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

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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 organellar 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* RNA 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 (*w1* and *iojap*). *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 *Hind*III 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 *Hind*III 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 *Hind*III 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 *Bam*HI 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

Fig. 1. Processing of the *petD* mRNA in the absence of chloroplast ribosomes. (A) Total leaf RNA from the indicated genotypes was analyzed by RNase protection, using a uniformly labeled probe that spanned the *petB*-*petD* intergenic region (see B). The *crs2* mutant, which lacks spliced *petD* RNA (Jenkins *et al.*, 1997), was used to identify bands corresponding to spliced *petD* transcripts. The protected probe fragments correspond to the following forms of the *petD* RNA: unprocessed, unspliced (U,U); unprocessed, spliced (U,S); processed, unspliced (P,U); and processed, spliced (P,S) (see B). Primer extension and S1 protection experiments revealed a single processed *petD* 5' end (Barkan *et al.*, 1994; and data not shown), indicating that the doublets detected here reflect heterogeneous cutting by RNase T1. A protected fragment corresponding to the processed *petB* 3' end was not detected because it accumulates to much lower levels than processed *petD* RNA (Barkan, 1988). (B) Map of the *petB*-*petD* intergenic region showing the probe used for RNase protection and the transcript forms it detects. The probe was transcribed from plasmid pB/D (Barkan *et al.*, 1994) and included a small amount of vector sequence (open boxes). The four transcript forms resolved in this experiment are drawn beneath the probe. Black box, protein-coding sequence; hashed box, intron sequence; thin line, intergenic RNA.

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

Fig. 2. Sites of *Mu* insertions in two mutant alleles of *crp1*. (A) Untranslated and translated portions of the *crp1* mRNA are represented by fine and bold lines, respectively. Clone A, derived from *crp1-m1*, included a portion of the *MuDR* transposon and 1033 bp of flanking genomic sequence. Clone B, derived from clone A, contained only the 1033 bp of genomic sequence. Comparison with cDNA clones revealed that this flanking sequence is transcribed and that it lacks introns. The *MuI* insertion in *hcf111* is 146 bp downstream of the *MuDR* insertion in *crp1-m1*. (B) PCR analysis illustrating the proximity of *Mu* insertions in the *crp1-m1* and *hcf111* mutant alleles. Arrows represent primer-binding sites. Primers 2 and 9 amplified a 498 bp fragment of wild-type DNA and did not amplify either mutant allele due to the presence of a *Mu* transposon between the primer-binding sites. Primers *Mu* and 2 amplified a 262 bp fragment from *crp1-m1* and a 116 bp fragment from *hcf111*. Primers *Mu* and 9 amplified a 353 bp fragment from *crp1-m1* and a 499 bp fragment from *hcf111*. (C) Nucleotide sequence of *Mu* 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

Fig. 3. RNA gel blot hybridization illustrating the absence of *crp1* mRNA in *crp1-m1* and *hcf111* mutants. Total leaf RNA (10 µg) from wild-type (WT), homozygous *crp1-m1* or homozygous *hcf111* mutants was gel-fractionated, transferred to nylon membrane and hybridized with a radiolabeled 1.6 kb fragment of the *crp1* cDNA. The lower panel illustrates the 18S and 16S rRNAs bound to the same membrane, detected by staining with methylene blue.

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 *MuI* member of the *Mutator* family. These results demonstrated that a *MuI* 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

A

(crp1-mi)
MuDR
↓
MPASLLPPTFLPHLLRRLAPAGCTTSSVTSSSVSIPASRYDFEPLLA YLSSPSVSASLTSPSPASVPAPEHRLAASYSAVPSHEWHALLRDLLAA SDVSL
MuI
(hcf111)
↓

