Thesis for the Master's degree in Molecular Biosciences

Main field of study in Molecular Biology

60 study points

Binding of recombinant protein disulfide isomerase I (PDII) from *Chlamydomonas reinhardtii* to the 5' untranslated region of *Chlamydomonas rbcL* transcripts

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UNIVERSITY OF OSLO 12/2010

Acknowledgements

This master project was performed at the department of Molecular Biosciences at the University of Oslo in Professor Uwe Klein's laboratory.

I would like to take this opportunity to thank my supervisor, Professor Uwe Klein for his guidance in the laboratory and support during the writing of this thesis. Professor Uwe Klein has been of great support during my thesis work and has always been available for any enquires I have had.

I would also like to thank my parents for their inspirational support and words of encouragement. They have been a source of inspiration for me and I am very grateful for their guidance and support.

Oslo, December 2010

Abstract

Chlamydomonas reinhardtii is a widely used model organism to study chloroplast gene expression and photosynthetic processes. In this project we worked to identify a novel trans-acting factor that is thought to contribute to the stability of *rbcL* mRNA. These types of factors are thought to bind to specific cis-sequence elements and protect the transcript from nuclease degradation.

Experimental studies have proven that the 5'untranslated region of *Chlamydomonas rbcL* mRNA plays a key role in the regulation of *rbcL* transcript stability. Changing nucleotides in the 5'UTR renders *rbcL* transcripts unstable, and it is thought that sequence elements within the transcripts recruit and bind proteins that functions as trans-acting factors.

In this project we cloned a protein (RB60) that is thought to have RNA-binding capabilities to *Chlamydomonas rbcL* 5'UTR. The protein was cloned into a transformation vector and introduced into *E.coli*.

UV cross-linking experiments showed that there is no conclusive evidence that RB60 can bind to *Chlamydomonas rbcL* 5'UTR. High concentration of RB60 protein yielded low affinity binding to *rbcL* 5'UTR sequences *in vitro*. Control experiments with *E.coli* extract without RB60 construct identified a bacterial protein that has high affinity for 5'UTR sequences *in vitro*.

Table of contents

1 Introduction

1.1 *Chlamydomonas reinhardtii*

1.1.1 *Chlamydomonas reinhardtii* as a model organism

Chlamydomonas reinhardtii is a species of eukaryotic unicellular green alga taxonomically located under the genus of *Chlamydomonas*. They are characterised by their two anterior flagella they use for motility as well as having a chloroplast that encompasses the photosynthetic apparatus. *Chlamydomonas* cells have in addition an "eyespot" that senses light and contributes to phototaxis (Figure 1-1). The presence of a pyrenoid as a centre for carbon dioxide fixation is essential for photosynthetic activity. The algae averages about 10μm in diameter and are frequently found in fresh water and damp soil. The laboratory strains of *Chlamydomonas reinhardtii* used for research (from now on *Chlamydomonas*) are isolates from 1945 that have been collected near Amerst, Massachusetts [1].

There are several advantages to using *Chlamydomonas* as a model organism. It has the ability to make its photosynthetic function dispensable by growing in the dark on organic carbon sources (i.e. acetate). It can grow photoautotrophically (light with CO_2 as carbon source), mixotrophically (light with acetate) or heterotrophically (dark with acetate) [2]. Mutants that are blocked in photosynthesis are viable if acetate is provided making them ideal candidates to study the photosynthetic machinery [1]. In addition there is the possibility to synchronize cell division cycles by subjecting the cells to alternate light and dark cycles. Like other photosynthetic eukaryotes, *Chlamydomonas* contains three autonomous genomes: nucleus, chloroplast and mitochondria. Mutations in each of these genomes can be readily distinguished in crosses. Haploid cells exist either as mating-type (+) or mating-type (-), while vegetative cells develop into gametes upon starvation and blue-light illumination (Figure 1-2) [2]. In addition there is the possibility to control the sexual life cycle through nitrogen starvation. The mating types of the *Chlamydomonas* cells are determined by genes that are expressed by the MT-locus that is initiated when nitrogen levels drop in the environment. The fertilization process produces a binucleate zygote that expresses zygote-specific genes that create a specific cell wall that makes the zygote more resistant to adverse conditions. An improved condition initiates the dormant zygote to undergo meiosis and release four haploid

vegetative cells [3] . Nuclear genes segregate according to Mendelian rules, while chloroplast and mitochondrial genes are subjugated to uniparental inheritance to progeny from mating-type (+) and mating-type (-) parent, respectively [4].

Several other important factors contribute to *Chlamydomonas* as a model organism is that it is easy to maintain and propagate under laboratory conditions requiring a simple medium of inorganic salts to grow. It has a well-defined genetics having its three genomes sequenced and so far being the only organism in which transformation techniques have been developed for all three genomes (nucleus, chloroplast and mitochondria) [5]. It has a short generation time (approx.7- 8 hours) and can grow on a petri dish making it easy to isolate mutants. Transformation of the nucleus and chloroplast are easily performed allowing for site-directed mutagenesis to both genomes [6]. *Chlamydomonas* has also several information rich databases dedicated to its genomic and organism delineation. There is an ongoing process in integrating these databases together to provide robust data retrieval and holistic information channel for the benefit of research communities dedicated to using *Chlamydomonas* as a research model organism [7, 8].
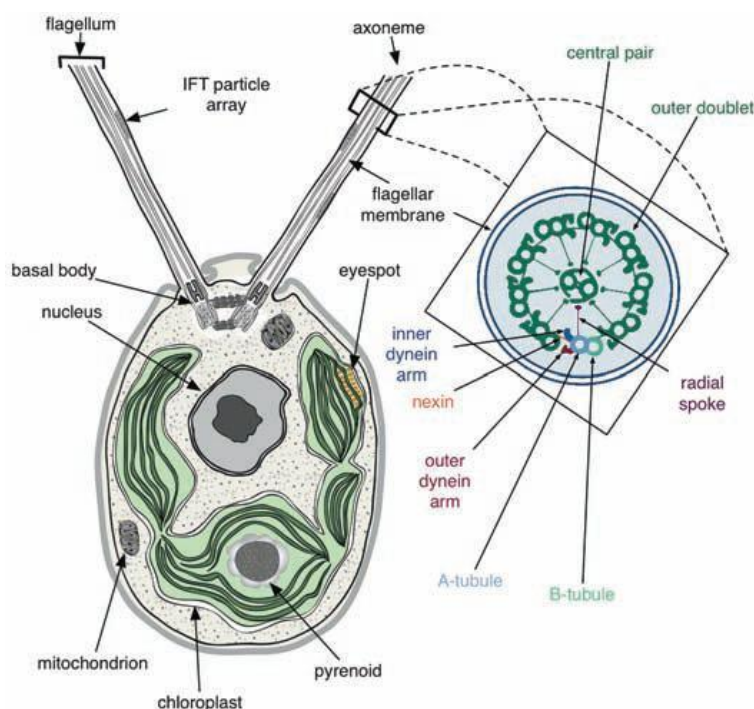
Figure 1-1: A schematic drawing of a *Chlamydomonas* cell. The chloroplast takes up almost half of the cell. The anterior flagella are rooted to the basal body of the cell (Merchant et al, 2007, ref nr. 9).

Evolutionary significance

Chlamydomonas as part of the *Chlorophyta* division that diverged from land plants over a billion years ago and together they constitute the green plant lineage. Many of the *Chlamydomonas* genes can be traced to an ancestral green plant and they share many genes with the angiosperms, especially those included in photosynthesis [9]. Molecular phylogeny studies of *Chlamydomonas reinhardtii* indicate that it shared a common ancestor with *V.carteri*, a multicellular green flagellate, rather than the hundreds of other *Chlamydomonas* species. This provides a unique insight into the development of multicellular eukaryotes, leaving scientist to hypothesize a twelve-step evolutionary program for the transition from unicellular to multicellular organisms [10].

1.1.2 Life Cycle of *Chlamydomonas*
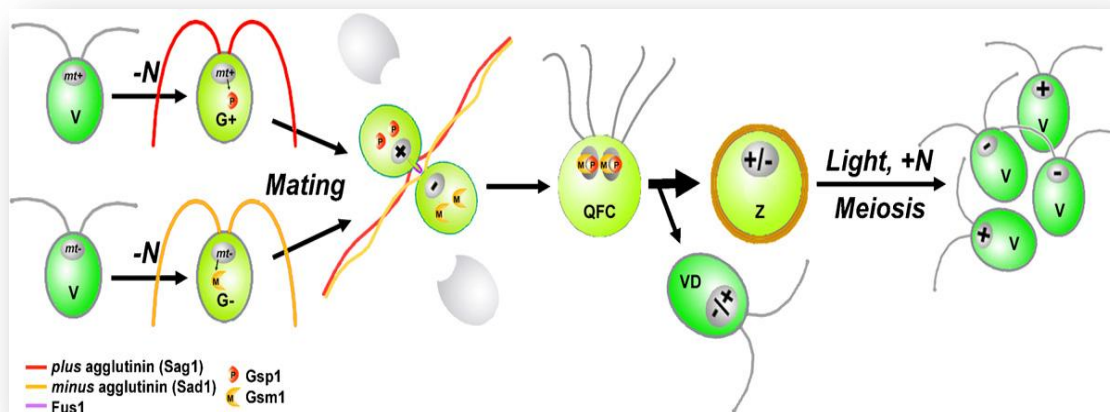
Figure 1-2: Life cycle *of Chlamydomonas reinhardtii*. Haploid vegetative (V) cells of two mating types (mt+ and mt-) divide mitotically. When exogenous nitrogen becomes limiting, they differentiate into gametes (G+ and G−), expressing mating type-specific gametic traits. In the laboratory, zygotes subjected to 5 days of dormancy in the dark and returned to light in N-containing media undergo meiosis to release four haploid meiotic products that resume vegetative growth. Occasional QFCs forego the meiotic pathway and instead resume vegetative growth as +/− vegetative diploids (VD) (Goodenough et al, 2007, ref nr. 3).

Chlamydomonas has provided important information on flagella function, cell-cell recognition, photosynthesis and secretion. Many of the genes identified have proven to have homologs in land plant as well as animals, enabling a greater understanding of fundamental biological processes [11].

1.2 The Chloroplast

1.2.1 The chloroplast – structure and function

Chloroplasts along with mitochondria are essential organelles that are a part of the eukaryotic cell. The former belong to the organelle group called plastids and are found in land plants, algae and some protists. Chloroplasts are specialized plastids that are responsible for photosynthesis. They are observed as flat discs that range from 2 - 10μm in diameter, contained by an inner and outer membrane. The outer membrane is permeable to molecules, while the inner membrane contains many integral membrane proteins that regulate the passage of small molecules in and out of the chloroplast. The material inside the chloroplast is called the stroma and contains one or more chloroplast DNA molecules. It also has grana (singular: granum) which are stacks of thylakoids. Photosynthesis occurs within the membrane of these sub-organelles (Figure 1-3).

Plastids of green alga evolved from a single primary endosymbiotic event where a free-living *cyanobacteria* was engulfed by a larger eukaryotic host. In four billion years this evolutionary event has only happened six times – highlighting its importance in evolutionary context [12]. The double membrane of these plastids can be viewed as evolutionary traces as they are derived from their ancestral prokaryote. Most of the organelle genes have been lost or transferred to the nucleus, and most of the chloroplast proteins are now encoded by the nucleus and exported into the chloroplast. Thus they contain much less DNA than their closest prokaryotic relatives. This loss of DNA can be attributed to the redistribution of genetic material between the nucleus, mitochondria and chloroplast [13]. The transfer of genetic material coincided with the development of the cells ability to return gene products to the organelles. Genes that are encoded by the nucleus that are destined to the plastids are tagged with an n-terminal signal that is recognized by receptors that are in the cytosol or bound to the outer membrane of the plastids. After recognition, the proteins are translocated across the plastid bi-layer through a translocation machinery before it is released [14].

The chloroplast is a semi-autonomous organelle that has the capacity to replicate and transcribe its own genome as well as to carry out protein synthesis. A core set of proteins present in all chloroplast (and mitochondria) have remained in the organelle, a common feature among these genes are that they code for hydrophobic proteins that are involved in

energy generating processes. Some genes are thus retained in the chloroplast due to their hydrophobicity or they must be retained to control the regulation of their expression [13].
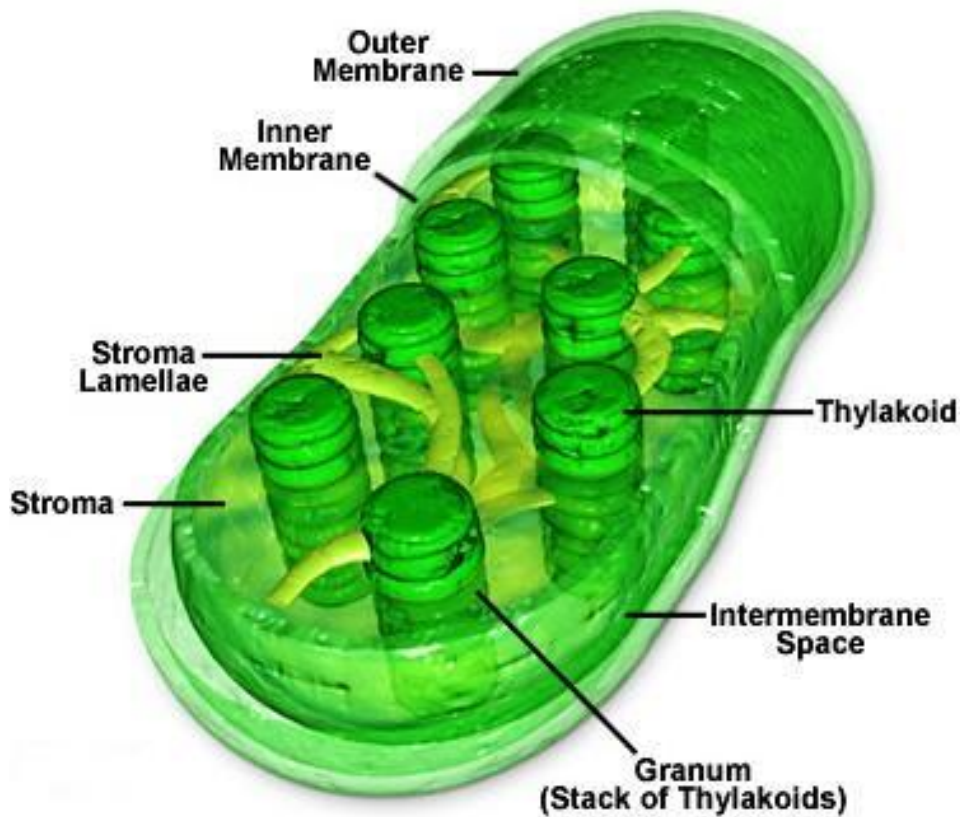
Figure 1-3: Structure of plant cell chloroplast. Photosynthesis occurs within the thylakoids membrane. Illustration taken from http://micro.magnet.fsu.edu

1.2.2 **The chloroplast genome of *Chlamydomonas reinhardtii***

Chlamydomonas contains a single cup-shaped chloroplast DNA molecule that takes up nearly half of the cell volume. There are approx. 80 copies of the circular chloroplast DNA molecules in the chloroplast and they are organized into 5-10 DNA-protein complexes called nucleoids [2]. The complete sequencing of the *Chlamydomonas* chloroplast genome was done in August 2001. It revealed a circular genome of 203,395bp possessing two copies of inverted repeat sequences (IR) that are separated by two almost equally sized regions of ~81 kb and ~78 kb (Figure 1-4). The plastome (chloroplast genome) contains 99 genes, including 5 rRNA genes, 17 ribosomal protein genes, 30 tRNA genes and 5 genes encoding the catalytic core of a RNA polymerase [15].

