that mask its ribosome-binding site (Barkan et al., 1994). This remains a viable model, and the processing defect may be the only factor contributing to the decrease in petD translation observed in crp1 mutants. It is also possible, however, that crp1 activates petD translation in some other fashion.

The translation of the petA and petD mRNAs could also be interdependent. A precedent for such an interaction has been discovered in Chlamydomonas chloroplasts, in which the translation rate of the petA mRNA is reduced 5-fold in mutants with deletions of the petD gene (Kuras and Wollman, 1994). By analogy, it is possible that the defect in petA translation in crp1 mutants is a consequence of the decrease in petD protein synthesis. However, several observations support the idea that CRP1 activates petA translation independently of any role it plays in petD expression. First, in crp1 mutants, the defect in petA translation (>20-fold reduction) is much more severe than the defect in petD translation (~10-fold reduction) (Barkan et al., 1994). Secondly, it has been found recently that deletion of the petD gene in tobacco chloroplasts does not lead to the dramatic loss of petA mRNA from polysomes that is seen in crp1 mutants (R.Monde and D.Stern, personal communication). It is likely, then, that in higher plants, petA translation initiation does not require concomitant petD protein synthesis, implying that the petA translation defect in crp1 mutants is not a consequence of the reduced rate of petD translation.

Taken together, these observations lead us to favor a model in which CRP1 activates petD mRNA processing and petA mRNA translation independently. There is ample precedent for organellar proteins functioning dually in RNA metabolism and translation. The yeast gene PET54 is required both for the translation of the mitochondrial cox3 mRNA (Brown et al., 1994) and for the splicing of
a group I intron in the *cox1* pre-mRNA (Valencik et al., 1989). The yeast gene *PET309*, which is related in sequence to *crp1*, is required both for the translation of the *cox1* mRNA and for the accumulation of *cox1* mRNAs derived from intron-bearing alleles (Manthey and McEwen, 1995). Two mitochondrial tRNA synthetases also participate in intron splicing (Lambowitz and Perlman, 1990). The evolution of such dual-function proteins was probably facilitated by the fact that in organelles, translation and mRNA biogenesis occur in the same compartment. Thus, components of the organellar translation machinery that interact with RNA could easily have been recruited to control mRNA metabolism (and vice versa).

The basis for the reduced level of PSI in *crp1* mutants has not been firmly established, but it is likely to be due to a defect in the expression of one or more of the seven chloroplast genes required for PSI accumulation (Maier et al., 1995; Ruf et al., 1997). Previously, we reported that the size, abundance and polysome association of the *psaA*, *psaB*, *psaI* and *psaL* mRNAs in *crp1* mutants are indistinguishable from wild-type (Barkan et al., 1994). Polysome analysis did suggest, however, that *crp1* may be required for the normal translation of *psaC* mRNAs (Barkan et al., 1994). We have now analyzed the expression of the *ycf3* and *ycf4* genes recently shown to be involved in PSI biogenesis (Boudreau et al., 1997; Ruf et al., 1997). No defect was detected in the size, abundance or polysome association of the *ycf3* mRNA (data not shown). Transcripts of *ycf4* are found solely on the same polycistronic mRNAs as *petA* (data not shown). These polycistronic mRNAs accumulate normally in *crp1* mutants but are associated with very few ribosomes (Barkan et al., 1994). Thus, it is plausible that *ycf4* may also be translated at reduced rates, contributing to the loss of PSI in *crp1* mutants.

**Relationship of CRP1 to other translational activators in organelles**

Nuclear genes that activate the translation of the *Chlamydomonas* chloroplast *psbA* (Girard-Bascou et al., 1992; Yohn et al., 1998), *psbC* (Rochaix et al., 1989; Zerges and Rochaix, 1994), *psaB* (Stampacchia et al., 1997), *atpA* (Drapier et al., 1992) and *psbD* mRNAs (Kuchka et al., 1988) have been identified on the basis of mutant phenotypes. RNA-binding proteins detected with *in vitro* binding assays have also been proposed to mediate chloroplast translation (Danon and Mayfield, 1991; Zerges and Rochaix, 1994; Hauser et al., 1996; Hiroyuki and Sugiuira, 1996). However, results presented here provide the first sequence and biochemical analysis of a protein with a genetically defined role in chloroplast translation.