B

crp1	302	Y N A L L K G Y V R I G S L K N A E Q V L D E M S Q C G . . . V A P D E A T Y S L L V D A I T R A G R W E S A R . I L L
cya5	482	Y Q K F L A F Y A G R G D T K S V T R L A N E Y R K H L P A E A A R D M S L I S S L M H V H A R N G N P E A A R K V L E
pet309	350	Y G I L M Y T M A R I G E L D S V N K L Y T Q L L R R G . . . M I P T Y A V L Q S L L Y A H Y K V G . . D F A A C F S H
crp1	358	K E M E A D G V K . P S B Y V F S R I L A G F R D R G D W Q K A F A V L R E M Q A S G V R . P D R H F Y N V M I D T F G
cya5	542	N C L E Q T G M G K P K T V H W N I L L N A Y A K A S D Y S G C L E A F E R L D E V G K . P D S Y S Y G T L M G M A A
pet309	404	F E L F K K Y D I T P S T A T H T I M L K V Y R G L N D L D G A F R I L K R L S E D P S V E I T E G H F A L L I Q H C C
crp1	416	K Y N C L G H A M D A F N K M R E E G . I E P D V V T M N T L I D A H C K G G R H D R A A E L F E E M R E S N C P P G .
cya5	600	V R G D L S F A L V L F N A A K A D G . I R P D V G M V D S L I E A Y C Q N D R F R E A E V I V K Q T V K K G T L P G K
pet309	464	K T T N H L I A Q E L F N L M T E H Y N I Q H T G K S I S A L M D V Y I E S N R P T E A I A L F E K H S K N L S W R D G
crp1	474	. T T T Y N I M I N L L G E Q E H W E G V E A M L S E M K E O G L V P N I I T Y T T L V D V Y G R S G R . Y K E A I D C
cya5	659	Y T V L F N T L L N H H A R R R D L K G L Y N L V E Y M H E N K I P S D N E T Y S H L L Y G L M Y C R Q . S H H A L N L
pet309	524	L I S V Y N K A I K A Y I G L R N A N K C E E L F D K I T T S K L A V N S E F Y K M M I K F L V T L N E D C E T A L S I
crp1	532	I E A M K A D . G I K P S P T M Y H A L V N A Y A Q R G L A D H A L N V V K A M K A D G L E V S I L V L N S L I N A F G
cya5	718	L R A S E K D N L F K P A P E H Y V L M A S F I H S R E P H M A L K L N E I S K R Q E P E M A E R T T R V I D A L G
pet309	584	I D Q L I K H S V I K V D A T H F E I T H E A Y D K E G Y R D G I N L Y K T M S Q N K V P A N S K I L Y Y I L K A V A

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 *cox1* translation and is required for the accumulation of *cox1* mRNAs from intron-bearing alleles. *CYA5* also activates *cox1* 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 copurifies with intact chloroplasts in Percoll gradients (Figure 5B) and is enriched in the chloroplast fraction to at least

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 μ g 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 cyt *b₆f* proteins (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 μ g) 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.

the same extent as the soluble chloroplast protein 28RNP (Schuster and Gruissem, 1991). The fractionation of the cytosolic protein phosphoenolpyruvate carboxylase (PEPCase) and the mitochondrial protein malate dehydrogenase (MDH) revealed that there was only minimal contamination of the chloroplast fraction with mitochondria and cytosol. These *in vivo* localization results were corroborated by *in organello* import assays. When the *in vitro* translation product of the *crp1* mRNA was incubated with isolated chloroplasts, a protease-resistant, lower molecular weight form accumulated (Figure 5C). Therefore, the *crp1* gene product is competent for import into chloroplasts.

To localize CRP1 further, chloroplasts were fractionated to separate the stromal, thylakoid and envelope compart-

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).

ments (Figure 6). CRP1 co-fractionated with the stromal marker cpn60 and separated from inner envelope (IM35) and thylakoid (OE33 and AtpB) markers. The fractionation of the thylakoid luminal protein OE33 demonstrated that only a small proportion of luminal proteins leaked into the stromal fraction. AtpB, a peripheral protein on the stromal face of the thylakoid membrane, remained bound to the thylakoid membrane, demonstrating that the fractionation procedure did not disrupt even weak membrane associations. These results indicate that CRP1 is a stromal protein with no detectable association with chloroplast membranes.

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 *w1* 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 *w1* 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 *w1* 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 *w1* 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

Fig. 7. Native CRP1 is a component of a high molecular weight complex. (A) Total leaf extracts were prepared under conditions that maintain polysome integrity (Barkan, 1998) and were sedimented in a sucrose gradient under conditions that resolve particles $>30S$. The gradient was divided into 26 fractions and one-tenth of each fraction was assayed for CRP1 by immunoblotting. The amount of CRP1 in fractions 11–26 was minute (data not shown). The direction of sedimentation is indicated by the arrow. (B) A CRP1-enriched extract was fractionated by size exclusion chromatography. Column fractions were separated by SDS-PAGE and transferred to nitrocellulose membrane. The filter was probed with antibodies to CRP1. Lanes containing 5 μ g of *crp1* leaf protein (lane 1), 5 μ g of WT leaf protein (lane 2) and 4 μ g of the CRP1-enriched extract (lane 3) were included for reference. The sizes of proteins in each fraction were determined by comparison with size standards. Lanes 4–20 show protein in fractions 10–26. The remaining 28 fractions (1–9 and 27–45) contained insignificant amounts of CRP1 (data not shown).

