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**Manipulation of Gene Expression in the
Chloroplast**

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Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted	0	0	0	0
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Licensed	0	0	0	0

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Visits/Meetings	2	3	1	6
Sabbaticals	0	0		
Postdoctorates	0	0		

- **Cooperation**, briefly explain whether synergistic, complementary or supportive.
Please, see description of cooperation in the following report _____

ABSTRACT

Manipulation of gene expression in the chloroplast: Molecular genetic analysis of mRNA degradation

The steady-state level of a given mRNA is determined by its rates of transcription and degradation. The stabilities of chloroplast mRNAs vary during plant development, in part regulating gene expression. Furthermore, the fitness of the organelle depends on its ability to destroy non-functional transcripts. In addition, there is a resurgent interest by the biotechnology community in chloroplast transformation due to the public concerns over pollen transmission of introduced traits or foreign proteins. Therefore, studies into basic gene expression mechanisms in the chloroplast will open the door to take advantage of these opportunities. This project was aimed at gaining mechanistic insights into mRNA processing and degradation in the chloroplast and to engineer transcripts of varying stability in *Chlamydomonas reinhardtii* cells. This research uncovered new and important information on chloroplast mRNA stability, processing, degradation and translation. In particular, the processing of the 3' untranslated regions of chloroplast mRNAs was shown to be important determinants in translation. The endonucleolytic site in the 3' untranslated region was characterized by site directed mutagenesis. RNA polyadenylation has been characterized in the chloroplast of *Chlamydomonas reinhardtii* and chloroplast transformants carrying polyadenylated sequences were constructed and analyzed.

Data obtained to date suggest that chloroplasts have gene regulatory mechanisms which are uniquely adapted to their post-endosymbiotic environment, including those that regulate RNA stability. An exciting point has been reached, because molecular genetic studies have defined critical RNA-protein interactions that participate in these processes. However, much remains to be learned about these multiple pathways, how they interact with each other, and how many nuclear genes are consecrated to overseeing them. *Chlamydomonas* is an ideal model system to extend our understanding of these areas, given its ease of manipulation and the existing knowledge base, some of which we have generated.

Results

Our experimental objectives and results from the proposal are as follows (since most of our results were already published in reviewed scientific journals, only a short description of the major points is described whereas the full description of the results and methods may be found in the reprints that are attached to this report):

Transcript 3' end formation and RNA stability

The 3' untranslated regions (UTRs) of most chloroplast transcripts can fold into stem-loop structures, which one of the PI's earlier showed to stabilize RNA but not terminate transcription in a spinach *in vitro* system, and to stabilize *Chlamydomonas* chloroplast *atpB* mRNA *in vivo*. One objective of the previous (1992-95) BARD proposal was to determine whether the 3' untranslated regions (UTRs) of *Chlamydomonas* chloroplast transcripts terminated transcription *in vivo*, and they did not (Rott et al 1996); we also confirmed and extended their role in RNA stability (Rott et al 1998a). In the work described in this manuscript, we showed that both the structure and the nucleotide sequence of the 3' UTRs are important for the accumulation of correctly 3' end processed mRNAs in *Chlamydomonas reinhardtii* chloroplasts. Using chloroplast transformation and genetic engineering, the 3' UTRs of several genes were placed at the 3' end of the *atpB* gene in either sense or antisense orientations. It was found that the 3' UTR was required for the correct 3' end processing of the *atpB* mRNA and that every 3' end tested, including one derived from an *E. coli* gene, was able to carry out this function. However, the correctly processed mRNA accumulated to the wild type level only when the 3' UTR was placed in the sense orientation. When the 3' UTR was placed in the antisense orientation, about half of the wild type amount of mRNA accumulated in the cell (Rott et al 1998a). This suggests that a correct 3' end formation sequence is essential for high level expression of chloroplast genes.

Alternating the endonucleolytic cleavage site effects 3' end processing *in vitro* but not *in vivo*

We next turned to a specific case of 3' end maturation, that of the *atpB* mRNA, which is processed first by a site-specific endonucleolytic cleavage, and second by exonucleolytic 3' to 5' resection to form the mature transcript. One objective was to mutate the recognition site of the endonuclease, in order to resolve its mechanism of

cleavage and to determine the precise nucleotide sequence of the cleavage site. Using site directed mutagenesis, the ECS sequence was varied. The corresponding *atpB* transcripts containing the mutated sequences were analyzed for *in vitro* 3' end processing assay using a chloroplast soluble protein extract. In addition, these sequences were introduced into the corresponding site of the gene in the chloroplast by transformation and the formation of a correctly 3' end processed and stable *atpB* transcript was analyzed in the transformants. These experiments revealed that while some base changes within the ECS altered processing *in vitro*, none altered processing *in vivo* (Rott et al 1999). We now hypothesize that both the 3' stem-loop itself and the downstream ECS play roles in RNA maturation.