Figure 1-4: *Chlamydomonas reinhardtii* **chloroplast genome.** Genes that have been disrupted are highlighted (Grossman et al. 2003, ref nr. 7).

The *Chlamydomonas* chloroplast genome shows some interesting and unique features compared to chloroplast genomes of land plant. High frequencies of short dispersed repeats (SDR) are located throughout the genome separated by the location of the genes. An estimated 19,500 SDRs are present in *C.reinhardtii* plastome which constitutes about 5% of the plastome. The function of the SDRs are largely unknown, but studies have shown that they play a role in the formation of stem loop structures in 3'end mRNA transcripts, adding a layer of flexibility in determining mRNA termini [16]. In addition there is an atypical organization of the genes encoding for RNA polymerase. An unusual gene tscA encodes an RNA that is involved in the trans-splicing process of *psaA* mRNA [17]. Experimental data shows that *Chlamydomonas* chloroplast genome exists both as circular and linear molecules [7].

1.2.3 **Uniparental inheritance**

The uniparental inheritance of the *Chlamydomonas* chloroplast genome from mt+ gamete was first described more than 50 years ago (Sager 1954). The first molecular evidence of reduction of mt- chloroplast DNA was done in 1980 [18]. The discovery of selective degradation of mt- chloroplast DNA provided evidence that this was an active process and has led to the stipulation of a 'destroyer-protector model' for the digestion of mt- chloroplast DNA. There are two distinct events to this model that explain the uniparental inheritance phenomenon. First is the 'protection' of mt+ chloroplast DNA and a 'destruction' of mt- chloroplast DNA during zygote development[19, 20]. This is supported by the detection of a nuclease that is imported into mt- gametes and degrades the chloroplast DNA [21]. Active digestion of male organelle DNA is observed in many species also animals, indicating that this is a universal mechanism among eukaryotes [22].

1.2.4 **Photosynthesis and RuBisCO**

Photosynthesis is the biological process in which carbon dioxide is transformed into chemical energy in the form of usable carbohydrates by using light energy and water. This biological reaction is the source of the O_2 we breathe and the carbohydrates all living organism depend on, thus making it the most important biological reaction on earth. Photosynthesis can be divided into two parts.

The *Light reaction* (light-dependent reaction) occurs within the chlorophyll where proteins that are organized in photosynthetic reaction centers get excited by light energy. There are two major photosynthetic reaction centers that contribute to the excitation of electrons – photosystem I and photosystem II. The excited electrons (e^-) are used in the electron transport chain to provide e^- to electron acceptors; this contributes to the reduction of NADP+ to NADPH, as well as converting ADP to ATP (Figure 1-4).

The *dark reaction* (also called Calvin Cycle or light-independent reaction) is where the carbon dioxide is converted to sugar, first in the form of 3-phosphoglycerate. The first step in photosynthetic carbon dioxide fixation is done by the enzyme RuBisCO (ribulose 1, 5-biphosphate carboxylase oxygenase) by capturing carbon dioxide from the atmosphere. RuBisCO is the most abundant protein in the world. It consists of eight large subunits that are encoded by the chloroplast genome, while the eight small subunits are encoded by nuclear rbc S-genes and are synthesized in the cytoplasm before they are imported into the stroma where they form complexes. The assembly of the holoenzyme is facilitated by the chloroplast chaperones cpn60 and cpn10 [23, 24].
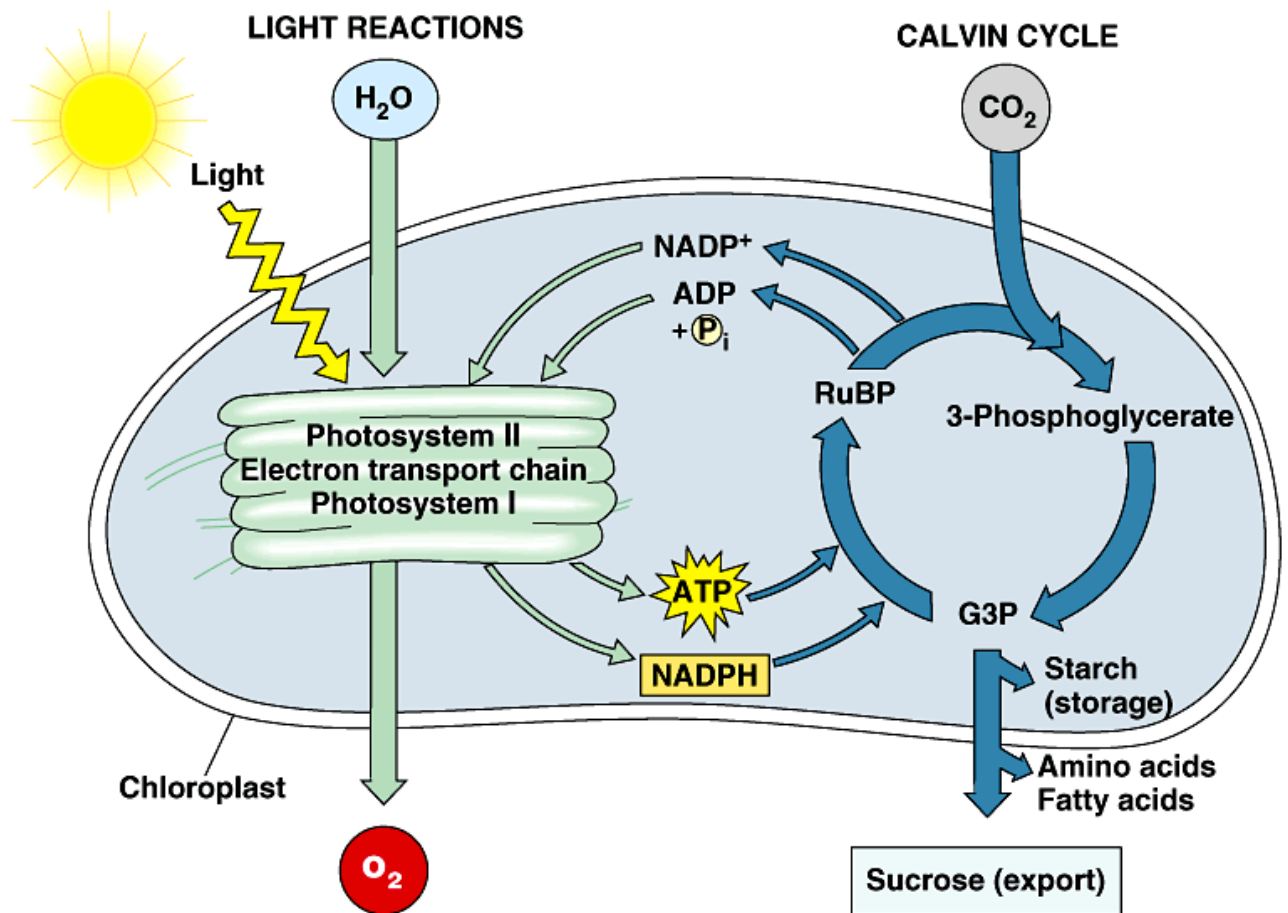
Figure 1-5: Light reactions and Calvin Cycle of photosynthesis. *ATP and NADPH produced in the light reactions are used in the Calvin Cycle. Water and carbon dioxide are used as substrates, while carbohydrate and oxygen are released as products. Illustration taken from: http://bglarochelle.pbworks.com/photosynthesis*

The catalytic limitation of Rubisco limits the efficiency of photosynthesis; research has been dedicated to increasing its activity for agricultural benefits [25]. There is also a growing interest in Rubisco as a tool to increase uptake of atmospheric carbon dioxide to combat climate change, as well as providing emission-free energy by using *Chlamydomonas* as biofuel producers [26].

1.3 Chloroplast gene expression

Due to the semi-autonomous nature of the chloroplast, most plastid proteins are encoded by the nuclear genome. The chloroplast proteins are first encoded as precursor proteins containing an N-terminal signal. The proteins are then imported into the chloroplast where they are cleaved and together with chloroplast-encoded proteins they form functional multiprotein complexes that govern and control the chloroplast gene expression [27]. Retrograde signaling ('plastid signals') from the chloroplast to the nucleus also plays a key role in the gene expression of nuclear-encoded plastid proteins. This bilateral communication between the chloroplast and the nucleus is important for optimizing chloroplast gene expression to changing environmental and metabolic states [28].

1.3.1 Chloroplast transcription

Chloroplast transcription is facilitated by two distinct RNA polymerases that are responsible for the transcription of chloroplast genes. They are called NEP (nuclear-encoded plastid RNA polymerase) and PEP (plastid-encoded plastid RNA polymerase). They recognize different set of promoters.

NEP is a single-peptide RNA polymerase that resembles the polymerase of the T3/T7 bacteriophage. Transcriptional activity has been detected in plants lacking PEP as well as in plants treated with PEP-specific inhibitors. The 10-nucleotide consensus sequence located upstream of transcription initiation sites are similar to T3/T7 phage-type promoters. NEP primarily transcribe plastid housekeeping genes [29, 30]. NEP has so far not been found in *Chlamydomonas*, suggesting that it only exists in higher plants. Experiments with addition of rimfampicin (which is an inhibitor for PEP through binding its β subunit) has shown that almost all transcription is terminated [31]. PEP is thus far the only RNA polymerase detected to be active in *Chlamydomonas* chloroplast genome.

PEP is a multi-subunit RNA polymerase complex that is encoded in the chloroplast by four genes *rpoA,rpoB,rpoC1 and rpoC2,* they code respectively for the peptide subunits *(α,β,β$'$ and β$''$)* making up the catalytic core of the enzyme. Homologs are found among the eubacterial RNA polymerase genes (*rpoA,rpoB,rpoC)* which make up the core of the PEP RNA polymerase similar in composition to the RNA polymerase of eubacteria. The latter gene *rpoC* is spliced into two separate genes in photosynthetic plants and algae [32]. In

Chlamydomonas the *rpoC1 gene* is split in two parts. Experiments demonstrating the lack of a single transcript as well as evidence of two linked coding regions confirmed this. These genes have thus been assigned *rpoC1a* and *rpoC1b* [15].

a) Plastid promoters

The nuclear encoded polymerase (NEP) recognizes promoters that share similarities with mitochondrial promoters in that they include a sequence motif 5´-YRTA-3´ near the transcription start site [30]. While the plastid encoded polymerase (PEP) initiates transcription from promoters resembling bacterial σ^{70} promoters, consisting of -35 (TTGACA) and -10 (TATAAT) consensus elements upstream the transcription start sites that resembles bacterial consensus sequences [29, 33].

b) Sigma factors

The RNA polymerase in bacteria is dependent on the nuclear-encoded promoter specificity factor sigma which is part of the RNA holoenzyme, for increase in promoter selectivity, specificity and the ability to initiate accurate transcription initiation. The sequence-specific binding of the holoenzyme occurs through contact between conserved amino acids in the sigma factor and specific nucleotides in the promoter sequence [34]. Based on this bacterial model, there has been extensive search for similar sigma-like factors (SLFs) in chloroplasts and several have been found in plants and algae. Higher plants express multiple SLFs and there is growing evidence supporting that this is in response to tissue-specific, light dependent and developmental signals. Providing an avenue for the nucleus to control expression of plastid genes under changing conditions [32].

In the case of *Chlamydomonas* there has only been discovered a single SLF. Two related studies provided evidence that *Chlamydomonas* contains a nuclear gene named RpoD, which encodes for an 80 KD protein. The protein has the conserved motifs of bacterial sigma-70 factors, as well as an N-terminal signal sequence [35]. Studies have confirmed that *Chlamydomonas* RpoD transcription exhibits circadian regulation, meaning that mRNA levels of RpoD levels fluctuates in light-dark cycling cells [36]. Being the sole sigma factor in *Chlamydomonas,* there is strong an indication that RpoD is the principal PEP transcription factor in *Chlamydomonas* chloroplast [36].
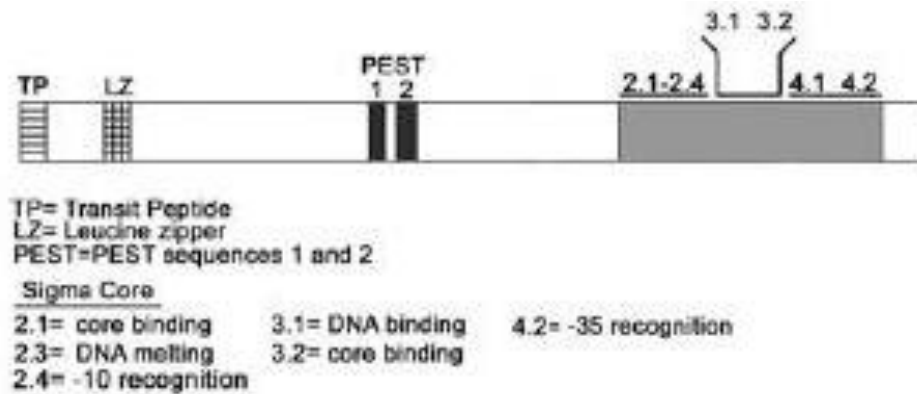
Figure 1-6: Motifs of the *Chlamydomonas RpoD* sigma factor protein. *(Carter et al. 2004, ref nr. 35)*

Transcriptional regulation through the circadian clock

Circadian rhythms are biological rhythms that occur with a period of 24 h under constant conditions of light and temperature [37]. Several studies have identified a correlated link between chloroplast and nuclear encoded gene's mRNA abundance over a circadian cycle. Numerous circadian rhythms of chloroplast-related mRNAs have been described in *Chlamydomonas* [38]. One group of genes called ROC (rhythm of chloroplast), encode several transcriptional regulators. One of them, ROC86 (XRN1) codes for a 5'-3' RNA exonuclease, suggesting a circadian role on the level of RNA turnover [39].

