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**TRANSPOSON-INDUCED NUCLEAR MUTATIONS THAT
ALTER CHLOROPLAST GENE EXPRESSION.**

**ANNUAL REPORT
FOR THE PERIOD
09/01/92-04/15/93**

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**PREPARED FOR THE
US DEPARTMENT OF ENERGY
DIVISION OF ENERGY BIOSCIENCES
OFFICE OF BASIC ENERGY SCIENCES
UNDER GRANT DE-FG06-91ER20054**

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MASTER

Annual Progress Report-April 20, 1993

Grant #DE-FG06-91ER20054

Transposon-Induced Nuclear Mutations that Alter Chloroplast Gene Expression
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Updated Project Description for Energy Biosciences Annual Report

The long term goal of this project is to use mutant phenotypes as a guide to nuclear genes that control the timing and cell-type specificity of chloroplast gene expression. Studies are being conducted with nuclear mutants of maize that are defective in the biogenesis or translation of chloroplast mRNAs. Currently, studies are focused on two nuclear mutants with specific and unique lesions in chloroplast RNA processing (*crp* mutants). *crp1* mutants (formerly called *hcf136*) fail to accumulate the cytochrome *f/b6* complex. The protein loss is due to a defect in the metabolism of transcripts encoding the *petB* and *petD* gene products, two subunits of the missing complex. Mutant seedlings lack the monocistronic *petB* and *petD* mRNAs, which both arise in normal plants by endonucleolytic cleavage of the polycistronic primary transcript of the *psbB* gene cluster. Precursor mRNAs accumulate normally in *crp1*, indicating that its defect is due either to a failure to cleave the precursors, or a failure to stabilize the fully processed mRNAs. We are interested in both the biochemistry of this site-specific RNA processing and in the role of the processing in generating translatable mRNAs. To address the latter, we are quantifying the rates of synthesis of the *petB* and *petD* gene products with the goal of determining whether the missing transcripts are more efficiently translated than their precursors. To address the biochemistry of the defect in RNA metabolism, the *crp1* gene is being cloned via the transposon tag.

crp2 (formerly called *hcf142*) lacks the predominant mRNA encoding *petA*, but appears to be otherwise unimpaired in chloroplast RNA metabolism. The precise role of *crp2* in synthesizing or stabilizing the *petA* mRNA is being investigated through biochemical studies. A molecular clone of the *crp2* gene will be obtained by transposon-facilitated cloning and will be used to more precisely define the role of the *Crp2* gene product.

PROGRESS REPORT- 8/92-4/93

A progress report was submitted in November of 1992. This report will therefore emphasize progress made since that time.

The long term goal of this project is to use mutant phenotypes as a guide to nuclear genes that play novel roles in chloroplast gene expression. The immediate goals of the funded grant proposal are to (1) identify transposon-induced nuclear mutants with defects in chloroplast gene expression, (2) characterize their phenotypes to identify those likely to be the most informative with regard to mechanisms of chloroplast gene expression, (3) clone the most interesting mutations by virtue of the transposon tag, (4) use the clones to further define the roles of these genes in chloroplast gene expression.

During this funding period we have finished our work with mutations causing global defects in chloroplast translation initiation. This work is described in a manuscript that is in press (see below). We are now focusing all of our attention on two mutants, *crp1* and *crp2*, isolated during the previous funding period. The nature of the phenotypes of these two unique mutants leads me to feel confident that they will reveal new aspects of chloroplast gene expression and its control by nuclear gene products. Our progress and goals are outlined in detail below.

Manuscripts accepted for publication (see Appendix).

A. Barkan (1993). Nuclear mutants of maize with defects in chloroplast polysome assembly have altered chloroplast RNA metabolism. *Plant Cell*, in press.

A. Barkan, R. Voelker, J. Mendel-Hartvig, D. Johnson, and M. Walker. (1993) Genetic analysis of chloroplast biogenesis in higher plants. *Physiologia Plantarum*, in press. (Invited review)

Mutants Defective in Chloroplast Translation.

Molecular and genetic studies of mutants with global defects in the incorporation of chloroplast mRNAs into polysomes are summarized in the attached *Plant Cell* pre-print. Since the last progress report, we have mapped the *cps1* locus to the long arm of chromosome 1.

