

PROGRESS REPORT

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**"Post Transcriptional Regulation of Chloroplast Gene Expression
by Nuclear Encoded Gene Products"**

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PROGRESS REPORT FOR GRANT NO. DE-FG02-90ER20009 - "POST-TRANSCRIPTIONAL REGULATION OF CHLOROPLAST GENE EXPRESSION BY NUCLEAR ENCODED GENE PRODUCTS" - Principal Investigator - Michael R. Kuchka, Ph.D.

The major ambition of the work carried out in this grant period was to define the ways in which nuclear encoded gene products affect the expression of specific chloroplast genes. The approach we have undertaken is primarily genetic, involving the characterization of nuclear mutant strains of *Chlamydomonas reinhardtii* which are blocked in the expression of a single chloroplast gene called psbD, encoding the D2 polypeptide of Photosystem II. A molecular analysis of psbD gene expression in these mutant strains will provide us with a clearer understanding of how nuclear encoded gene products function in this specific case of chloroplast gene expression and will also advance our overall appreciation of the interaction between these two distinct genetic compartments within eukaryotic plant cells. This report will be organized into four sections concerning 1) psbD Translation/D2 Protein Turnover Mutants, 2) psbD mRNA Stability Mutants, 3) In Vitro Mutagenesis of psbD, and 4) Additional Studies.

psbD Translation/D2 Protein Turnover Mutants

Much of the work on these mutant strains is described in the accompanying preprint entitled, "Nuclear Factors Affect the Expression of the Chloroplast-Encoded D2 Protein of Photosystem II at a Late Stage of Translation and/or at the Post-Translational Level in *Chlamydomonas reinhardtii*." The results of this study will be summarized briefly here. Four nuclear mutant strains of *Chlamydomonas* were subjected to comparative genetic and molecular biochemical analysis. All four strains (nac 1-11, nac 1-18, ac-115, and 6.2u) are incapable of growing photosynthetically because they fail to synthesize the major Photosystem II protein D2 (Kuchka et al., 1988). Intercrosses of the four strains defined at least two (and possibly three) distinct nuclear genes. D2 synthesis is blocked post-transcriptionally in these mutants. Northern analysis showed that normal levels of psbD mRNA are present in mutant cell chloroplasts, yet no D2 protein is detectable by protein pulse-labeling experiments. In all mutants, psbD mRNA is associated with large classes of polysomes, suggesting that translation of this message is initiated in these strains. This implies that mutant gene products may normally function in steps of translation elongation and/or in the stabilization of the nascent D2 protein. This is the first example of nuclear encoded gene products participating at these stages in the expression of a single chloroplast gene. We feel strongly that a continued examination of these nuclear mutants, and a detailed definition of the function of these gene products in D2 synthesis is warranted. Experiments are proposed in the Renewal Application which will address these issues.

Phenotypic revertants of the original mutant strains were generated by demanding for growth on minimal media. A collection of revertants derived from each mutant background has been generated. Some of these strains have been backcrossed to wild type to determine the genetic nature of reversion. In at least two distinct cases, reversion is caused by an extragenic suppressor which overcomes the affects of the

original mutation. We are focusing on these suppressors, and working on the isolation of additional ones, to identify new genes whose products might interact with the original mutant gene products in the process of psbD gene expression. A suppressor called sup4b, which was isolated from nac 1-18 mutant background, has been studied. Besides its ability to suppress, the sup4b mutation has no phenotype in an otherwise wild type genetic background. The sup4b mutation was crossed into other mutants and was shown to be able to suppress the affects of two different mutations in NAC1, as well as the mutant phenotype of ac-115, a strain mutated in another nuclear gene required for D2 expression. We find this "bypass" suppression intriguing. We would like to understand the molecular nature of this suppression and ultimately identify the Sup4b locus. Experiments directed towards these goals are outlined in the Renewal Application.