The RNA binding protein CHLAMY 1 recognizes specific UG-repeats that are present in 3'-untranslated regions (UTRs) of several mRNAs and its binding activity is regulated by the circadian clock. Experiments with introduction of UG-repeats within 3-UTRs of reporter genes have triggered circadian rhythms [37]. CHLAMY 1 has strong affinity to two proteins that are involved in metabolism of CO_2 , one of them being the small subunit of rubisco (rbcs) [38].

1.3.2 Post-transcriptional processing

The accumulation of plastid mRNA transcripts changes during chloroplast development and differentiation while the relative transcription rate is stable. The independence of transcription activity and mRNA accumulation suggests that mechanisms exist that can fluctuate the stability of mRNA transcripts. Transcriptional regulation plays a minor role in modulation of plastid gene expression indicating that this is mostly controlled at the post-transcriptional level [40]. Experiments with light-induced chloroplast development in different plant species

revealed that transcription activity and RNA accumulation act independently for most of the genes studied. Providing support for the hypothesis that post-transcriptional control of plastid gene expression is the main mechanism by which differential accumulation of most mRNA transcript occur [41]. Post-transcriptional mechanisms consist of RNA editing, intercistronic processing, intron splicing, RNA processing of terminal ends (5' and 3'end) and RNA stability. Nucleus-encoded factors participate in all steps of chloroplast gene expression, including in post-transcriptional processes. The expression of small subunits of chloroplast genes as well as functioning as regulatory factors that modulate chloroplast gene expression are needed in response to environmental and developmental signals [42].

RNA editing

RNA editing is found in all major groups of land plants and consist of nucleotide conversion of cytosine (C) to uracil (U) and to a lesser extent from U to C [43]. RNA editing events are essential for expressing functional proteins through modifying the amino acid sequence, or creating a translational start codon and stop codon. In tobacco, the functional *psbL* mRNA coding for a subunit of photosystem II is created by editing an ACG codon to create an AUG translational initiation codon. The most variable editing sites are usually in the third-codon position where editing does not alter the corresponding amino acid [44]. RNA editing is required for correct gene expression, proteins translated from edited transcripts are different from the ones that are deduced from the gene sequences [45]. RNA editing does not occur in *Chlamydomonas*. This was demonstrated by introducing an altered codon in *Chlamydomonas* that is edited in maize and tobacco, following transformation no editing was observed and the alga became non-photosyntethic [46]. There has also been suggested that RNA editing plays a partial role in transcriptional regulation of PEP polymerase through editing of a key residue in *rpoA* [47].

No consensus sequence has been identified near nucleotides destined for editing. However, certain proximal cis-elements are required for editing. In the case of *psbL* mRNA it was discovered that editing occurred within a 22 nucleotide fragment around the edited cytosine both upstream and downstream (-16 to +5) [48]. While studies on *ndhb* transcripts showed that vital sequences were located -12 and -2 positions from editing site [49]. In tobacco there are 34 nucleotides that undergo editing to U. Even though there is no common consensus

region for the editing sites, there is a possibility to cluster the recognition regions into groups that share common short sequences [50].

The cis-elements are binding sites for nuclear encoded trans-factor proteins. The first chloroplast-editing trans-factor to be identified was found in *Arabidopsis* mutants defective in NAD(P)H dehydrogenase [51]. These site-specific factors appear to be in limiting quantities, because high-level expression of transcripts carrying editing sites result in reduced editing of the endogenous gene that carries the same editing targets [52].

Intercistronic processing

Chloroplast genes are often organized into operons in higher plants and subsequently transcribed into polycistronic primary transcripts that contain the information for several proteins. They are often processed into monocistronic transcript units (mRNA) that code for a single protein. The enzymes that are involved are considered to be both endo- and exonucleases [53]. An example is the *psbB* operon of spinach which is transcribed as a polycistronic unit and is translated to more than a dozen different proteins [54].

In *Chlamydomonas* very few chloroplast genes are organized into operons, one of them is the *petA-petD* operon that is transcribed to a dicistronic mRNA or a *petD* monocistronic mRNA. Studies with deletions of the 5' region in *petD* identified a sequence that is essential in determining whether *petD* is transcribed as a monocistronic unit or with a *petA-petD* dicistronic unit [55]. Mutants with defective intercistronic processing machinery often lead to translational blockage [53].

Intron splicing

Chloroplast genes are interrupted by noncoding intervening sequences called introns. The chloroplast introns of plants and algae are divided into two main subclasses, group I and group II, which are defined by their characteristic secondary structures and splicing mechanisms [56]. The first chloroplast gene containing introns was described in *Chlamydomonas* (Rochaix and Maloe, 1978). Following the complete sequencing of the *Chlamydomonas* chloroplast genome, five group I introns have been identified in *Chlamydomonas* chloroplasts. One is located within the *rrnL* gene that codes for the chloroplast 23S rRNA, while the other four are located within the *psbA* gene that codes for an

essential protein (D1) of photosystem II. Splicing of *psbA* increases when cells are transferred from dark to light, indicating a regulatory mechanism of *psbA* splicing [57].

All of the group I introns in *Chlamydomonas* self-splice *in vitro*, meaning that the precursor RNAs are also ribozymes [58].

Chlamydomonas also contains two group II introns, these are located within the *psaA* gene and are spliced in trans, meaning that the mature psaA transcripts consists of exons joined together from three different precursor mRNA transcripts [58]. The process of compiling a mature *psaA* mRNA requires at least 14 nuclear encoded factors as well as one chloroplast-encoded co-factor (*tscA* RNA) [56]. This highlights the role nuclear-encoded splicing factors play in complex splicing mechanisms in chloroplasts. Several of these nuclear genes have been identified [59].

Processing of mRNA terminal ends (5' end and 3' end)

RNA processing is a general term to describe modifications of a synthesized RNA molecule. The transcriptome in chloroplasts contains primary and processed transcripts. In plants and *Chlamydomonas*, the longer transcripts are primary, while the shorter transcripts are cleavage products of the former. There are no current examples of primary transcripts in *Chlamydomonas* chloroplasts, indicating that most transcripts undergo processing in this species [60]. The latter go through forms of processing to form mature 5´ and 3´ends. Ribonucleases are the enzymes that carry out RNA maturation and degradation. These processes are mostly carried out by two types of ribonucleases, **exoribonucleases** that remove nucleotides either from 5'ends or 3'ends, and **endoribonucleases** that cleave transcripts internally. Endonuclease cleavage products are sometimes substrates for exonucleases [61].

There are two possible mechanisms that may lead to the formation of mature 5'ends as a result of RNA processing. A 5´ → 3´ exonucleolytic pathway or a site-specific cleavage by an endoribonuclease [60]. 5'end processing of *psbA* mRNA includes removal of nucleotides and a stem loop structure indicating that this has a role in ribosome association and subsequent translation [62].

Chloroplast mRNAs undergo 3´end processing because transcription termination is inefficient and typically continues beyond the 3´end of the gene. Chloroplast genes are like bacterial genes flanked at their 3´end by inverted repeats (IR) that can form stem loop structures. The

3´end are located downstream of the inverted repeats. In the case of *atpB* mRNA 3´end formation involves a two-step process, an endonucleolytic cleavage followed by a exonuclease trimming [63]. Experiments indicate that stem-loop structures do not serve as terminators, but play a role in RNA 3´end processing [64]. Disruption of IR *in vivo* leads to unstable transcripts, due to exposing them to exonuclease activity [16].

1.3.3 mRNA degradation and stability

The abundance of RNA at any given time is determined by its synthesis and degradation. RNA stability is expressed in units of half-life, meaning the amount of time (hours or minutes) that is required for half of the initial amount of the RNA being measured to disappear [53]. A distinct feature of chloroplasts compared to prokaryotes is their long RNA half-lives, which can range from 30 minutes to several days.

A general hypothesis describes chloroplast RNA degradation as a three step process:

1. Endonucleolytic cleavage producing two RNA molecules, a distal and proximal fragment with the latter having removed the protective 3´ IR. This produces structures for the second step by eliminating secondary structures and RNA-binding proteins.

2. The proximal fragment is efficiently polyadenylated, adding a tail of several hundred nucleotides. The distal fragments are subjected to further rounds of endonucleolytic cleavage, creating additional fragments that would undergo polyadenylation.

3. Polyadenylated RNA fragments are rapidly degraded by polynucleotide phosphorylase (PNPase) which has a high affinity for poly(A) sequences or other exonucleases [65].

In vitro studies of RNAs with stem loop structure were found to be poorly polyadenylated, so an alternative mechanism for the degradation of the distal fragment can be through 5´→ 3´ exonuclease activity [53]. Depletion of PNPase in *Chlamydomonas* left few transcripts with poly(A) tails in chloroplast transcripts, indicating that PNPase is the major enzyme responsible for synthesis of A-rich tails for this species [66]. Experiments where chloroplasts were treated with the polyadenylation inhibitor cordycepin, showed that exonucleatic degradation was inhibited. Leading to accumulation of endonucleolytic cleaved products,

demonstrating that polyadenylation is an important post-transcriptional process in targeting transcripts for degradation [67].

Studies on chloroplast mutants have revealed that both 5´-untranslated and 3´-untranslated regions (UTRs) of chloroplast mRNAs contain elements required for the stability of the transcripts [53].

The role of 3´- untranslated regions

Deletion of stem-loop structures located within the 3´ UTRs of *atpB* and *psaB* mRNAs lead to significant reduction in the accumulation of these transcripts [68, 69]. Deletion of these structures in *atpB* mRNA lead to significant reduced levels compared to wild-type levels without affecting the transcription rate of the gene. Indicating that the inverted repeats in proximity to the 3´end play a key role in the stability of *atpB* mRNA [69]. However, the 3´-unstranslated region of *rbcL* and *psaB* and their associated inverted repeats showed that their removal does not affect RNA decay and therefore play no major role in RNA stabilization [70]. Recent studies of the two inverted repeats close to the 3´end of *rbcL* showed that deletion of either IR showed no change in their accumulation rate, but deletion of both resulted in loss of *rbcL* mRNA [71]. This indicates that the distinct features of the 3´end of each chloroplast transcript and their formation determine their significance in the stability of the transcript. Functional substitutions of different RNA elements have the capability to protect RNA regions from 3´→5´ exonucleolytic attack in *Chlamydomonas* chloroplasts [72].

The role of 5´- untranslated regions

The 5´ ends of transcripts do not generally feature stem-loops, but are rather protected by specific proteins. The 5´ UTRs of several chloroplast mRNAs have been shown to contain RNA cis-elements that are required for translation. In *Chlamydomonas*, the protein MCA1 protects *petA* mRNA by binding its 5´ end and protecting it from 5´→ 3´ degradation. The abundance of the protein seems to play a function in regulating the accumulation of *petA* mRNA [73]. Experiments with chimeric chloroplast genes containing different 5´ and 3´ UTRs indicate that the accumulation of chimeric mRNA was largely independent of the 3´-UTR, suggesting that mRNA accumulation of many *Chlamydomonas* chloroplast transcripts is mainly determined by the promoter and 5´ UTR [74]. Stability determinants for mRNAs of *psbB*, *psbD* and *petD* have been examined, and for these three chloroplast genes there have

been found nuclear-encoded proteins that promotes mRNA stability by binding to cis-elements in proximity to the 5´UTR. This has been confirmed for *psbD*, where deletions or changes of a few nucleotides at the gene's 5´ UTR can destabilize the transcript. The same was identified for *petD* where mutations of two to nine nucleotides downstream of the 5´ end caused RNA instability (Figure 1-7) [75, 76].

Transcript	Sequence	Position	Reference
atpB	5'-A U A A G C G U U A G U-3'	+ 31 - + 42	this study
rbcL	5'-A U U U C C G G A C-3'	+ 38 - + 47	this study
petD	5'-U U A G C A U G-3'	+ 2 - + 9	Higgs et al., 1999
psbD(1)	5'-C A C A A U G A U U A A-3'	+ 1 - + 12	Nickelsen et al., 1999
psbD(2)	5'-U G A G U U G-3'	+ 43 - + 49	Nickelsen et al., 1999

Figure 1-7: Example of sequences in 5´UTRs of transcripts of *Chlamydomonas* chloroplast genes that have been identified to be important for mRNA stability *(I.L. Anthonisen et al, 2001 ref nr.92)*

The three regulatory sequences in the 5´ UTR of chloroplast *petD* mRNA seems to be conserved among the different *Chlamydomonas* species, even if the overall *petD* 5´UTR are quite different. Indicating that orthologs to regulatory sequences in the 5´UTR along with their trans-acting factors are highly specific among the *Chlamydomonas* species [77].