Our finding that CRP1 is not associated detectably with chloroplast membranes is of particular relevance to current models for translational control in chloroplasts. Several RNA-binding proteins in *Chlamydomonas* are tightly associated with a chloroplast membrane fraction (Zerges and Rochaix, 1998). This has led to hypotheses that invoke the targeting of mRNA to the membrane via membrane-bound RNA-binding proteins as an essential step in the translation and/or assembly of integral thylakoid membrane proteins (Rochaix, 1996; Yohn et al., 1998; Zerges and Rochaix, 1998). Extensive data in support of an analogous model exist for yeast mitochondria. Translation of mitochondrial mRNAs encoding inner membrane proteins requires gene-specific translational activators that are tightly associated with the inner membrane (reviewed in Fox, 1996). The accumulation but not the synthesis of the inner membrane proteins Cox2p and Cox3p depends upon determinants in the 5′-untranslated regions (5′-UTRs) of their mRNAs (Sanchirico et al., 1998). Because it is the 5′-UTRs that interact with the membrane-bound translational activators, it has been proposed that the translational activators play a dual role, functioning also to target the nascent polypeptides, via their mRNAs, to the membrane for assembly with their partners.

*Pet309p*, which is required for *cox1* translation and is related to CRP1, likewise is associated with the inner mitochondrial membrane (Manthey et al., 1998). However, the predicted transmembrane domain in *Pet309p* lies outside of the region of similarity to CRP1, and the amino acid sequence and fractionation behavior of CRP1 indicate that CRP1 is a soluble protein. This difference between CRP1 and its fungal counterparts is consistent with the notion that the targeting of the *petA* and *petD* gene products to the thylakoid membrane does not involve an mRNA targeting step. Given that chloroplasts have retained three ancestral, prokaryotic mechanisms for targeting proteins to the thylakoid membrane (reviewed by Schnell, 1998), there would seem to be no need for a thylakoid membrane targeting mechanism involving mRNA–protein interactions. It is intriguing that the evolution of an RNA-based targeting mechanism in yeast mitochondria correlates with the loss of the ‘Sec’ and ‘SRP’ membrane targeting mechanisms found in bacteria and chloroplasts (Glick and von Heijne, 1996). Perhaps the evolution of an RNA-based targeting mechanism in mitochondria allowed these ancestral prokaryotic mechanisms to be safely discarded. Whether chloroplasts have also evolved an mRNA-based targeting mechanism despite the maintenance of ancestral Sec and SRP membrane targeting mechanisms remains to be determined.

We found that CRP1 is a component of a multisubunit complex. It is not known yet whether this is a hetero- or homo-multimer. The fact that no more than a minute fraction of CRP1 is associated with ribosomes suggests that associations with the translation machinery are either transient, or are indirect and mediated by other factors. It will be very interesting to discover how the CRP1 complex influences interactions between mRNAs and the translation machinery, and whether the related mitochondrial proteins *Pet309p* and Cya5p function similarly.

**Materials and methods**

**Plant material**

The origin of *crp1-m1* and evidence that it is allelic to *hcf111* were described previously (Barkan et al., 1994). *wil/crp1* double mutants were constructed by intercrossing *wil/+* and *crp1-m1/+* plants, followed by self-pollination of the *F1* progeny. Pale green and albino mutants segregated in the *F2* progeny, which were homozygous for the *crp1* and *wil* mutations, respectively. Double mutants were identified from among the albino (*wil/wil*) progeny by using a PCR assay to genotype the *crp1* locus. Each PCR reaction contained three primers: primers 9 (5′-ACATTGCAATTCTGCTTTCAAC) and 10 (5′-GACAGCCGGAG-3′) flank the Mu insertion and amplify a 426 bp fragment specifically from the wild-type allele. Primer MuTIR (5′-CGCTTCCATTTGCAGCTAATCC) corresponds to the Mu terminal inverted repeat and, in conjunction with primer 9, amplifies a 350 bp fragment.