Transcript 3' end formation and translation initiation

In the course of the experiments described in the previous paragraph, a surprising finding was made, namely, that 3' end maturation had a role in translation initiation. Normally, chloroplast translation initiation is thought to be regulated by 5' UTR sequences, not by the 3' UTR. However, a careful analysis showed that certain incorrectly 3' processed, or unprocessed, mRNAs present in *Chlamydomonas* chloroplasts are not associated with polyribosomes, suggesting that they are not translated or are inefficiently translated (Rott et al. 1998). These findings are reminiscent of the better-characterized situation in the cytoplasm of eukaryotic cells, where the 3' poly(A) tail enhances translation by promoting the binding of translation initiation factors to the 5' cap.

Polyadenylation of chloroplast transcripts

One focus of the proposal was on the role of polyadenylation in chloroplast gene expression. Previous work confirmed early reports that chloroplast transcripts can be polyadenylated, and further showed that this polyadenylation destabilized chloroplast mRNA *in vitro* (Lisitsky et al 1996; Kudla et al 1996; Schuster et al 1999). To see whether polyadenylation also destabilized chloroplast mRNA *in vivo*, chimeric gene fusions with "pre-added" poly(A) tails were introduced into *Chlamydomonas* chloroplasts. These tails did destabilize transcripts, although the results were complicated by additional effects on transcription and translation.

An additional and necessary objective was to carry out a descriptive and functional study of polyadenylation in *Chlamydomonas* chloroplasts, since all previous work was carried out in spinach. To this end, we have found that all three classes of RNA (mRNA, tRNA, rRNA) can be polyadenylated in *Chlamydomonas* chloroplasts, and found some other aspects unique to this system (Komine et al 2000).

Insertion of poly(dA) sequences into the intergenic region increases transcription in *Chlamydomonas reinhardtii* chloroplasts.

Poly(dA:dT) was reported to be an ubiquitous promoter element that stimulates transcription via its intrinsic DNA structure, and to influence gene expression when inserted into intergenic regions of yeast nuclear genes. Does the poly(dA) tract inserted into the intergenic region of a chloroplast gene affect transcriptional activity? Our strategy was to insert a poly(dA) tract or control sequences between two genes, *atpB* and *aadA*. We found that transcription was increased for the downstream *aadA* gene leading to the accumulation of elevated amount of this transcript (Lisitsky et al, 2000). These results demonstrate that the poly(dA) tract can function in the chloroplast as a transcriptional enhancer. The results also suggest that dA-rich intergenic sequences commonly found in the chloroplast genome may have heretofore unanticipated functions.

Cooperation

The two investigators have collaborated for 7 years on the processing, stability and translation of chloroplast mRNAs. This fruitful collaborative has resulted in a considerable number (7) of publications in peer-reviewed journals, reciprocal visits by the PI's, and travel by a graduate student to the U.S. Because the PI's have presented scholarly seminars during their visits (the U.S. PI has given 6 seminars at different institutions in Israel since the BARD cooperation started), this collaboration has contributed to broader scientific interchanges. In addition to publications, there have been 3 visits by co-PI Stern to Israel (1994, 1998, 1999), and 4 visits by co-PI Schuster to the U.S. (1994, 1997, 1999, 2000). These visits have stimulated contact not just between our two laboratories, but between both co-PIs and the broader scientific communities in the countries visited, through the presentation of seminars and at larger meetings.

While both *Chlamydomonas* and spinach are in use in both the Stern and Schuster laboratories, which is an advantage, the techniques and emphases are in many ways

complementary. The Stern lab emphasizes molecular genetic approaches to gene expression problems, and has a substantial (4-5 person) *Chlamydomonas* group. This provides critical mass and an excellent environment in which to design and carry out the objectives envisioned with this organism. The Schuster laboratory conducts largely *in vitro* biochemical work, including protein purification, enzyme assays and gene cloning. Their expertise with separation technologies, and the excellent protein analytical facilities at the Technion, makes this group ideally suited to carry out protein purification and related *in vitro* experiments. At the same time, it should be emphasized that there has been, and is anticipated to be, a frequent exchange of experimental materials such as antibodies and *Chlamydomonas* strains. This occurs because of ideas generated through email conversations and visits, and because of the complementary interests of the two PI's.

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