1.4 RNA-binding proteins as trans-acting factors

Several RNA binding proteins (RBPs) play an essential role in chloroplast gene expression. The interaction between RNA binding proteins and the corresponding RNA elements found within mRNA have been shown to be an essential part of mRNA stability and translational regulation [78]. The majority of RNA binding proteins analysed to date contain β sheets and α helices that together form RNA recognition motifs (RRM) that bind to target RNA [78]. The 5´ UTR of *PsbA* mRNA binds four nuclear-encoded proteins. *In vivo* experiments reveal that the 5´ UTR contains specific RNA sequences, including a stem-loop that function as binding sites for the proteins. Three nuclear-encoded genes have been cloned from *Chlamydomonas* that encode proteins mediating their function through chloroplast 5´ UTRs. They have been found in high-molecular weight complexes also containing RNA [79, 80]. RNA affinity

experiments of the *psbA* gene expression in *Chlamydomonas* revealed that the *psbA* 5´ UTR was interacting with a protein complex consisting of four subunits (RB47, RB60, RB55 and RB38). Through UV cross-linking assays it was identified that only the RB47 had direct contact with the *psbA* mRNA. The genes were cloned for the RB47 and RB60, revealing that they encode for a poly(A)-binding protein and a protein disulfide isomerase, respectively [81]. *In vitro* studies indicate that phosphorylation and redox reactions modulate the protein-binding to the *psbA* 5´UTR through RB47 and/or RB60 [82, 83]. A similar mechanism has been reported in *Arabidopsis* [84]. Comprehensive experiments were also performed on the chloroplast gene *psbD*, revealing several cis-acting elements required for stabilization or translation of the *psbD* mRNA. Deletion mutations on the *psbD* 5´ UTR resulted in the inability of a 40kD protein (RB40) to bind *in vitro* [75].

Nucleus-encoded proteins can also bind to 3'-untranslated regions as trans-acting factors and several studies have confirmed this in *Chlamydomonas*. The nucleus-encoded protein MCD4 participates in the degradation of nucleotides in proximity to a cleaved pre-mRNA, possibly by facilitating the endonucleolytic cleavage step in 3´end maturation of *atpB* mRNA [85].

The pentatricopeptide repeat proteins (PPR) are known as sequence-specific RNA-interacting protein, participating in many RNA processes, including RNA stabilization and modification [86]. They contain consensus sequences of tandem repeats consisting of conserved 31-36 amino-acid motif that is related to the tetratricopeptide repeats (TPR). This consensus sequence occurs in tandem repeats 2-26 motifs per polypeptide. Their structural variation coincide with their function in RNA binding, even though the details of the protein-RNA binding remain vague [87]. In *Chlamydomonas* the PPR family consists so far of a dozen members, one of them being required for chloroplast mRNA stability [73]. Nac2 and Mbb1 are nucleus-encoded TPR-like proteins contributing to the protection, protein recruitment and stability of their target mRNAs [80, 88].

1.5 The 5'end of the *Chlamydomonas rbcL* gene

The first indications of the role of chloroplast RNA 5´ regions were obtained through studies of *Chlamydomonas rbcL* gene [89]. In tobacco, 5´ UTR-controlled regulation of *rbcL* turnover has been reported, where lower rates of transcription is compensated by greater mRNA stability [90]. The *rbcL* 5´ UTR of *Chlamydomonas* is compromised of 92 nucleotides and is predicted to fold into two stem-loop structures. The first stem-loop closest to the 5´end consists of 41 nucleotides, while the other is smaller at 21 nucleotides (Figure 1-7). The promoter of the *rbcL* gene consists of a canonical -10nt sequence (TATAATAT), in addition a transcription-enhancing element downstream is able to increase transcription of the *rbcL* gene about 10-fold [91].

 In vivo studies have shown that *rbcL* 3´ UTR does not contribute when it comes to the stability of *rbcL* transcripts, but rather functions as RNA processing or transcription termination site. The degradation process of chimeric genes have shown to be independent of their respective *rbcL* 3´ UTR [70]. The stability of *Chlamydomonas rbcL* mRNA has been shown to be mainly attributed to sequences at the 5´UTR [89].
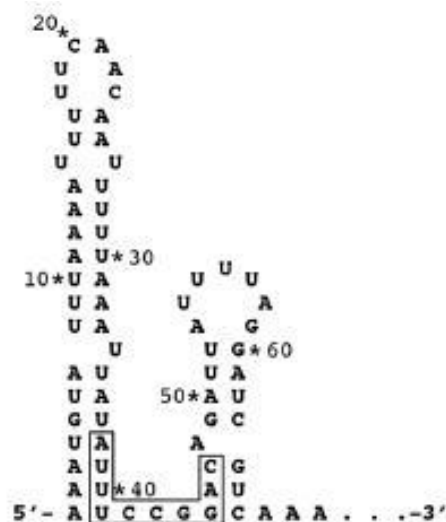
Figure 1-8: Predicted RNA secondary structure of the first 69 nucleotides of the *Chlamydomonas rbcL* mRNA. Nucleotides that comprise the cis-acting stability element (+38 to +47) are boxed. *(Suay et al.2005, ref nr. 94)*

Studies with reporter gene constructs with *rbcL* 5´ regions of *Chlamydomonas* identified a 10-nt sequence (5´-AUUUCCGGAC-3´) located +38 to +47 relative to transcription start site, that is crucial for transcript longevity. The -10nt RNA stabilizing sequence element

participates in the short single-stranded region between the two stem-loops and extends by a few nucleotides on each side into the bottom of both stem structures. Replacement of single nucleotides within this sequence led to a reduction of transcripts abundance of more than 95% [92]. Chimeric *rbcL*:GUS (β-glucoronidase) transcripts containing changes in the beginning of the 5´UTR that affect RNA secondary structure are estimated to enhance transcript degradation 50-fold [93].

Chloroplast transcript containing a stable 5´ stem-loop can be degraded rapidly, implying that a 5´ terminal stem-loop does not provide a structural hindrance for mRNA ribonucleases. Recent studies have shown that the role for the *rbcL* 5´- terminal stem-loop is to provide a stable RNA secondary structure, placing the crucial 10nt RNA stabilizing element in a specific conformation, consisting of a helical and single-stranded portion. The specific sequence and structure for the stability of *rbcL* transcripts suggests the involvement of a trans-factor to this element [94].

The lack of sequence and structural consensus for cis-acting stabilizing elements of chloroplast mRNA indicate that the diversity of these elements function as target site for different trans-acting factors functioning in multi-protein complexes, the 5´UTR of *psbA* is an example of this [81].

A recent study detected a novel PPR protein that binds to rbcL 5'UTR in *Chlamydomonas* and *Arabidopsis*. MRL1 is a conserved nuclear-encoded pentatricopeptide repeat (PPR) protein that is required for the stabilization of *rbcL* mRNA in *Chlamydomonas* and *Arabidopsis*. A mutation of this gene does not affect *rbcL* transcription, but *mrl* mutants lack *rbcL* mRNA in *Chlamydomonas* indicating that its primary role is to stabilize *rbcL* mRNA through binding its 5´ UTR. MRL1 is predominantly located in a high molecular mass complex [95].

Preliminary studies have identified a protein disulfide isomerase of 60kD that binds to *Chlamydomonas psbA* 5'UTR. The *psbA* mRNA is thought to be regulated by redox signals, and there is evidence that RB60 functions as a redox-sensor subunit. In vitro experiments show that RB60 is imported into the *Chlamydomonas* chloroplast [96]. Biochemical studies have shown that RB60 is associated with another protein (RB47), contributing to the formation of disulfide bridges between cysteins [97].

1.6 Aim of this study

The aim of this project was to determine whether the protein RB60 binds to the 5´-untranslated region of *rbcL* transcripts of *Chlamydomonas*. RB60 has been detected in multi-protein complex that binds to the 5´ UTR *psbA* complexes as a disulfide isomerase.

- First objective was to amplify the RB60 gene through PCR from and a different construct received from California, USA.

- Second objective was to clone the RB60 PCR product into a vector containing an N-terminal his-tag.

- Third objective was to express the gene in *E.coli*, isolate the RB60 protein and purify it.

- Fourth objective was to test the sensitivity of binding of anti-RB38 and anti-RB60 received from GenScript by western blotting. To use them co-immunoprecipitation experiments.

- Fifth objective was to test *in vitro* binding of isolated RB60 protein to *rbcL* 5´UTR sequences.

2 Material and methods

2.1 Working with bacterial cells

In order to minimize contamination when working with bacteria, instruments and solutions were sterilized by autoclaving for 20 min at 120 °C.

The bacteria used in this work were competent bacteria cells of *Escherichia coli* (*E.coli*) TB1 strain. *E.coli* has a long history as an experimental model because it can be easily manipulated and grown in laboratory settings.

2.1.1 Preparation of competent bacterial cells and growth plates

E.coli cells were made competent through $CaCl_2$ treatment [98].

Frozen competent cells were melted on ice prior to adding DNA. Exposure to heat shock (42°C, 90 seconds) followed by immediate cooling on ice was used for transformation procedure. Transformed cells were selected from ampicillin-containing LB plates.

The bacteria were grown in Luria/Bertani (LB) medium (10g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) and incubated with rotation or shaking at 37°C. *E.coli* cells were plated onto agar plates to obtain single colonies. Growth medium for LB plates consist of LB medium with 1.5% agar (15g).

2.1.2 Preservation and growth of bacteria

For long-storage of bacteria cultures, approx. 1.5ml of bacteria culture was stored in 1.5ml eppendorf tubes at -80 °C.

Bacteria from a culture were grown on agar plates by streaking on agar surface with a sterile glass pin. Cells usually grow very much after overnight incubation (O.N) at 37°C and reach a stationary phase. Bacteria colonies from an agar plate were harvested by scraping with a sterile pipette tip on the surface of agar plates. The pipette tip was inoculated with 3 ml LB-medium containing ampicillin in a 15mL sterile plastic tube. Cells were grown O.N. at 37°C with rotation.

For large-scale growth, 1ml of cell culture was inoculated in a 100ml LB medium flask containing 100μl ampicillin (final concentration: 16mg/ml) and put on shaker for 2 hours. Absorbance was checked until an OD of around 0.5 could be measured by a spectrophotometer. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to induce protein expression. LB flask was then put back on shaker for another 6 hours. Cells were then harvested with two tubes and centrifuged for 5min, 5000rpm (Beckman Coulter, Avanti J-25). Supernatant was discarded, while pellet was resuspended completely with buffer A (see appendix II). Cells were centrifuged again and spun down. Supernatant discarded again and pellet was resuspended again in 1ml buffer A. Cell solution was transferred to two 1.5ml eppendorf tubes before frozen and stored at -80°C.

2.1.3 **Plasmid isolation from *E.coli***

Miniprep

Small scale plasmid isolation of DNA from *E.coli* cells was performed according to protocol [98]. Transformed *E.coli* cells were grown overnight in ampicillin-containing (60μg/ml) LB medium prior to isolation. Plasmid isolation from 1.5ml cell culture yields approx. 1 - 3μg DNA.

Maxiprep

For large-scale plasmid isolation of DNA, maxi-prep procedure was used. A CsCl density gradient centrifugation was performed according to protocol [98]. Transformed *E.coli* cells were grown overnight in ampicillin-containing (60μg/ml) LB medium prior to isolation. Plasmid isolation from 100ml cell culture yields approx. 150 – 400 μg DNA.

2.1.4 **Cell disruption by ultrasound**

Frozen cell cultures were melted on ice (4°C) prior to ultra-sonication (Ultrasonic VCX). The homogenization of the cells is done through high frequency waves from the probe that disrupts the cells and releases the intracellular components. The probe should be submerged in the solution, but not be in contact with the tube wall. 5 seconds of high frequency burst is accompanied by a 10 sec break for a total of 1 min. Fractionation of the cells is done through

cold centrifugation (4°C) for 10 min at 13.2 rpm (Beckman Coulter, Avanti J-25). Supernatants are transferred to new tubes.

2.2 DNA methods

2.2.1 Agarose gel electrophoresis

This method is used to separate DNA molecules according to their size[1]. A gel of 0.7% or 1% containing ethidium bromide in TAE buffer was used to give a good separation of DNA molecules between 0.5 and 10 kb. A 1kb standard ladder was used to estimate the size of the DNA fragments.

2.2.2 Isolation of DNA fragments by gel electrophoresis

DNA fragments from gel electrophoresis (see section 2.2.1) were extracted by cutting out a piece of the gel right below the DNA fragments. A dialysis-membrane was inserted into the well and filled with TAE buffer. This ensured that a barrier was created for the movement of DNA fragments. Electrophoresis was resumed and the DNA fragments moved into the well and were stopped by the dialysis-membrane, creating a sharp DNA band that can be visualized under mild UV-light. The DNA fragments in the TAE buffer can be collected by a pipette and purified through phenol-chloroform extraction and precipitated by ethanol.

2.2.3 PCR amplification of RB60 gene

Polymerase chain reaction (PCR) is a technique used to amplify a specific region of a DNA strand. The method provides an opportunity to scale up miniscule amounts of starting material.

The procedure consists of three main steps:

1. The **denaturation** step that is performed at a high temperature (~94°C) allows for the separation of complimentary DNA strands.

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[1] From: http://www.methodbook.net/dna/agarogel.html

2. The **annealing** step requires lowering of the temperature to 55-62°C, which gives the primers the opportunity to anneal to their complimentary sequences. The primers are designed to bracket the DNA region to be amplified.

3. Primer **extension** is usually performed at 68-72°C, according to optimal temperature range for the polymerase. Extension time varies according to the length of PCR product of interest.

We decided to use a construct (pET-RB60) containing our gene of interest (RB60) that had already been made in the lab as a template DNA for PCR amplification. This construct contained an incorrect RB60 gene inserted that was too large (containing additional nucleotides). The correct RB60 gene is approx. 1600bp[2].

Following primers (obtained from MWG Biotech AG) were used:

Forward primer: 5´-CTAGGAGTACGTTTACGCCATGAAC-3´

Reverse primer: 5´-CGGATCACGCGCTGCCGGTGCT-3´

The forward primer contained an *NcoI* restriction site, while the reverse primer contained a *HindIII* restriction site. The primers were 100% complementary with the RB60 sequence located within the vector.

PCR product was checked on an agarose gel to verify if our RB60 gene had been amplified. 10µl Sodium acetate and 200µl 96% EtOH was added to the PCR sample and precipitated ON.

2.2.4 Restriction digestion of PCR product and vector

The restriction enzymes *NcoI* and *HindIII* (New England Biolabs) were used for restriction cutting of PCR product and pEcoli-Cterm 6xHN expression vector (Clontech) prior to ligation. Restriction digestion is performed according to New England Biolab guidelines.