We have found that mutants of this type arise rather frequently. Their phenotypes are consistent with the possibility that they have defects in nuclear genes encoding ribosomal proteins or translation factors. Because two recently isolated mutants have phenotypes that seem more certain to provide unique insights into chloroplast gene expression (see below), I do not plan to perform further studies with global translation mutants at this time. Analysis of this type of mutant has nonetheless proved useful. These mutants were used to unmask an association between translation and the metabolism of the *rbcL* mRNA (see *Plant Cell* preprint). In addition, the fact that such mutants share some easily-assayed properties (altered metabolism of rRNAs and *rbcL* mRNA) can be used in the future to quickly determine whether newly isolated mutants are likely to fall into this category.

At this time we have no evidence for the existence of any nuclear genes in maize that promote the translation of specific chloroplast mRNAs. During this funding period we looked in detail at five mutants lacking single complexes but with normal chloroplast mRNAs to determine whether the failure to translate individual mRNAs could be responsible for the protein losses. All five mutants (four of which lack the cytochrome *f/b6* complex and one of which lacks the photosystem II complex) synthesize all of the missing proteins at the normal rate (see the attached review). It appears from the published literature that nuclear genes that facilitate the translation of specific chloroplast mRNAs in *Chlamydomonas* are rather common. Our failure to uncover such mutants in maize may reflect a fundamental difference between plants and algae regarding the control of chloroplast gene expression by the nucleus. Of course, we have so far analyzed only five mutants adequately to address this issue, and so may simply have not looked far enough. Therefore, we will continue to assay the rates of synthesis of individual proteins in newly isolated mutants lacking single thylakoid complexes.

Mutants Defective in Chloroplast RNA Metabolism

We are now focusing on two mutants, *crp1* and *crp2*. As described in the previous report, *crp1* mutants fail to process or stabilize two specific chloroplast mRNAs; the loss of these mRNAs correlates with the loss of the cytochrome *f/b6* complex. Our progress in unraveling the connection between RNA processing and translation in this mutant is described below.

The *crp2* mutant was referred to as *crp3* in the previous progress report. This new nomenclature will be permanent, since it is being published soon in the attached review. Our progress in studies of this mutant are described below. Recent results have led us to stop working with the mutant formerly called *crp2*. This mutation causes aberrant metabolism of exised group II introns. While this in itself is interesting, the most interesting thing about that mutant had been that the intron defect was associated with a global loss of chloroplast-encoded proteins. However, we have recently determined that the intron phenotype is not obligatorily associated with a

photosynthetic defect. That is, there appear to be two mutations segregating independently in these stocks. One mutation causes a global loss of chloroplast proteins, the other causes an alteration in the metabolism of excised introns. Because the intron defect does not appear to have any consequences with regard to the ultimate functioning of the photosynthetic machinery, and because we cannot be certain it is caused by a transposon insertion, we do not plan to pursue this mutant.

Progress and future directions in studies of *crp1*

The photosynthetic defect in *crp1* is due to the absence (<5% WT levels) of the cytochrome b6f complex and a reduction (10% WT levels) in the PSI complex. We had previously found that the loss of the cytochrome complex is correlated with a defect in the biogenesis of *petB* and *petD* mRNAs: the monocistronic derivatives of shared polycistronic precursors do not accumulate in this mutant. Since the polycistronic precursors are unaffected by the mutation, these results suggest that (1) the mutation blocks the activity of a site-specific endonuclease or a factor that stabilizes the processed transcripts; and (2) that processing to a monocistronic form might be important to increase the translational efficiency of one or both of the *petB* and *petD* open reading frames.

Recently, we have used the *crp1* mutant to investigate the relationship between the complex maturation of transcripts of the *psbB* gene cluster and the generation of translatable mRNAs. The rates of synthesis of the *petB* and *petD* gene products were assayed during *in vivo* pulse-labelling studies of mutant and normal leaves. The *petB* protein was synthesized at the normal rate in the mutant. The absence of the monocistronic *petB* mRNA therefore has negligible effect on the rate of *petB* translation, indicating that polycistronic mRNAs encoding *petB* serve efficiently as templates for *petB* translation. In contrast, the accumulation of radiolabelled *petD* protein during 20 minute labelling periods was reduced at least 10-fold relative to WT. Because this loss of *petD* translation correlates with the absence of the monocistronic *petD* mRNA, these results suggest that the monocistronic *petD* mRNA is the primary functional template for *petD* translation *in vivo*.