One of our major objective is to determine whether the nuclear mutations described above are directly affecting translation of psbD mRNA or are shortening the half-life of the nascent D2 polypeptide. Currently, we are using immunoprecipitation experiments of pulse-labeled or pulse-chased proteins to distinguish between these two possibilities. We have acquired a D2 specific antiserum from Dr. John Mullet, Texas A&M University, and are using this to assay D2 synthesis in mutant cells. An example of an immunoprecipitation experiment using this antiserum is shown in Figure 1. In this experiment, extracts from pulse-labeled cells were reacted with the D2-specific antibody. Immunoprecipitated proteins were electrophoresed and visualized by autoradiography. It is clear from this figure that strains ac-115 and 6.2u synthesize an appreciable amount of mature-sized D2 protein. In contrast, nac 1-18 makes only trace amounts of D2. From this experiment it also appears that the NAC 1 gene is epistatic to AC115 because the phenotype of the nac 1-18; ac-115 double mutant resembles nac 1-18. This result has important implications concerning the relative functions of the two nuclear gene products. One possibility is that the NAC 1 gene product works earlier than the AC115 gene product in D2 translation. Alternatively, the product of the NAC 1 gene may associate more closely with the nascent D2 polypeptide and influence its half-life more directly than AC115. To distinguish between these possibilities, we plan to pursue immunoprecipitation experiments using pulse-labeled and pulse-chased protein samples. It is also intriguing that a lower molecular weight band of approximately 15 kDa is precipitated from the wild type sample and also from ac-115 and 6.2u mutant cell extracts. It appears from Figure 1 that the amount of this low molecular weight protein is equivalent in wild type and ac-115 extracts, while the mature-sized D2 protein is present at one-fifth to one-tenth of wild type levels in ac-115. Might this polypeptide represent a D2 translation intermediate which accumulates in ac-115? We plan to continue this line of experimentation to clarify nuclear gene product function in the synthesis of the D2 protein.

psbD mRNA Stability Mutants

Another nuclear mutant of our collection, nac 2-26, fails to synthesize the D2 protein because the psbD mRNA encoding the protein is unusually unstable in this strain (Kuchka et al., 1989). By

transcription run-on experiments, we have recently shown that the psbD gene is initially transcribed to completion (data not shown), but the message is unstable and undetectable in mutant cells. In this case, a nuclear encoded factor apparently works to stabilize a specific chloroplast encoded mRNA. We have been studying this mutant to understand the cis- and trans-acting elements which are important determinants of psbD mRNA turnover. A number of different lines of experimentation suggest that the 5' untranslated (UTR) region of psbD mRNA is an important cis element (Kuchka et al., 1989; Rochaix et al., unpublished results). We are interested in identifying proteins which bind to this site. One of these proteins may be the nuclear NAC 2 gene product. We have generated by polymerase chain reaction a 220 base pair sequence of DNA which encodes the entire 5' UTR as well as the first several codons of the psbD mRNA. This sequence has been cloned into an in vitro transcription vector for the generation of radiolabeled transcripts. These labeled RNAs are used for gel mobility shift assays as well as for uv-crosslinking experiments with wild type and mutant cell extracts, to identify proteins which bind to this sequence. An example of a gel mobility shift assay is shown in Figure 2. It is clear from this figure that both wild type and mutant cell extracts include components which bind to the psbD 5' UTR RNA and retard its electrophoretic mobility in the gel. We seek to identify these components by uv-crosslinking analyses. Ultimately, we hope to be able to identify the NAC 2 gene product by this sort of experimentation. It is reasonable to suppose that the NAC 2 factor is a nuclear encoded protein which binds to the 5' end of the psbD mRNA to confer stability. UV-crosslinking experiments may well lead us to this protein. Such experiments are described in the Renewal Proposal.

In Vitro Mutagenesis of psbD

A careful comparison of all strains affected in psbD gene expression, including the nuclear mutants described above and a chloroplast mutant carrying a psbD frameshift mutation (FuD47, Erickson et al., 1988), reveals that the biochemical phenotypes of these strains differ in interesting ways. The most striking variation among these strains is in the level of accumulation of different PS II polypeptides. In nuclear mutants affecting psbD message translation, as well as in FuD47, proteins of the oxygen evolving complex (OEE proteins) are present in normal quantities, while PS II core components are greatly reduced. In contrast, nac 2-26 chloroplasts are completely deficient in all PS II proteins, core and OEE proteins included. It is not clear whether these differences relate to pleiotropic effects of the various mutations or whether they relate directly to different levels of D2 protein expression in these strains. It should be noted that strain FuD47 is capable of synthesizing a half-sized D2 protein. On the other hand, nac 1-11, nac 1-18, ac-115, and 6.2u synthesize reduced but detectable quantities of D2, while nac 2-26, because it has no psbD mRNA, makes no D2. We would like to understand if these differences in the levels of D2 might affect the assembly and stability of PS II proteins directly. Another way in which mutants affecting psbD gene expression differ is in their synthesis of D1, a related PS II polypeptide. It has been claimed that translation of D2 and D1 are