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[2] Data obtained from PubMed, updated 18/9-10:
http://www.ncbi.nlm.nih.gov/pubmed?Db=gene&term=%28rb60[gene]%29%20AND%20%28*Chlamydomonas* %20reinhardtii*[orgn]%29%20AND%20alive[prop]%20NOT%20newentry[gene]&sort=weight

After restriction digestion, both PCR product and vector are identified and collected by gel electrophoresis (see section 2.2.2).

pEcoli expression vector is 5.7kb (see appendix I), while PCR product (RB60) is 1.6kb.

2.2.5 Ligation reaction

Approx. 1:3 ratio between vector and insert (PCR product) is needed to get the correct amount of DNA molecules in relation to each other. Following reagents were added in an eppendorf tube:

Vector (pEcoli)	1.5μl (200ng)
Insert (RB60)	2μl (120ng)
Buffer 10X	1μl
T4 DNA ligase	0.5μl
PEG	2μl
dH$_2$0	3μl
Total	10μl

Prior to ligation, DNA fragments were heated to 45°C to facilitate melting of hydrogen bonds, avoiding that fragments ligate to themselves (re-ligation). Ligation reaction was set at 16°C for 3 hours, before being moved to room temperature for 30min.

2.2.6 DNA quantification

Dot spot method was used to estimate DNA concentrations [98].

2.2.7 DNA sequencing

Sequencing of RB60 gene cloned into the pEcoli-Cterm 6xHN expression vector was done by Sanger dideoxy method (MWG Biotech, Martinsried, Germany) to verify whether the gene was cloned correctly into the vector.

2.3 Protein methods

The construct (pEcoli-Cterm 6xHN_RB60) encodes the RB60 protein along with a histidine tag (6xHN). Immobilized metal ion chromatography (IMAC) exploits the interaction between side-chains of certain amino acids (in particular histidine) on proteins and chelated transition metal ions (i.e. Ni^{2+}). Ni^{2+} is the preferred metal ion for purification of histidine-tagged proteins. The IMAC medium Ni Sepharose consists of beads of highly cross-linked agarose that have been charged with nickel (Ni^{2+}) ions (GE Healthcare).

2.3.1 Preparation of Ni-Sepharose column

1. Gently shake the bottle containing Ni-Sepharose until medium is homogenous.

2. Take out 400µl of slurry into an eppendorf tube.

3. Centrifuge tube for 5min, 0.5 rpm.

4. Discard supernatant carefully and add 1ml of distilled water ($dH_2 0$).

5. Gently shake the tube to mix the solution well for approx. 3min.

6. Repeat step 3 – 6 using buffer A instead, 1ml.

7. Pipette out buffer (clear liquid) and add 200µl of buffer A again, mix well.

8. Pour slurry into column. Let slurry solution get into column well (30min).

9. Wash column with 1ml buffer A.

10. After preparing Ni Sepharose column, cell solution after centrifugation (see section 2.1.5.) was poured into the column. Prior to this 60µl was taken out to a new tube ('crude extract'). 30µl SDS gel loading buffer was added.

11. Load the rest of the cell solution to the Ni sepharose column.

12. Let first few drops drip through (mainly buffer A). Collect 60µl of flow through (~3drops) in a new tube ('flow through'), add 30µl SDS gel loading buffer.

13. After flow through of supernatant is finished, add 3ml of buffer A into the column. Collect fractions in eppendorf tubes. Add another 3ml of buffer A and collect again. Take out 60µl from the last tube and transfer to a new tube ('wash'), add 30µl SDS gel loading buffer.

14. Elute the column with 3ml of **buffer B** (appendix II) and collect fractions in separate tubes (~800µl pr. tube). Take out 60µl from each fraction and transfer to new tubes ('purified RB60'). Add 30µl SDS gel loading buffer.

15. Denature all tubes containing SDS gel loading buffer by heating the samples at 95°C for 5min. Freeze samples at -20°C.

16. Store samples of purified proteins in cold environment (4°C).

2.3.2 Filtration

Protein samples were concentrated through filtration. This was done by applying the protein solutions to tubes containing columns with filters that have a molecular cut off points of 30 kD (Millipore), thus excluding proteins larger than this. This was done by brief (5 min) centrifugation (5000rpm) of the tubes to let buffer and proteins less than 30 kD run through the filter. Volumes of the samples were reduced from 2.5ml to ~300µl.

2.3.3 Determining protein concentration

The concentration of RB60 protein was determined by following a standard procedure. (*Bio-RAD protein assay protocol*). Different concentrations of bovine serum albumin (BSA) were used as standard. Absorbance was measured at 595nm.

2.3.4 **SDS-PAGE**

In sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins are separated according to their molecular mass under denaturing conditions. Treatment with the anionic detergent SDS in heating environment (95°C) destabilizes secondary and tertiary structures, thus denaturing proteins and applies a uniform net negative charge to the proteins. Ensuring that movement of proteins through the gel depends solely on their size.
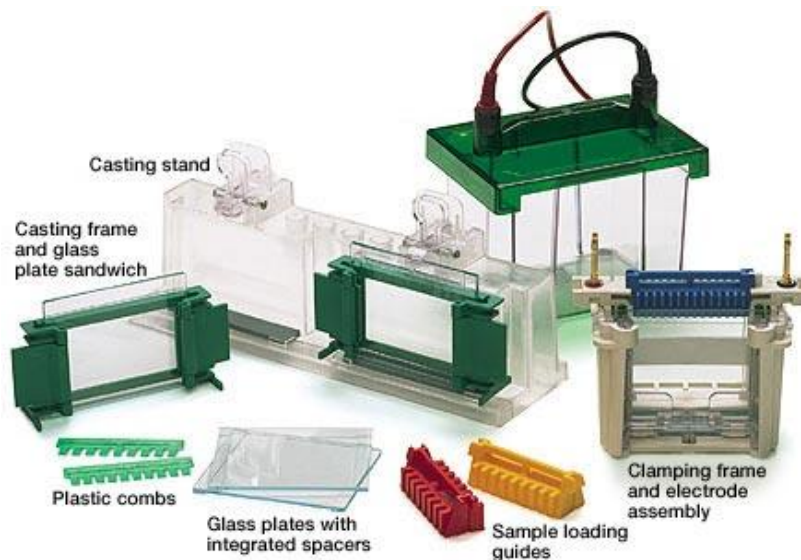
Figure 2-1: Equipment of the Mini-PROTEAN 3 electrophoresis system.

Illustration from: http://www.proteomicsnijmegen.nl/FTMS_pages/Documents/protean3.pdf

Preparation of SDS-PAGE

A 10% polyacrylamide solution was prepared in a 15ml plastic tubes. **Solution A, B and B'** (appendix II) are the main components of the polyacrylamide gel:

Seperating gel		Stacking gel	
Distilled water (dH$_2$0)	3.4ml	Distilled water (dH$_2$0)	3.61ml
Solution A	2.75ml	Solution A	800µl
Solution B	2.06ml	Solution B'	1.5ml
Ammonium persulfate (10%)	33µl	Ammonium persulfate (10%)	60µl

Ammonium persulfate polymerizes the solutions and is thus added last. The separating gel is added between the assembled plates and layered with ~1ml distilled water in order to even out the upper surface. The separating gel polymerizes completely after $30 - 40$min. The water layer is removed before adding the stacking gel solution. A plastic comb is placed between the assembled plates to create wells.

Protein samples were denatured for 5 min at 95°C in a heat block and cooled down on ice. 15µl of sample was loaded into the wells along with 8µl protein standard ladder (Bio-Rad).

Electrophoresis was performed according the Mini-PROTEAN 3 instruction manual [3] (Bio-Rad).

After electrophoresis the gel was floated of the glass plate and into a staining solution containing coomassie blue for about $5 - 10$ min on a rotating shaker. After staining, the staining solution was poured off and the gel washed with distilled water. The gel was then transferred to a de-staining solution for $15 - 30$ min to remove excess coomassie blue.

2.3.5 Western blotting

Western blotting is an analytical method used to detect specific proteins of interest. Proteins from bacteria cells were first separated by SDS-PAGE. Proteins were then subsequently transferred from the polyacrylamide gel to an immobilized membrane through electrophoresis [99].

A primary antibody was added and bound with high specificity to the protein of interest immobilized on the membrane. A secondary antibody, an enzyme-conjugated antibody binding the primary antibody is used to visualize and detect the protein of interest through chemiluminescence.

The western blotting was done with the use of nitrocellulose membrane using the *Mini-Trans-Blot® Electrophoretic Transfer Cell* (BIO-RAD).

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[3] Manual can be found at: http://www.proteomicsnijmegen.nl/FTMS_pages/Documents/protean3.pdf

The following steps were taken:

1. Preparation of 1L western transfer buffer (appendix II).

2. Nitrocellulose membrane (BIO-RAD) was cut according to the size of the gel.

3. Fiber pads, membrane and filter papers were soaked in transfer buffer.

4. Assemble of the gel sandwich with gel and membrane sandwiched between fiber pads and filter papers (see figure 2-2). Close gel sandwich properly.

5. Gently insert the gel sandwich into the electrode module and put into buffer tank filled with transfer buffer. Include a cooling unit in the buffer tank to keep the environment cold during electrophoresis. Also a small stir bar is added to help maintain even buffer temperature and ion distribution.

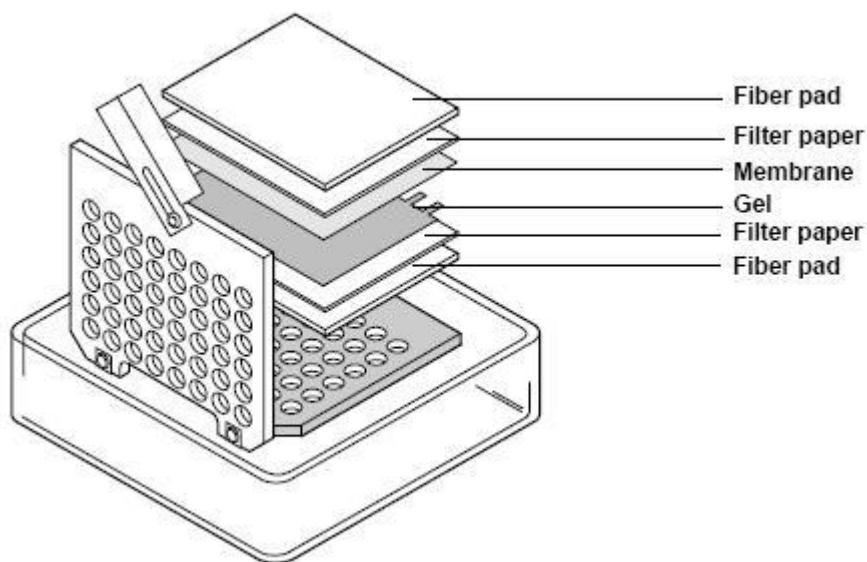6. Electrophoresis was run at 100V for 10 min and 20 min at 60V.

Figure 2-2: Assembly of gel sandwich containing gel, membrane, filter papers and fiber pads.

Illustration from bio-rad instruction manual (http://mcdonald.ucdavis.edu/Manual/MiniTransblotCell.pdf).

7. After electrophoresis the membrane was removed from the gel sandwich and non-specific binding sites were blocked by immersing the membrane with blocking buffer containing TBS, Tween (0.05%) and non-fat dry milk (5%) (Appendix II) for 1 – 2 hours on a rotating shaker at 4°C.

8. After blocking, the membrane was rinsed with distilled water before incubation with the primary antibody (appendix III) O.N. at 4°C.

9. The membrane was washed with washing buffer (appendix II) in between incubation with primary and secondary antibodies (appendix III).

10. Incubation with secondary antibody (appendix III) for 1.5 – 2 hours, before final washing of membrane.

11. Membrane was developed with a chemiluminescence solution (appendix II) that reacts enzymatic with the secondary antibody to produce visible bands.

2.4 RNA-binding experiments

2.4.1 Plasmids

The starting vector for construction of plasmids SP64-UTR and SP64-+54, were used for *in vitro* transcription of complete and short (+1 to +54) versions of the *rbcL* 5' UTR sequence, respectively, was the pSP64 poly(A) vector (Promega, appendix I). The vector sequence was altered (QuickChange Site-directed Mutagenesis kit, Stratagene) to create plasmid pSP64-SwaI (G-G $\Rightarrow$ A-A in positions 3019-3020 of the SP6 promoter sequence) containing a *Swa*I site that allowed easy oligonucleotide cloning of the *rbcL* 5' UTR sequence in frame with the SP6 promoter sequence. The complementary oligonucleotides SP64-1F and SP64-1R (appendix III), containing the *rbcL* 5' UTR sequence from +1 to +54, were annealed and cloned into *Swa*I/*Sac*I-digested pSP64-SwaI to create plasmid pSP64-+54. The complementary oligonucleotides SP64-2F and SP64-2R (appendix III) were annealed and cloned into *Bsp*EI/*Sac*I-digested pSP64+54 to create plasmid pSP64-UTR. Transcripts from plasmids pSP64-UTR and pSP64-+54 have a 4 nucleotide GAAT extension at their 5'

terminus which does not affect folding of the *rbcL* 5' UTR nucleotide sequence into its predicted native conformation. The extension provided the G nucleotide in position +1, required by SP6 RNA polymerase for efficient initiation of transcription.

2.4.2 *In vitro* transcription

Labeled transcripts of the *rbcL* 5' UTR and of the UTR sequence from position +1 to +54 were synthesized using the MAXI script kit (Ambion) with SP6 RNA polymerase and *Ecl136II*-digested plasmids pSP64-UTR and pSP64-+54.

2.4.3 Electrophoretic mobility shift

The gel electrophoretic mobility shift assay (EMSA) is used to detect protein complexes with nucleic acids. The technique is premised on the knowledge that electrophoretic mobility of protein-nucleic acid complexes is less than the corresponding free nucleic acids [100].