**Nucleus-encoded chloroplast regulatory protein**
specifically from the mutant allele. Both fragments are amplified from heterozygous tissue. PCR's were carried out in a volume of 50 μl and contained 50 mM KCl, 10 mM Tris–HCl pH 9, 1% Triton X-100, 1.5 mM MgCl₂, 0.2 mM dNTPs, 1 μg of genomic DNA and 250 ng of each primer. The following profile was used for these reactions: 94°C/5 min, followed by 30 cycles of 94°C/45 s, 60°C/1 min, and a final extension at 72°C/15 min.

**Nucleic acid extraction and analysis**

RNA was extracted with TRIzol reagent (Bethesda Research Laboratory) according to the manufacturer’s instructions. RNase protection and RNA gel blot hybridizations were performed as described previously (Barkan et al., 1994). Genomic DNA was extracted from young maize seedlings and analyzed by Southern hybridization with digoxigenin-labeled Mu probes as described previously (Voelker et al., 1997). The site of insertion of Mu in the hcf111 locus was determined by using primers 2 (CGGGCGGGTTGAGGAAG) and 9 in conjunction with the MuTR primer (see above for sequences). PCRs were carried out in a volume of 50 μl and contained 50 mM KCl, 10 mM Tris–HCl pH 9, 1% Triton X-100, 1.5 mM MgCl₂, 0.2 mM dNTPs, 300 ng of genomic DNA and 250 ng of each primer. The following profile was used for these reactions: 94°C/4 min, followed by 30 cycles of 94°C/45 s, 60°C/1 min, 72°C/1 min, and a final extension at 72°C/5 min. The PCR products were cloned into a Bluescript vector and used as templates for DNA sequence analysis.

**Molecular cloning methods**

To clone the 7.7 kb HindIII–MdR fragment linked to crpi, 30 μg of DNA from a homozygous crpi-m1 mutant was digested with HindIII and fractionated in an agarose gel. A gel slice containing DNA fragments of 3.5–5 kb was excised and DNA was extracted from the gel with QIAEX beads, according to the manufacturer’s instructions (Qiagen). The DNA was ligated into Bluescript SK + plasmid (Stratagene) and calf intestine phosphatase, and electroporated into XL1-Blue MRF’ cells (Stratagene). Colony lifts (Sambrook et al., 1989) were probed with a radiolabeled MdR probe, leading to the identification of clone A (see Figure 2). The maize sequence flanking the cloned MdR element was subcloned by digesting clone A with AaeII and HindIII. This yielded a fragment of 1068 bp that included the entire 1033 bp of flanking DNA together with 35 bp of the Mu terminal inverted repeat. This fragment was ligated into a modified Bluescript vector and named clone B.

A cDNA library was generated from mRNA purified from the leaves of 14-day-old, greenhouse-grown maize seedlings (inbred line B73, Pioneer Hi-Bred). The library was prepared with the Uni-Zap XR cloning kit (Stratagene) and was screened with genomic clone B, yielding a 2.4 kb cDNA, ~300 nucleotides shorter than the 2.7 kb RNA detected in the wild-type tissue. This cDNA has been deposited in DDBJ/EMBL/GenBank (accession No. AF073522). Sequence alignments were calculated using ClustalW 1.7 (Thompson et al., 1994) and Boxshade (Bioinformatics group, ISREC, Lausanne, Switzerland), with default parameters.

**Extraction and analysis of protein**

Methods for the extraction of total leaf protein, SDS–PAGE and immunoblot analysis are described in Barkan (1998). intact chloroplasts were purified from the leaves of 10-day-old seedlings as described in Voelker and Barkan (1995) and separated into stromal, thylakoid and envelope fractions as described by Keegstra and Yousif (1986).

Chloroplast import assays were performed as in Bruce et al. (1994), with the following modifications: intact pea chloroplasts were purified as in Voelker and Barkan (1995), radiolabeled CRP1 was synthesized with a Novagen STP3 coupled transcription-translation kit, and 2×10⁸ c.p.m. were used per import reaction.