closely coupled, and that D2 is essential for D1 synthesis (Erickson et al., 1988; deVitry et al., 1989). Protein pulse-labeling experiments of FuD47 showed that this strain is reduced in D1 synthesis (Erickson et al., 1988). In contrast, nuclear mutants affected in D2 synthesis make the D1 protein normally (Kuchka et al., 1988, 1989). We have recently confirmed these results by immunoprecipitation experiments using a D1 specific antibody (data not shown). To clarify the D2 protein's role in PS II polypeptide accumulation and D1 expression, and to distinguish D2's function from the function of nuclear encoded factors, we plan to generate a true D2 null mutant by in vitro mutagenesis and transformation, and to study the biochemical phenotype of this strain.

We have generated a number of plasmid constructs which carry mutated versions of the Chlamydomonas psbD gene together with a selectable marker for chloroplast transformation, aadA (Goldschmidt-Clermont, 1991). The aadA gene confers streptomycin and spectinomycin resistance to cells when expressed in chloroplasts. When introduced into chloroplasts by the particle bombardment transformation method, we expect that this plasmid DNA will recombine into psbD gene sequences, integrating the aadA marker and disrupting the gene. One plasmid has been used to transform wild type Chlamydomonas cells. Four putative transformants that are antibiotic resistant have been identified. These putative transformants exhibit an unusual fluorescence phenotype, typical of nonphotosynthetic mutants. We are currently analyzing these putative transformants by Southern blotting, probing with aadA and psbD specific sequences. We hope to identify a strain which is mutated in psbD and to use this strain for studies of PS II protein accumulation and D1 protein expression. These transformation experiments are done in collaboration with Dr. Michel Goldschmidt-Clermont, University of Geneva. This is necessary because our laboratory does not have the equipment for chloroplast transformation or for the measurement of fluorescence induction kinetics. We plan to continue this collaboration. Our laboratory will generate clones for transformation and analyze putative transformants, while Dr. Goldschmidt-Clermont will perform the transformations and the initial characterization of transformant candidates (see attached letter).

Additional Studies

Our characterization of psbD expression requires a D2 specific antibody. Recently, we have benefitted from the use of a D2 specific antiserum raised against the maize polypeptide. This antiserum cross-reacts well with the Chlamydomonas polypeptide, as assayed by immunoblot experiments. As we have a limited quantity of this antibody, we need to generate our own. Towards this end, we have cloned a psbD restriction fragment carrying the majority of the D2 coding sequence into a protein expression vector. At present, clones are being analyzed. We will use the suitable clone for expression of large quantities of D2 protein in bacteria and use isolated protein as an immunogen for antibody production in rabbits.

Summary Statement

Many individual chloroplast genes require the products of a collection of nuclear genes for their successful expression. These nuclear gene products apparently work with great specificity, each committed to the expression of a single chloroplast gene. We are interested in probing this level of interaction between the two genomes in Chlamydomonas cells. We have chosen as a model nuclear mutants of Chlamydomonas affected in different stages in the expression of the chloroplast encoded Photosystem II polypeptide, D2. We have made the most progress in understanding how nuclear gene products affect the translation of the D2 encoding mRNA. At least two nuclear genes are required for this process. Two of these genes have been mapped genetically. In contrast to other examples of nuclear control of translation in the chloroplast, here, nuclear gene products appear to be required either for specific stages in translation elongation or for the post-translational stabilization of the nascent D2 protein. We have not yet been able to distinguish between these two possibilities. However, it is important to note that this is the first example of nuclear encoded factors working at these stages in the expression of a specific chloroplast gene. Pseudoreversion analysis has led us to at least one new interesting locus which may also be directly involved in D2 expression. The collection of mutants at hand also provides us with a means of understanding how nuclear gene products work to stabilize individual chloroplast encoded mRNAs. We have made considerable progress in pursuing the molecular basis of psbD mRNA stabilization. psbD 5' UTR specific transcripts have been synthesized in vitro and used in gel mobility shift assays. UV-crosslinking studies are underway to identify the trans-acting factors which bind to these sequences. The continued examination of these mutants will help us to understand how nuclear gene products work in this specific case of chloroplast gene expression, and will elucidate how two distinct genomes can interact generally.