The standard mobility shift assay contained 100 - 500ng recombinant RB60 protein and ≈ 0.2 pmol radiolabeled 5' UTR sequences (≈ 70 counts sec^{-1}) in 15 µl of 15 mM Tris, pH 7.5, 66 mM potassium acetate, 0.13 mM EDTA, 1.6 mM MgCl$_2$, 66 ng yeast tRNA, 13% glycerol. After mixing, the samples were incubated for 30 min on ice. At the end of the incubation period 1 µl of RNA gel loading buffer (0.25% bromphenol blue, 0.25% xylene cyanole FF, 1 mM EDTA, 50% glycerol) was added and samples were fractionated in a 10% polyacrylamide/10% glycerol gel at 100 V. The gel was briefly (5 min) fixed in 20% methanol/5% acetic acid, dried, and exposed to x-ray film (Kodak Biomax MS) for 10-30 min.

2.4.4 UV cross-linking assays

UV irradiation of protein-nucleic acid complexes causes the formation of covalent bonds between proteins and nucleic acids. This technique can be used to identify protein-nucleic acid binding sites and quantify the weight of proteins involved [101].

For UV cross-linking the 15 µl samples were pipetted, after the 30 min incubation period, onto parafilm on ice and irradiated for 3 min with UV light (254 nm) at 180 mJ cm^{-2} (CL-1000 UV crosslinker, UVP, Inc., Upland, CA, USA). Following digestion with 1 µg RNase A

for 1 h at 37 °C, samples were mixed with 0.5 volumes of 3X SDS gel loading buffer (0.2 M Tris, 0.6 M 2-mercaptoethanol, 6% SDS, 30% glycerol, 0.08 g bromphenol blue) and proteins separated by SDS polyacrylamide (10%) gel electrophoresis. The gel was briefly (5 min) fixed in 20% methanol/5% acetic acid, dried, and exposed to x-ray film (Kodak Biomax MS) overnight.

3   Results

3.1 Molecular cloning of RB60

3.1.1 Amplification, restriction digestion and ligation of RB60

The first objective was to amplify a gene for RB60 (1600bp) from a construct (pET-RB60). Amplification was done through PCR with primers specifying the boundaries for the RB60 gene, as well as introducing restriction sites for *HindIII* and *NcoI* restriction enzymes (as described in section 2.2.3).

The PCR product (RB60) was subsequently restriction digested and ligated into a *HindIII/ NcoI* digested pEcoli-Cterm 6xHN expression vector (section 2.2.4 and 2.2.5). Transformation was performed and the constructs were introduced into competent *E.coli* cells, transformants were selected on the basis of ampicillin resistance (section 2.1.2).

3.1.2 Verifying the ligation

Transformed *E.coli* cells were selected and grown in 3ml liquid cultures. Plasmids were isolated by miniprep procedure (section 2.1.4). Verification of the constructs was checked by cutting with specific restriction enzymes (section 2.2.4) and run on agarose gel (2.2.1).

The restriction enzymes *HindIII* and *NcoI* were used to cut the pEcoli-Cterm 6xHN_RB60 construct. This would release the insert (RB60) and vector (pEcoli) to give recognizable DNA fragments.

As seen in the photo of the agarose gel (figure 3-1) the expression vector was used as a control (nr.2), confirming its size of being above 5000bp. The vector containing the insert (nr.3) was cut with *HindIII* to release a linear DNA fragment considerably larger than the vector, indicating that the RB60 insert had been cloned into the vector.

The mini-prep isolate containing the construct cut with *HindIII* and *NcoI* (nr.4) released two distinct DNA fragments. The lower fragments being the RB60 insert of 1600bp, while the upper band being of identical size as the vector (nr.2) of 5.7kb.

1: 1 kb DNA plus ladder

2: pEcoli-Cterm 6xHN expression vector

3: Construct (pEcoli_RB60) cut with *HindIII*.

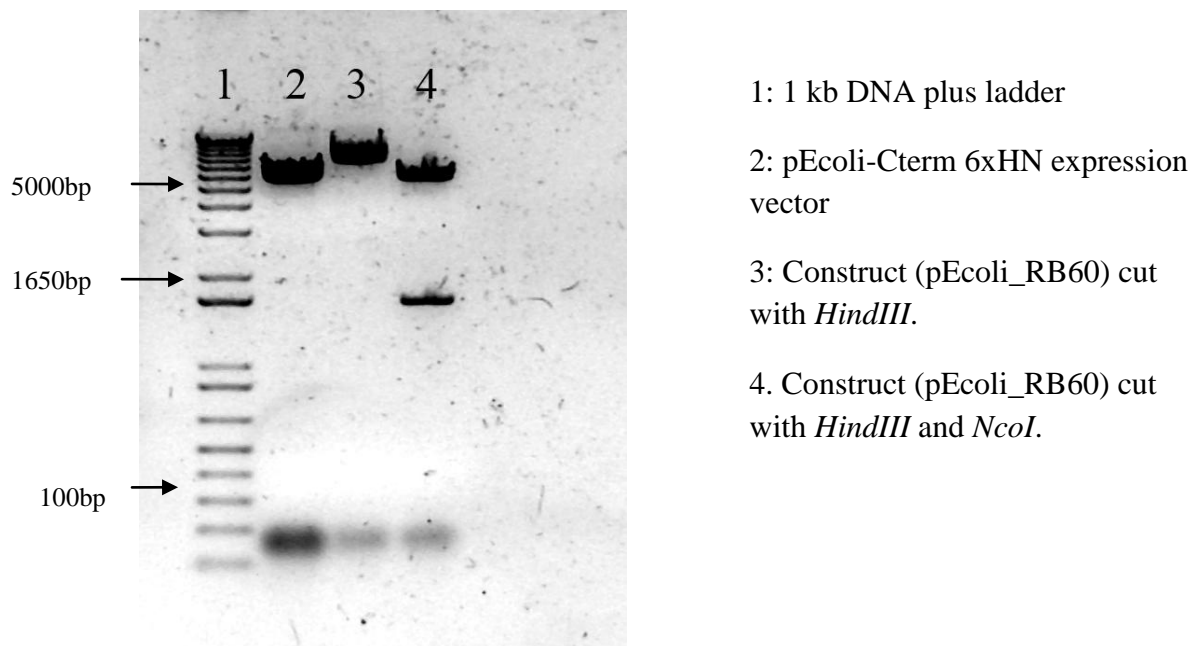4. Construct (pEcoli_RB60) cut with *HindIII* and *NcoI*.

Figure 3-1: Mini-prep DNA isolation and restriction enzyme digestion to verify the cloning of PCR amplified RB60 into expression vector pEcoli-Cterm 6xHN. Checking the size of vector and construct digested with the restriction enzymes *HindIII* and *NcoI*.

3.1.3 **Sequencing**

The samples were sent for sequencing after verification of transformants was done by restriction cutting (2.2.7).

3.1.4 **Growth of transformed *E.coli* cells**

Ampicillin-resistant bacteria colonies were selected for growth and protein expression of RB60 gene was induced (section 2.1.3).

3.2 Protein isolation

Frozen bacterial cells containing expressed RB60 were melted on ice prior to sonication and centrifugation (2.1.5). Supernatants from the *E.coli* cells were processed through *immobilized metal ion affinity chromatography* (IMAC) to isolate our specific protein of interest (RB60) through a Ni-Sepharose column (2.3.1). Different fractions were isolated through the column and run on a SDS polyacrylamide gel (2.3.4).
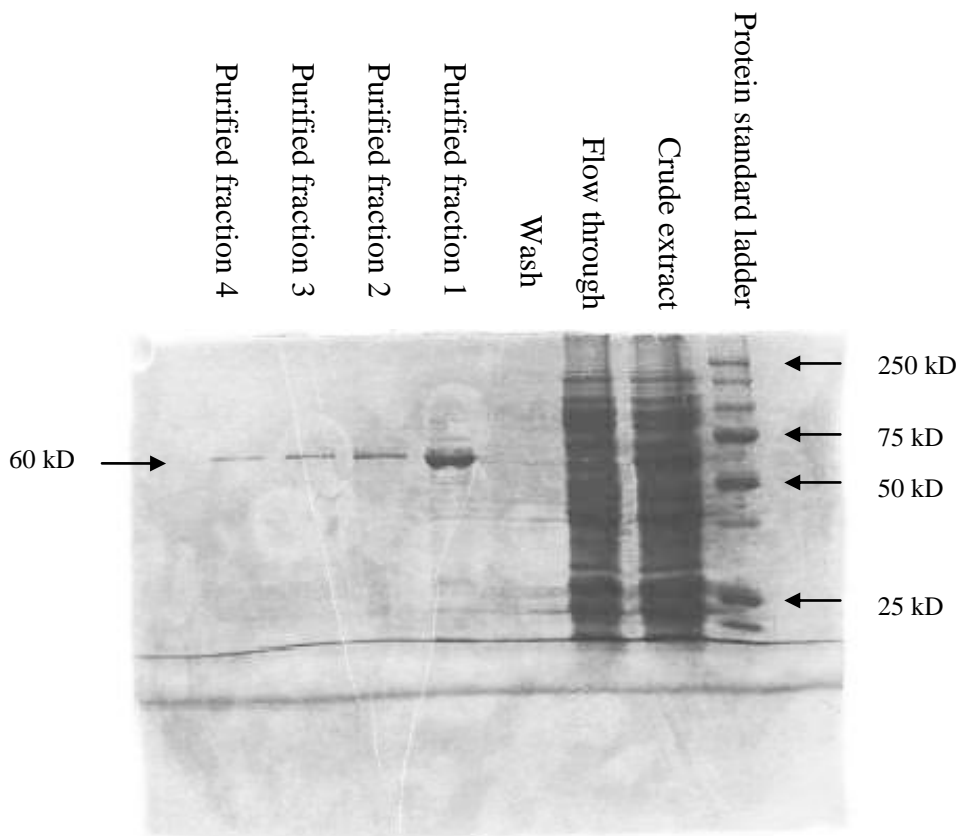

Figure 3-2: Different fractions of RB60 isolated from protein expressing *E.coli* cells through IMAC and run on SDS-PAGE gel.

The gel picture (figure 3-2) shows that the protein of interest was isolated with a relatively high specificity, indicating that the IMAC procedure exclusively binds amino-acids (histidine) that interact with the Ni^{2+}-ions in the column. The 'wash' fraction underlines this, as there is no protein binding here of RB60.

The isolated protein from the purified fractions was identified to have a size of 60 kD, with the highest levels of protein accumulation in fraction 1 and a steady reduction of protein

levels in the subsequent fractions (2-4). Some visible contamination is left in the purified fractions, with the highest contamination levels in the first purified fraction.

3.2.1 Protein purification

The purified fractions of RB60 were transferred to a single eppendorf tube. Further purification was conducted through gel filtration, enabling the reduction of contamination levels and increasing the protein concentration of RB60 in the samples (2.3.2).
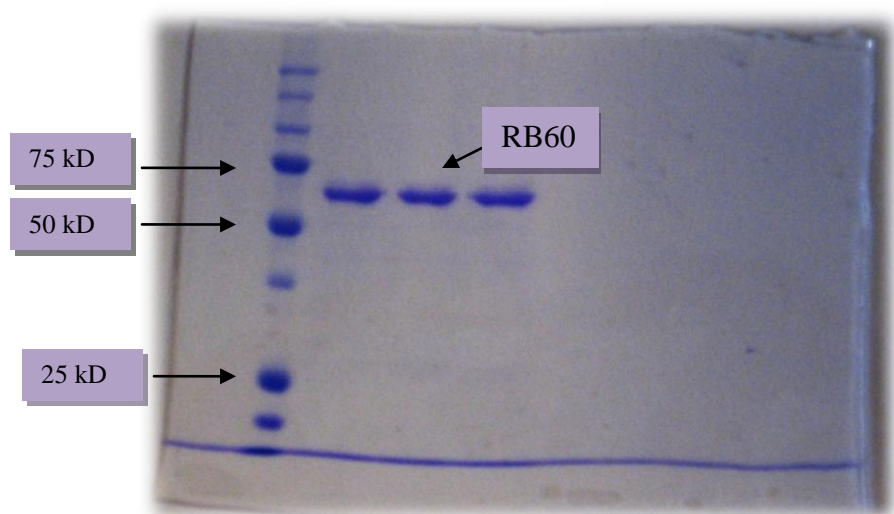

Figure 3-3: Purification of isolated RB60 protein sample. Contamination levels decreased through gel filtration.

Three identical samples with the purified RB60 were run on SDS-PAGE. The gel picture (figure 3-3) shows there was a reduction in the contamination levels after gel filtration, though some contamination is still detectable as a smear. The volumes of protein samples for the purified RB60 were significantly reduced from 2.5ml to 300µl.

3.2.2 Protein concentration

The concentration of purified RB60 protein samples were determined (2.3.3). Concentration levels of the samples ranged from 0.5 – 0.9 mg/ml.

3.3 Antibody-binding assays

Antibody binding assays were performed for RB38 and RB60. This was done in preparation for possible co-immunoprecipitation experiments with these two proteins in identifying novel proteins that bind to *Chlamydomonas rbcL* 5'UTR.

3.3.1 Antibody binding assay for RB38

RB38 has been identified in protein complexes that bind to *Chlamydomonas psbA 5'UTR* (see section 1.4). Later studies identified RB38 also to bind to the 5'UTR of *psbD* mRNA and contribute to the initiation of D2 protein synthesis [102].

The sensitivity of anti-RB38 primary antibody (GenScript) was assayed by western blotting. To investigate the antibody sensitivity, several dilutions of RB38 were made to determine the sensitivity levels of anti-RB38.

Dilutions:

Tube nr.	1	2	3	4	5	6	7	8
RB38	Protein standard ladder	5µg	2.5µg	1.25µg	625ng	312.5ng	156.25ng	78.1ng

Table 3.4: Dilutions prepared of RB38 protein samples for detection of antibody sensitivity.