For sucrose gradient analysis of polyosomes, 0.4 g of wild-type or w1 leaf tissue were treated as described previously (Barkan, 1998) except that the heparin concentration was reduced to 0.1 mg/ml. One-tenth of each gradient fraction was fractionated by SDS–PAGE, and CRP1 was detected by immunoblotting.

The CRP1-enriched extract used for size exclusion chromatography was prepared as follows. Intact chloroplasts isolated from 400 g of leaf tissue were lysed by suspension in lysis buffer (30 mM HEPES–KOH pH 7.7, 10 mM magnesium acetate, 60 mM potassium acetate, 2 mM dithiothreitol (DTT), 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μM phenylmethylsulfonyl fluoride (PMSF)), vortexing and incubation on ice for 30 min. Membranes were pelleted by two rounds of centrifugation at 30 000 g for 30 min at 4°C. The supernatant was diluted to a protein concentration of 250 ng/ml and crushed ammonium sulfate was slowly added to a final concentration of 40%. The solution was precipitated at 4°C for 30 min. Precipitate was collected by centrifugation at 10 000 g for 10 min and was resuspended in 500 μl of buffer D (20 mM HEPES–KOH pH 7.9, 60 mM potassium chloride, 12.5 mM MgCl₂, 0.1 mM EDTA, 2 mM DTT) and filtered (S&S Unifo-13, 0.2 CA). The filter was washed with an additional 100 μl of buffer E, bringing the volume to 400 μl. A 250 μl aliquot of this material (50 μg of protein) was loaded onto the column.

Size exclusion chromatography was performed as per the manufacturers’ recommendations using a Pharmacia Superdex 200 HR 10/30 column with a Waters Protein Purification System. Fractions were eluted with buffer E + 5% glycerol, at 4°C at a rate of 4 ml/min; 200 μl fractions were collected. The molecular weight range in each fraction was estimated by comparison with size standards included in Pharmacia’s Gel Filtration Calibration Kits. Protein in each fraction was precipitated by addition of 1600 μl of ice-cold ethanol, incubation at –20°C overnight, and centrifugation at 3000 g at 4°C for 15 min. Pellets were washed with 70% ethanol, air dried briefly and resuspended in 1× SDS sample buffer/0.5 M Tris pH 8.0.

**Antisera**

A recombinant CRP1 fragment was generated by ligating a 1.5 kb EcoRI fragment of the cDNA clone into the Pet28 A(+) vector (Novagen), to yield an in-frame fusion with sequences encoding a his tag. This fusion protein included amino acids 86–583 of the predicted crpi gene product. The fusion protein was purified on a nickel column and injected into rabbits, for the production of polyclonal antiserum.

Antibody to OE33 was described previously (Voelker and Barkan, 1995). Antibody to spinach AtpB was raised in rabbits against purified spinach AtpB protein included amino acids 86–583 of the predicted crpi gene product. The fusion protein was purified on a nickel column and injected into rabbits, for the production of polyclonal antiserum.

**Acknowledgements**

We are grateful to Don Miles for providing hcf111 seed, to Peter Gegenheimer for providing AtbP antigen, and to the following individuals for their donations of antibodies: Kathy Newton for anti-MDH, W.Taylor for anti-PEP carboxylase, W.Gruissem and Gadi Schuster for anti-PEP carboxylase, W.Gruissem and Gadi Schuster for anti-28RNP, Danny Schnell for anti-IM35, and Tony Gatenby for anti-cpn60.

We also wish to thank Vicki Chandler for providing clones of members of the Mutator family, and Dennis McCormac, Bethany Jenkins and Brad Tall for helpful comments on the manuscript. This work was supported by grant DE-FE-0001820 from the Department of Energy. D.G.F. was supported in part by NIH Training Grant 5 T32 GM07759.

**References**


Received December 7, 1998; revised March 4, 1999; accepted March 5, 1999

Note added in proof
The CRP1 sequence includes multiple tandem copies of the tetratricopeptide repeat (TPR) motif, a motif that mediates protein–protein interactions in proteins with diverse functions.