References

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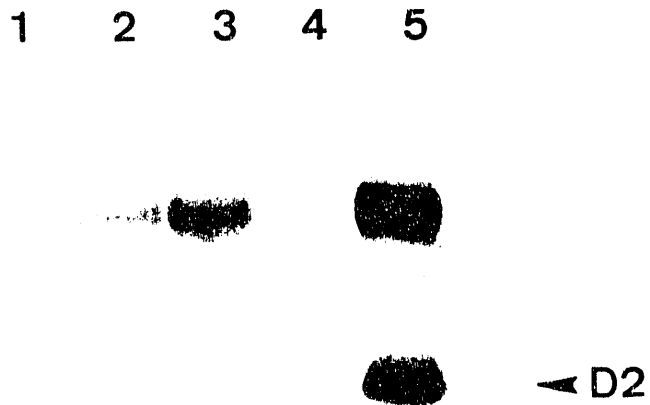


Figure 1. Autoradiogram of translation products from $^{35}\text{SO}_4$ pulse-labeled cells immunoprecipitated with a D2 specific antiserum. (lane 1 - nacl-18;ac-115 double mutant, lane 2 - 6.2u, lane 3 - ac-115, lane 4 - nacl-18, lane 5 - wild-type)

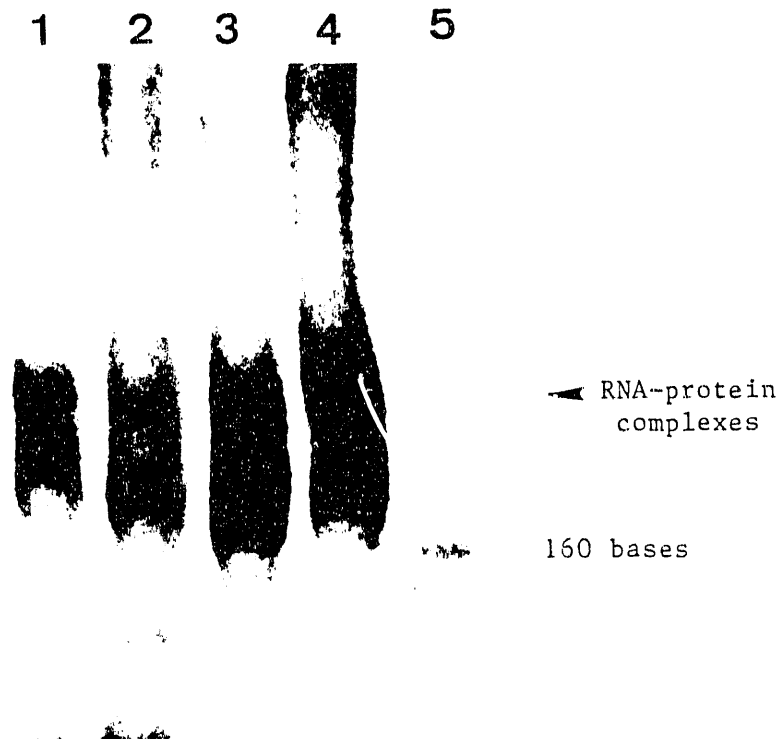


FIGURE 2: Gel Mobility Shift Assay of ^{32}P labeled psbD 5' UTR reacted with $40\mu\text{g}$ of wild type or nac2-26 soluble protein preparations. Lane 1- labeled psbD 5' UTR, nac2-26 protein and a 200 fold excess of unlabeled psbD 5' UTR; Lane 2-labeled psbD 5' UTR, nac2-26 protein; Lane 3- labeled psbD 5' UTR, wild type protein and a 200 fold excess of unlabeled psbD 5' UTR; Lane 4- labeled psbD 5' UTR, wild type protein; Lane 5- labeled psbD 5' UTR.



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Geneva, January 21, 1992

Dear Mike,

Following our e-mail discussions, I am sending you this letter to confirm that I would be very happy to continue our collaboration. I will bombard the appropriate Chlamydomonas strains with the DNA samples that you will send me. In particular it will be interesting to see whether we can obtain a homoplasmic psbD disruption with the new constructs that you planned.

Looking forward to these pleasant and fruitful interactions.

Best wishes,

Michel Goldschmidt-Clermont

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