Distilled water (dH$_2$0) and SDS were added to tubes containing RB38 protein prior to SDS-PAGE (2.3.4) and subsequent western blotting (2.3.5).
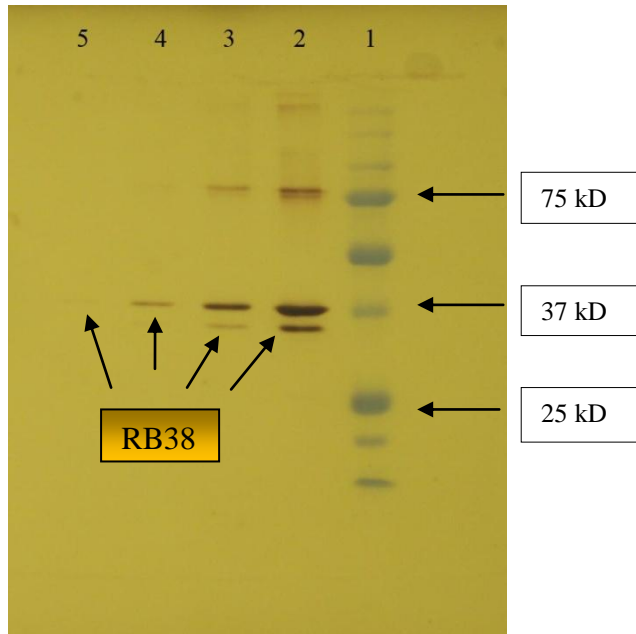
Figure 3-5: Antibody-sensitivity assay of RB38. Dilutions of RB38 containing different concentration were tested for sensitivity.

Clear detection is visible down to 1.25µg of RB38 (4), a weaker signal is detectable at 625ng of RB38 (5). Significant contamination is visible as well, in particular a protein of approx.75 kD is seen to have specificity for the antibodies used (figure 3-5).

3.3.2 **Antibody binding assay for RB60**

Several experiments were performed with an oligopeptide primary antibody *anti-RB60* (GenScript) containing a peptide sequence that should in theory have specificity for the RB60 protein. However, no detection was recorded using this antibody. Instead the use of a complete anti-RB60 antibody isolated from rabbit serum was used to provide visible detection of RB60. The sensitivity of this complete anti-RB60 from rabbit serum was tested by western blotting (2.3.5).

Dilutions were made from isolated RB60 protein sample after determining the concentration (2.3.3). Distilled water (dH_2O) and SDS were added to tubes containing RB60 protein prior to SDS-PAGE (2.3.4) and subsequent western blotting (2.3.5).

Dilutions:

Tube nr.	1	2	3	4	5	6
RB60	Protein standard ladder	3µg	1.5µg	750ng	375ng	Control (no RB60)

Table 3.6: Dilutions prepared from isolated RB60 protein sample after determining concentration levels.
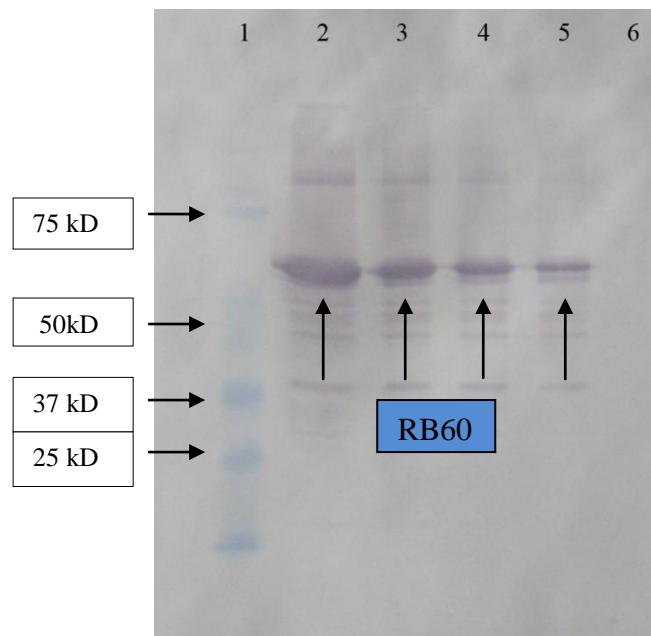

Figure 3-7: **Antibody-sensitivity assay of RB60.** Dilutions of RB60 containing different concentration were tested for sensitivity. Contamination is detected in all the samples.

The expressed RB60 protein is detected in all dilutions (figure 3-7). The control (6) with no RB60 protein does not provide a signal. The strongest signal is detected with the highest concentration (3µg) of RB60 (2), while the lowest concentration of 375ng (5) is detected as well.

The contamination level is quite visible in the samples, this decreases as the samples are diluted. This indicates that the antibodies used have specificity to other proteins besides RB60, and that the purification of RB60 was not sufficient to clear the sample of contaminants.

3.4 Gel electrophoretic mobility shift assay

Two plasmid constructs (SP64-UTR and SP64-+54) were used to synthesize labeled transcripts to verify whether RB60 contains binding capacity to *rbcL* 5'UTR. The synthesized transcripts contain nucleotides +1 to +54, which constitute the first stem loop and the cis-acting stability element (+38 to +48) of *rbcL* 5'UTR, as well as the complete 5'UTR sequence (2.4.2).
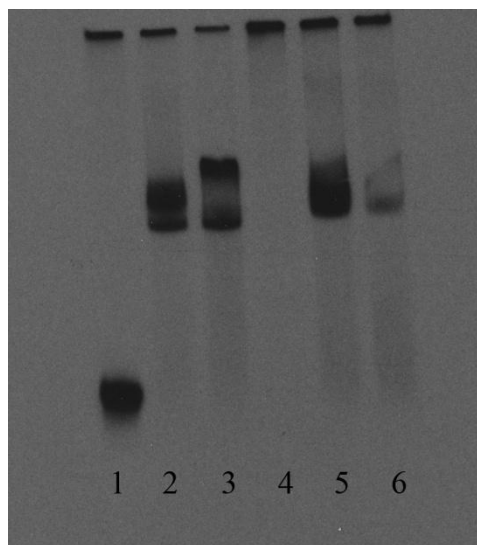
Figure 3-8: Autoradiogram of gel mobility shift assay of RB60 binding to *rbcL* 5'UTR sequences. Radiolabeled 5' UTR sequences were added with RB60. UTR sequences with absent RB60 proteins were used as control (1, 4).

Lanes	Content in autoradiogram
1	Complete transcribed *rbcL* 5'UTR. Control (no RB60).
2	Complete transcribed *rbcL* 5'UTR with RB60 (C-term his-tag) expressed from *E.coli* cells.
3	Complete transcribed *rbcL* 5'UTR with RB60 (N-term his-tag and fewer amino acids) expressed from *E.coli* cells.
4	Transcribed *rbcL* 5'UTR sequence from +1 to +54. Control (no RB60).

| 5 | Transcribed *rbcL* 5'UTR sequence from +1 to +54 with RB60 (C-term his-tag) expressed from *E.coli* cells. |
| 6 | Transcribed *rbcL* 5'UTR sequence from +1 to +54 with RB60 (N-term his-tag and fewer amino acids) expressed from *E.coli* cells. |

The autoradiogram (figure 3.8) illustrates that no band shift occurred in our control samples were RB60 is absent (1, 4). A band shift can be detected with the samples containing the shorter *rbcL* 5'UTR sequences (+1 to +54) containing isolated RB60 expressed from *E.coli* cells containing either old or new constructs (5,6). The samples with the complete *rbcL* 5'UTR bound with RB60 (2, 3) also created a band shift, indicating specificity for RB60 to these UTR sequences. These band shifts were of similar size indicating no major difference between the isolated RB60 proteins expressed with an N-terminal or C-terminal His-tag, or that specificity was altered due to differences in the length of the transcribed *rbcL* 5'UTR sequences.

3.5 UV cross-linking assay

The samples with transcribed *rbcL* 5'UTR and isolated RB60 were UV cross-linked and subsequently treated with endonuclease RNase A (2.4.4). The UTR sequences that covalently bind RB60 will be protected from degradation by the RB60 proteins, while unbound UTR sequences will be subject to degradation by the endonuclease.
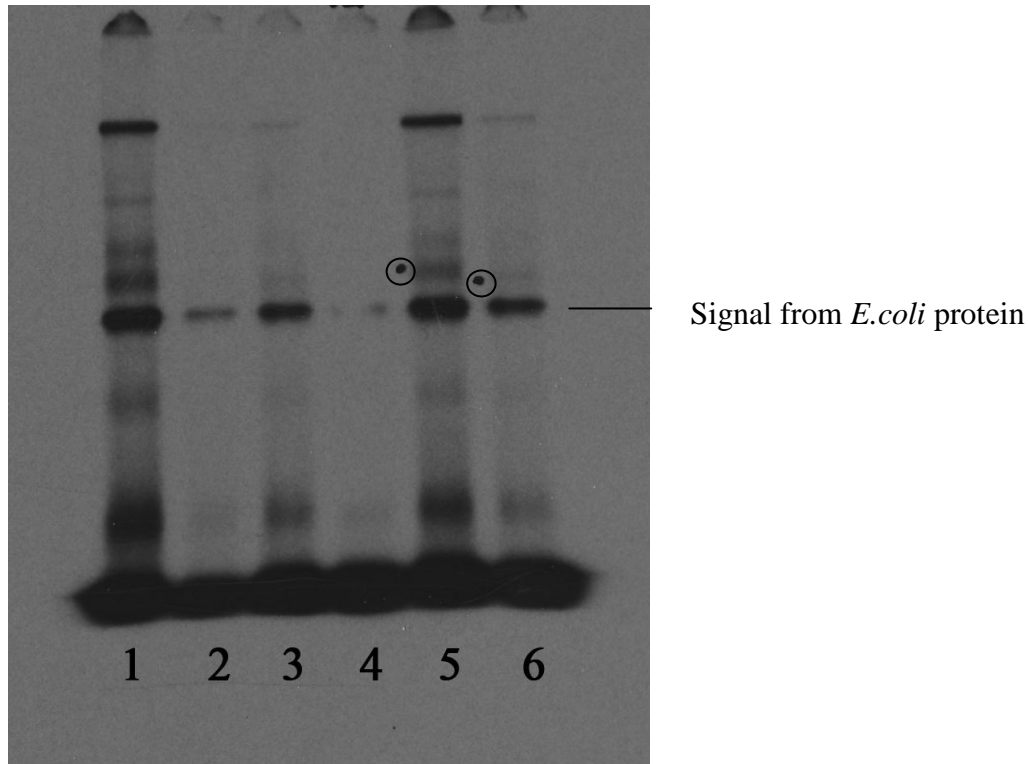
Figure 3-9: Autoradiogram of UV cross-linking of *rbcL* 5'UTR sequences with RB60. Isolation of RB60 proteins was done from *E.coli* cells containing old or new constructs. Circled areas indicate location of RB60 protein from SDS-PAGE gel. Signal from E.coli protein is highlighted.

The autoradiogram indicates that there is an RNA-binding protein of approx. 60kD that covalently binds to complete and short (+54) 5' UTR *rbcL* sequences. No difference is detected whether the RB60 proteins were isolated from cells containing old or new constructs.

A SDS-PAGE with only RB60 was performed to verify whether the proteins that crosslinked to the 5'UTR sequences were correct in size. Comparing the SDS-PAGE gel to the autoradiogram (figure 3-9), showed that the protein providing the signal from the UV cross-linked assay were of a smaller size than 60kD. The RB60 protein from the SDS-PAGE was situated slightly higher than anticipated (circled area).

The RB60 protein binds with a low affinity to the 5'UTR sequences. Another protein expressed in *E.coli* that binds to the 5'UTR transcripts with a much higher affinity than the isolated RB60. This high affinity binding of a different protein to 5'UTR sequences prevents any insight to whether RB60 binds to 5'UTR sequences due to the interference of the protein from *E.coli*.

51

Conclusion

Gel mobility shift assay and UV cross-linking experiments indicate that isolated recombinant RB60 proteins expressed in *E.coli* cells do not bind *in vitro* to 5'untranslated regions of *Chlamydomonas rbcL* mRNA. This was established through an SDS-PAGE experiment with RB60 proteins that noted that the binding site was different for this protein compared to the signals detected from the UV cross-linking experiment. The protein providing the signal in the UV cross-linking assay was of a different size than the 60 kD predicted. This protein is most likely expressed in *E.coli* cells and has a very high affinity for the *in vitro* transcribed *rbcL* 5'UTR sequences.

Due to the high concentration levels of isolated recombinant RB60 and the absent of binding capacity to the 5'UTR sequences *in vitro*, suggest that RB60 proteins do not bind to the 5'UTR of *Chlamydomonas rbcL* mRNA *in vitro*.

4   Discussion

The stability of *Chlamydomonas* chloroplast mRNA have been shown to be determined by secondary structures that functions as binding sites for trans-acting factors. Experimental studies have identified several RNA-binding proteins that play a key role in regulating RNA transcript stability through binding of 5'UTR sequences [75, 76].

The stability of *Chlamydomonas rbcL* mRNA has been shown to be mainly attributed to sequences at the 5´UTR [89]. Studies have shown that modifications in the nucleotide sequence of *rbcL* 5'UTR destabilize chimeric *rbcL*-GUS transcripts and reduce the accumulation of *rbcL* mRNA [103].

 A recent study detected a novel protein that binds to *Chlamydomonas* and *Arabidopsis* the 5'UTR of *rbcL* mRNA. MRL1 is a conserved nuclear-encoded protein that is required for the stabilization of *rbcL* mRNA in *Chlamydomonas* and *Arabidopsis* [95].

The aim of this project was to determine whether a 60 kD protein identified as containing RNA-binding capabilities can specifically bind *in vitro* to 5'UTR sequences of *Chlamydomonas rbcL* mRNA. A novel construct containing the protein of interest (RB60) was made containing an N-terminal histidine-tag for *immobilized metal ion affinity chromatography* (IMAC) isolation. Isolated proteins binding capacity to *rbcL* 5'UTR sequences of different lengths were tested through *in vitro* experiments (electrophoretic mobility shift assay and UV cross-linking).

4.1   Methodological considerations

The purification procedure of the RB60 protein through filtration was not efficient, leading to visible contamination in downstream experiments. In addition *E.coli* expresses a high affinity protein that binds to *in vitro* transcribed sequences of the *rbcL* 5'UTR, interestingly this bacterial protein is of similar size to our protein of interest (see section 3.5). *In vitro* experiments with RNA-binding proteins expressed in bacteria can be problematic, since bacteria codes for thousands of proteins. Inevitably a few might have specificity for RNA sequences of interest, as was the case in the UV cross-linking and electrophoretic mobility shift assay experiment (fig.3-8 and fig. 3-9).