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 *psaJ* 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 modulate chloroplast translation (Danon and Mayfield, 1991; Zerges and Rochaix, 1994; Hauser *et al.*, 1996; Hirose and Sugiura, 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 mRNA-based membrane targeting 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 mRNA-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). *w1/crp1* double mutants were constructed by intercrossing *w1/+* 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 *w1* mutations, respectively. Double mutants were identified from among the albino (*w1/w1*) progeny by using a PCR assay to genotype the *crp1* locus. Each PCR reaction contained three primers: primers 9 (5'-ACATTGCAGTTCTGGCTTCAAC) and 10 (5'-GACAGCCGAGTAAGAC) flank the *Mu* insertion and amplify a 426 bp fragment specifically from the wild-type allele. Primer MuTIR (5'-CGCCTCCATTTCGTCGAATCC) corresponds to the *Mu* terminal inverted repeat and, in conjunction with primer 9, amplifies a 350 bp fragment

specifically from the mutant allele. Both fragments are amplified from heterozygous tissue. 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, 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/45 s, 72°C/2 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 *MuI* in the *hcf111* locus was determined by using primers 2 (GGTGGCGGTGGAGGAAG) and 9 in conjunction with the *MuTIR* 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 3.7 kb *HindIII*-*MuDR* fragment linked to *crp1*, 30 µg of DNA from a homozygous *crp1-m1* mutant was digested with *HindIII* and fractionated in an agarose gel. A gel slice containing DNA fragments of 3.5–4 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) that had been treated with *HindIII* and calf intestine phosphatase, and electroporated into XL1-Blue MRF' cells (Stratagene). Colony lifts (Sambrook *et al.*, 1989) were probed with a radiolabeled *MuDR* probe, leading to the identification of clone A (see Figure 2). The maize sequence flanking the cloned *MuDR* element was subcloned by digesting clone A with *XmnI* 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 by RNA gel blot hybridization. A cDNA fragment corresponding to the 5' portion of the mRNA was then obtained by 5' RACE (Frohman *et al.*, 1988) as follows. cDNA synthesis was catalyzed by Superscript II reverse transcriptase (Gibco-BRL) and primed with primer 2 (sequence listed above). The products were treated with RNase H and free dNTPs and primers were removed with the QIAquick PCR purification kit (Qiagen). The cDNA was then tailed by incubation with dATP and terminal deoxynucleotidyl transferase. The cDNA was amplified in a PCR involving an *EcoRI*-oligo(dT) primer [CGGAATTC(T)₁₇] and primer 10, which is nested with respect to primer 2 used for cDNA synthesis. The 5' RACE product was cloned in-frame with the cDNA to reconstruct the full-length cDNA. DNA sequence analysis was performed by Yanling Wang, in the Institute of Molecular Biology DNA Sequencing Facility. The nucleotide sequence of the full-length *crp1* 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 × 10⁵ c.p.m. were used per import reaction.

For sucrose gradient analysis of polysomes, 0.4 g of wild-type or *w1* leaf tissue was 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 mM 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 stirred 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, 17% glycerol) + protease inhibitors (2 µg/ml aprotinin, 0.5 µg/ml leupeptin and 0.7 µg/ml pepstatin). To remove ammonium sulfate, the extract was placed in a Pierce slide-A-lyzer and dialyzed against 1 l of buffer D at 4°C for 48 h. The extract was then aliquoted and frozen at -80°C. Before loading onto the column, one 100 µl aliquot was diluted with 200 µl of buffer E (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 Uniflo-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 *crp1* 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 recombinant AtpB that was generously provided by Peter Gegenheimer (University of Kansas). MDH antibody was donated by Kathy Newton (University of Missouri), PEP carboxylase antibody by W.Taylor (CSIRO, Canberra), 28RNP antibody by W.Gruissem and Gadi Schuster (University of California, Berkeley), IM35 antibody by Danny Schnell (Rutgers University) and cpn60 antibody by Tony Gatenby (DuPont).

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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.