The synthesis of the *petA* and *petC* gene products were assayed in these same labelling experiments. Since mRNAs encoding both of these proteins were unaltered in size and abundance, we expected to find that both proteins would be synthesized at the normal rate. This expectation was fulfilled for the *petC* gene product. But, we were surprised to discover that the accumulation of radiolabelled *petA* protein during the 20 minute labelling period was reduced at least 10-fold, relative to WT. Several explanations are being considered. First, the *petA* protein may be drastically destabilized (half-life of less than 5 minutes) as a consequence of the failure to translate *petD*. This explanation seems unlikely because the stability of the *petA* gene product in other mutants lacking the cytochrome f/b6 complex is rather long: it has a half-life of at least 45 minutes in four non-allelic mutants that fail to assemble the cytochrome f/b6 complex due to post-translational defects (see attached review).

A second possibility is that translation of the *petA* open reading frame is blocked if the *petD* gene product is not synthesized. That is, the synthesis of these two proteins, which must ultimately assemble with one another, is concerted. To distinguish between these possibilities, we are currently performing very short pulse-labellings (5 minutes) in isolated mutant and WT chloroplasts. If the incorporation of radiolabel into the *petA* gene product is substantially reduced (relative to WT) even during 5 minute labelling periods, this will provide strong evidence that the translation of the *petD* and *petA* proteins is interrelated- i.e. the failure to synthesize the *petD* gene product results in an inability to start or to complete synthesis of the *petA* gene product. These results would lead us next to an investigation of the mechanism by which the translation of the *petD* and *petA* gene products is coordinated. For example, we will determine whether ribosomes are associated with the *petA* open reading frame in *crp1*, and, if so, whether they might be stalled at specific sites.

In addition to using *crp1* to investigate the role of RNA processing in generating translatable mRNAs, we are also interested in the biochemical events that facilitate the accumulation of processed chloroplast mRNAs. For example, the phenotype of *crp1* is consistent with a defect

either in a site-specific ribonuclease or in a factor that stabilizes the fully processed transcripts. Towards defining the primary biochemical function of the *Crp1* gene, we are mapping the 3'-termini of monocistronic *petB* transcripts and the 5'-termini of monocistronic *petD* transcripts in WT chloroplasts. We speculate that a single endonucleolytic cleavage between *petB* and *petD* simultaneously generates these two termini. However, it remains possible that the two ends are generated by independent nucleolytic events. Knowledge of the precise locations of RNA termini in this intergenic region will help us to distinguish between these models. Finally, we are searching for a *Mu* transposon that is genetically linked to the *crp1* mutation, and plan to use this transposon to clone the *Crp1* gene. The clone will be critical for future biochemical and developmental studies.

Progress and future directions in studies of *crp2*

Crp2 mutants lack the predominant *petA* transcript and therefore fail to synthesize or stabilize this mRNA. Other chloroplast mRNAs appear to be unaltered, so the defect in mRNA metabolism is quite specific.

What remains difficult to explain is the connection between the photosynthetic defect and the mRNA defect. The photosynthetic defect results from global protein losses: a 5 to 10 fold reduction in the levels of all thylakoid complexes and of Rubisco. This fact, together with the fact that most chloroplast mRNAs are unaltered in size or abundance suggest a global defect in chloroplast translation. However, unlike the *cps* mutants, which fail to assemble chloroplast mRNAs into polysomes, mRNAs in *crp2* chloroplasts are associated with a normal number of ribosomes. I suspect, therefore, that *crp2* chloroplasts may have a decreased rate of translation elongation.

Perhaps the specificity of the defect in RNA metabolism can be related to the global defect in protein accumulation by considering the nature of the transcript that is affected by the mutation. This transcript was originally detected with a *petA* probe, but appears to include two conserved open reading frames of unknown function (ORF185 and ORF230). Although the loss of *petA* mRNA can explain the loss of the cytochrome *b6f* complex, it would not appear to explain the loss of the other complexes. One possibility is that the global loss of chloroplast proteins in *crp2* is due to the loss of proteins encoded by ORF185 and/or ORF230.

During the coming year, we will look further into the biochemistry of the RNA defect in *crp2*: We will assess whether the defect is transcriptional or post-transcriptional, and whether the absence of specific RNA binding proteins are correlated with the defect. We will also determine when the *Crp2* gene acts during chloroplast development by determining whether the defect is expressed in etioplasts, in proplastids, and in root plastids. Finally, we will search for a particular *Mu* transposon that is genetically linked to the mutation, and use this to start the cloning process.

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