An alternative purification procedure that could be of interest is the *TAG affinity purification (TAP) method.* This is a method that allows rapid purification of protein or protein complexes under native conditions, even when expressed at their natural level. This method involves a fusion of a TAP tag to the protein of interest, and subsequent introduction into host cell or organism. A key feature to this technique is that the TAP tag consists of two IgG binding domains of *Staphylococcus aureus* separated by a TEV protease cleavage site, which gives two specific affinity purification steps. Experimental studies have shown that the two affinity tag chosen for this method are highly efficient in their recovery of proteins present at low concentration, which is often the case inside cells [104, 105].

4.1.1 Electrophoretic mobility shift assay (EMSA)

EMSA is a rapid and sensitive method to detect protein-nucleic interactions. This technique has several advantages, among this is that it is simple to perform and is highly sensitive. This makes it possible to use small amounts of protein and nucleic acids concentrations. However, there are several limitations to consider when using this method and analyzing the results. A crucial aspect to consider is that rapid dissociation during electrophoresis can prevent the detection of complexes. In addition, many complexes are more stable in the gel than what is the case in their native condition [100].

4.1.2 UV cross-linking assay

UV cross-linking is a standard method used to detect RNA-binding proteins. The UV irradiation triggers the formation of covalent bonds between the RNA and proteins in the vicinity that interact with the sequence. Subsequent treatment with an RNase is to degrade sequences that have no protection that bound proteins will offer, producing short oligoribonucleotides. In general, UV cross-linking is relatively inefficient, in particular if the interaction of protein and RNA sequence is weak. This requires extensive UV- irradiation, the short wavelength UV light that is needed can be damaging to the protein [106].

4.1.3 **Alternative method**

An alternative method that can be used instead of *in vitro* experiments for identification of RNA-binding proteins might be to use *in vivo* methods. This will enable us to directly use *Chlamydomonas* cells and not fear contamination from bacterial expressed proteins that might bind to our RNA transcripts of interest.

A novel technique called *Photoactivatable-Ribonucleoside-Enhanced Cross-linking and Immunoprecipitation* (PAR-Clip) is a method that provides the option to combine UV cross-linking with immunoprecipitation of the investigated RNA-binding proteins and recover the cross-linked RNA. These RNA sequences can then be converted to a cDNA library for deep sequencing, thus providing information on a transcriptome-wide basis the binding sites for the RNA-binding proteins and their respective RNA recognition elements [107, 108].
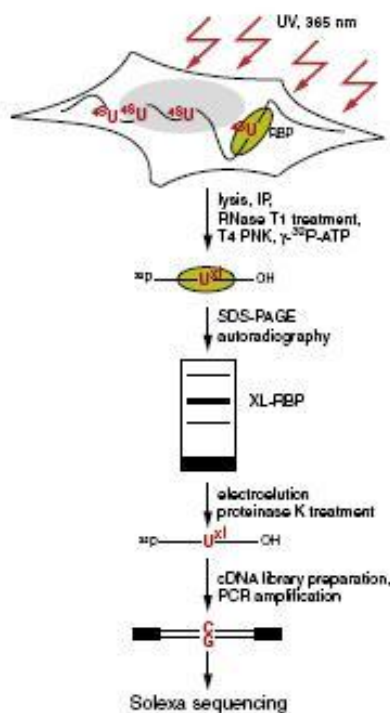

Figure 4-1: **Illustration of PAR-CLIP method.** (M. Haftner et al. 2010)

An important feature of this method is that it enables us to differentiates between crosslinked and non- crosslinked RNA sequences, the latter being more readily reverse transcribed [108].

Future perspective

Future studies of *Chlamydomonas rbcL* mRNA stability might elucidate other proteins that contribute to the increased half-lives of *Chlamydomonas* mRNA. Co-immunoprecipitation experiments with crosslinked RB38 which has been documented to bind 5'UTR of *rbcL* mRNA might identify additional factors that bind to 5'UTR cis-elements of *rbcL* mRNA.

In vivo experiments with *Chlamydomonas* cells might prove to be a more fruitful approach in identifying novel trans-acting factors that contribute to the increased half-lives of *Chlamydomonas* transcripts.

In addition there is an increasing interest in *Chlamydomonas* as a model organism for biohydrogen production as well as a system for vaccine production.

Rubisco research has in recent years focused on the rate-limiting step of CO_2 fixation of this enzyme. Strategies to improve crop yield potential and uptake of excess atmospheric CO_2 for agricultural processes and combating climate change, underlines the potential of *Rubisco* research to provide important answers through basic scientific research.

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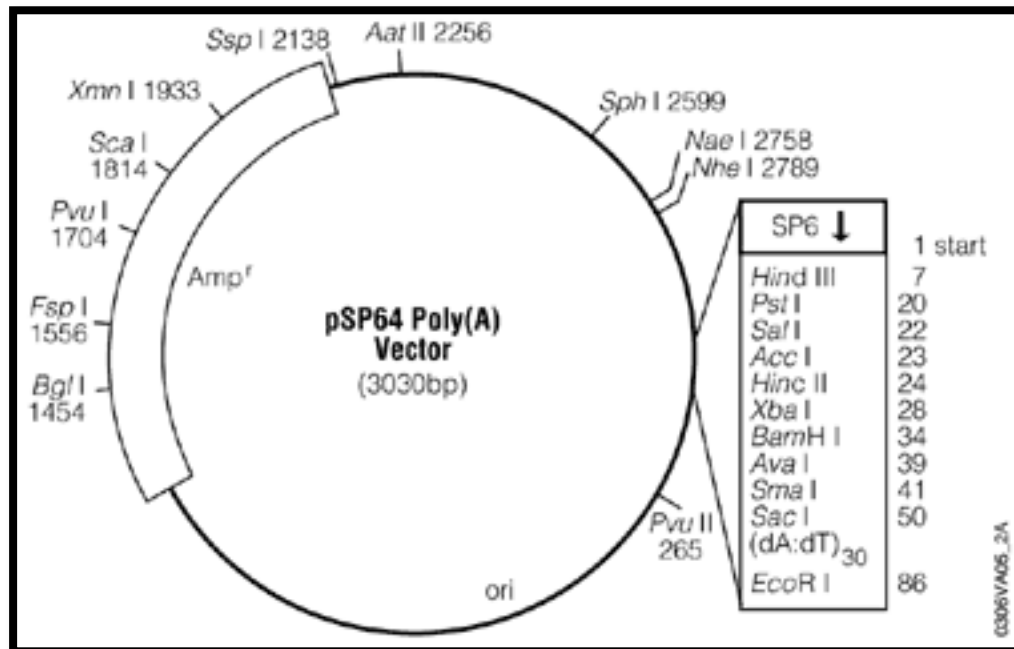
Appendices

Appendix I – Plasmid maps

pEcoli-Cterm 6xHN Linear Vector Information
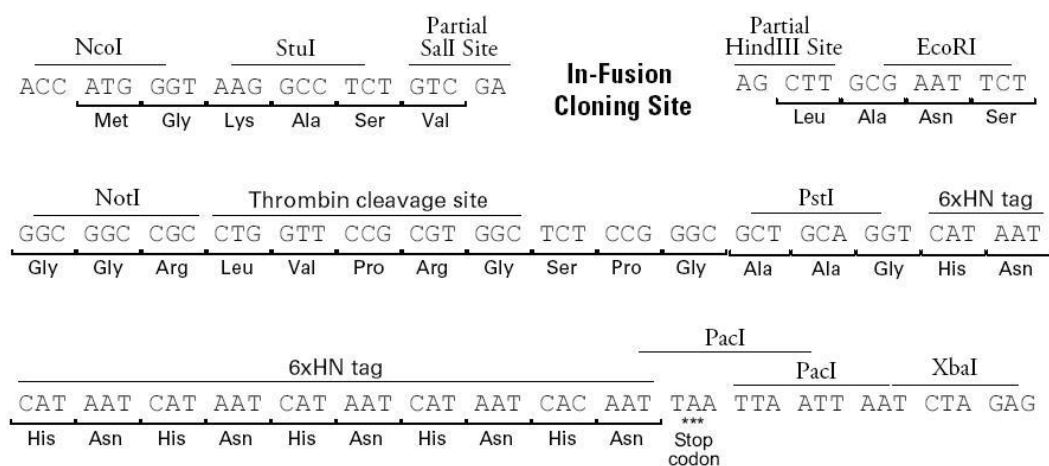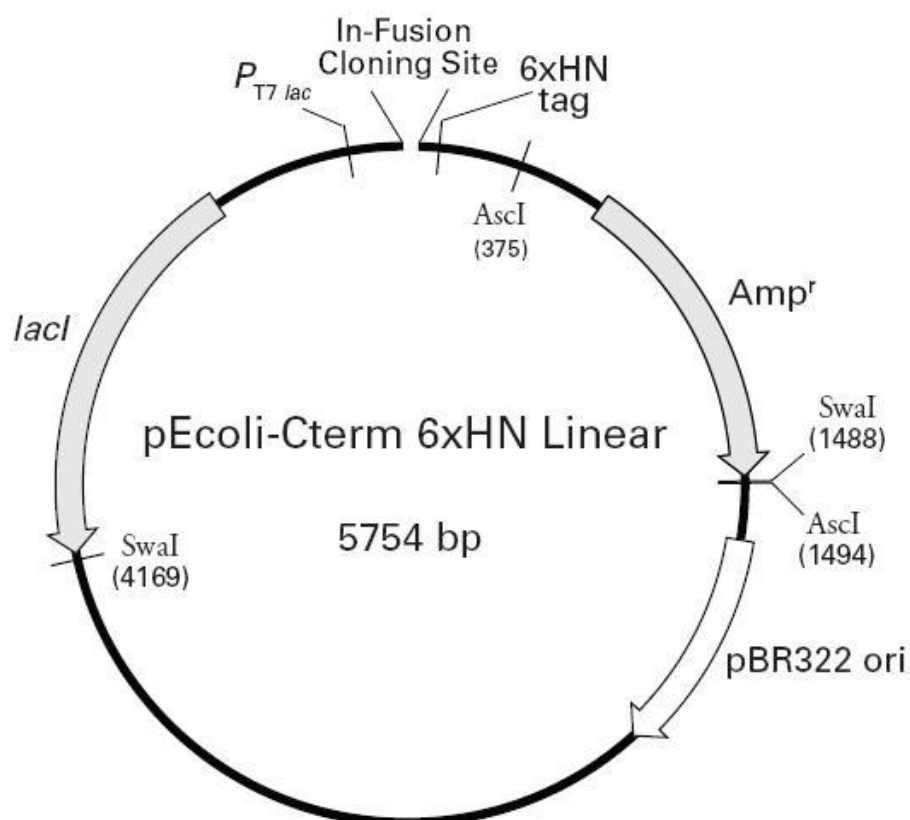
pEcoli-Cterm 6xHN Linear Vector Map and In-Fusion™ Cloning Site.

pSP64 Poly(A) plasmid vector

Appendix II – Composition of buffers

TAE buffer (50X)

Tris base (242g)

Cons. Acetic acid (57ml)

0.5M EDTA (100ml)

Adjust with dH_2O to a final volume of 1L

Store: room temperature

Electrophoresis buffer (10X)

Tris base 30g

Glycine 146g

Sodium dodecyl sulfate (SDS) 10g

Dissolve in 1L dH_2O

Dialysis buffer

0.1M Tris base (12.11g)

0.5M k-acetate (49.1g)

0.001M EDTA (0.37g)

Add 800ml dH_2O and adjust PH to 7.5

Add 200ml dH_2O to a final volume of 1L.

Buffer A (binding buffer)

20mM Sodium phosphate (1.78g)

1M Sodium chloride (29.22g)

20mM Imidazole (0.68g)

Adjust PH to 7.4 with HCl

TBS (10X)

Tris base 121,1g

NaCl 90g

Dissolve in 800ml dH_2O.

Adjust PH to 7.5 with HCl

Add remaining 200ml dH_2O to a final volume of 1L.

Western transfer buffer

Tris base 5.8g

Glycine 29.9g

Sodium dodecyl sulfate (SDS) 0.37g

200ml methanol

Solve in 800ml dH_2O to a total volume of 1L

Store: room temperature

Solution A (acrylamide 30%)

Acrylamide 29.2g

N',N-bis-methylene-acrylamide 0.8g

Dissolve with 100ml dH_2O

Store: 4°C in the dark

Buffer B (elution buffer)

20mM Sodium phosphate (1.78g)

1M Sodium chloride (29.22g)

0.5M Imidazole (17.2g)

Adjust PH to 7.4 with HCl

Western developing buffer

Tris base 6.05g

1M $MgCl_2$ 3ml

Dissolve in 1L dH_2O.

Western developing solution

9ml developing buffer

1ml NBT

100μl BCIP

NBT: 2mg in 2ml 96% EtOH

BCIP: 10mg in 2ml DMF (dimethylformamide)

Solution B (1.5M Tris-HCl, PH 8.8)

1.5M Tris (18.15g)

Adjust PH to 8.8 with HCl

Add 4ml SDS and 200μl TEMED

Dissolve in 100ml dH_2O

Store: room temperature

Solution B' (0.5M Tris-HCl, PH 6.8)

Tris 6.17g

Dissolve in 60ml dH_2O

Adjust PH to 6.8 with HCl

Add 4ml SDS and 800μl TEMED

Dissolve in 100ml dH_2O

Store: room temperature

Appendix III – Primers and antibodies

<u>**Oligonucleotides**</u>

SP64-1F	5'-AGGTGACACTATAGAATAAATGTATTTAAAATTTTTCAACAATTTTTAAATTATATTTCCGGACAGATTATGAGCT-3'
SP64-1R	5'-ATAATCTGTCCGGAAATATAATTTAAAAATTGTTGAAAAATTTTAAATACATTTATTCTATAGTGTCACCT-3'
SP64-2F	5'-CCGGACAGATTATTTTAGGATCGTCAAAAGAAGTTACATTTATTTATATAAGAGCT-3'
SP64-2R	5'-CTTATATAAATAAATGTAACTTCTTTTGACGATCCTAAAATAATCTGT-3'

<u>**Antibodies**</u>

Primary antibody	Peptide sequence	Dilutions	Secondary antibody	Dilutions
Anti-RB38	QRDSESGDERGGRGC	1:10000	Anti-rabbit IgG alkaline phosphatase	1:10000
Anti-RB60	CLPKKGSDGDEGTSD	1:10000		