Thesis for the Master's degree in Molecular Biosciences
Main field of study in Molecular Biology

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Destabilization of *Chlamydomonas* chloroplast *rbcL* transcripts after adding 19 nucleotides to their 5` untranslated region

60 study points

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Oslo, November 2007

Per-Bjarne Mikalsen

Abstract

The gene *rbcL* in the *Chlamydomonas* chloroplast codes for a mRNA of about 1.6 kb including a 5`UnTranslated Region (5`UTR) of 92 bp.The beginning of the 5`UTR has two stem-loops as part of its secondary structure. A 10-nucleotide element found to be crucial for transcript stability lies at the base in between these two stem-loops. Addition of nucleotides to the 5`terminus of *rbcL* transcripts has in some cases made the transcript unstable depending both on the number and type of nucleotides added. In this study a 19 nucleotide sequence was added to the 5`terminus of the *rbcL* 5`UTR in a chimeric reporter gene construct to study its effect on RNA stability.The 19 added nucleotides were not predicted to fold into any secondary structure or to influence folding of the original 5`terminus in any way. Due to the added nucleotides accumulation of transcripts was completely abolished suggesting that transcripts became susceptible to RNase attacks.

Contents

1 Introduction

1.1 Short on *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii is a eukaryotic, unicellular green alga, living in fresh water and soil. Its shape is usually oval or ellipsoid and the average diameter is approximately 10 µm. Figure 1.1.1 shows a schematic view of a *Chlamydomonas* cell. The algae move around by breast stroke motions from two 10-12 um long flagellas located in the anterior part of the cell. Inside a cell wall that encapsules the cell are the different compartments amongst them the nucleus with nucleolus, chloroplast, stigma, endoplasmatic reticulum and golgi bodies. A *Chlamydomonas* cell contains a single cup-shaped chloroplast which occupies around two thirds of the basal part of the cell. Fig 1.1.2 shows a model of the chloroplast. The stigma functions as an eyespot sensing light, using the flagellas and the eye spot the algae can orientate themselves relative to a light source. The eyespot of *C.reinhardtii* consists of two to four layers of granules and is 100-140 nm in diameter (Goodenough et al., 2007; Maul et al., 2002; Harris, 2001; Harris, 1989; Sager and Palade, 1957).

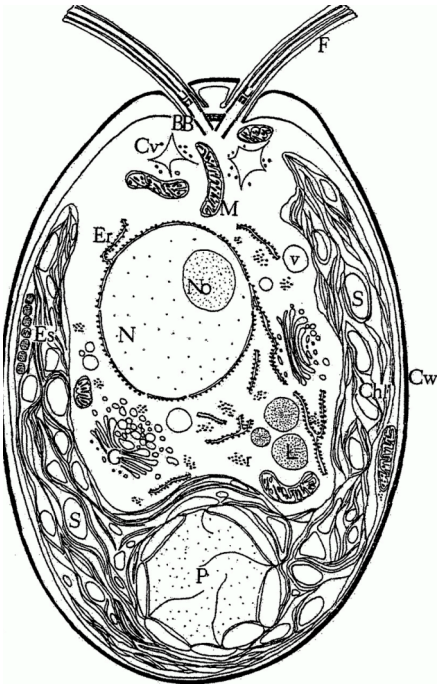

Figure 1.1.1 : A semidiagrammatic representation of an interphase *Chlamydomonas* cell. Cell length, 10 µm; BB, basal bodies; Chl, chloroplast; Cv, contractile vacuole; Cw, cell wall; Er, endoplasmic reticulum; Es, eyespot; F, flagella; G, Golgi apparatus; L, lipid body; Mi, mitochondria; N, nucleus; No, nucleolus; P, pyrenoid; r, ribosomes; S, starch grain; v, vacuole (Harris, 2001).

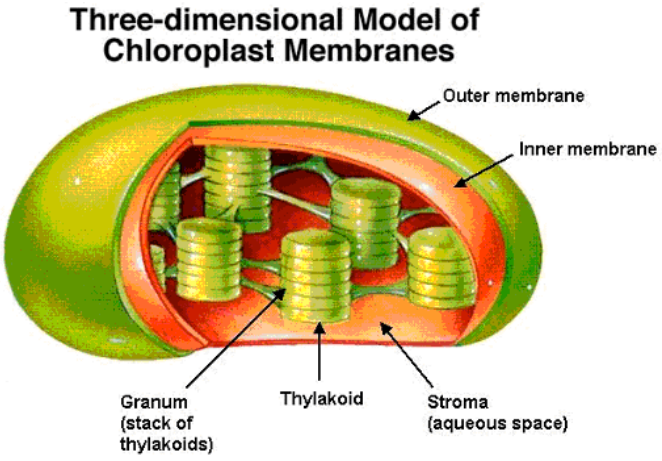

Fig 1.1.2 : Model of a chloroplast. Source : http://www.ualr.edu/botany/chloroplast.jpg

1.2 *Chlamydomonas* life cycle

A mature *C.reinhardtii* cell is haploid. When growth condtions are satisfactory *C.reinhardtii* grows vegetatively and divide asexually by mitosis. When such cells are grown in light-dark cycles the cells will divide during the dark phase. Two or three mitotic divisions then take place in rapid succession (Harris, 2001). Alternative alleles of a gene in the nuclear genome determine the mating type, either mt+ or mt-. During ordinary mitosis the nuclear genome is transmitted to the offspring in a classical Mendelian fashion. In contrast, during the sexual life cycle chloroplast and mitochondrial genes transmit uniparentally from the plus and the minus mating-types respectively (Harris, 1989; Proschold et al., 2005).
If a culture of *C.reinhardtii* is starved for nitrogen, gametogenesis may occur (Harris, 2001). In this situation gametes are released from the mother cell. Gametes of different mating types and from different cells will mate with the help of their flagellas. A gamete of mating type mt+ can mate with a gamete of mating type mt- thanks to adhering of their flagellas (Harris, 2001). Clumps of gametes are formed since one gamete can adhere to more than one cell of opposite mating type. In this clump two gametes fuse and a diploid zygote is formed which will swim away from the rest of the clump (Goodenough, 2007). Thirty minutes after zygospore formation all the mt- ChloroPlastDNA (cpDNA) may be destroyed (Kuroiwa et al., 1982; Nishimura et al., 1999). The mitochondrial genome is on the other hand inherited mostly from the mt- gamete (Goodenough. 2007). Methylation pattern on chloroplast genes are involved in its breakdown or survival (Harris, 1989). The zygote then becomes a zygospore with a heavy wall. Once nitrogen is introduced into the medium the zygospores can germinate with the occurence of meiosis. Four haploid meiotic products are then released. A mitotic division may occur after meiosis inside the zygospore. Then eight instead of four progeny cells are released. In the laboratory zygospores may germinate within a few days but in soil the zygospores can remain viable for several years (Harris, 2001).
Fig 1.2.1 shows the life cycle of *Chlamydomonas reinhardtii*.
Fig 1.2.2 shows three *Chlamydomonas* cells that are adhering to each other.

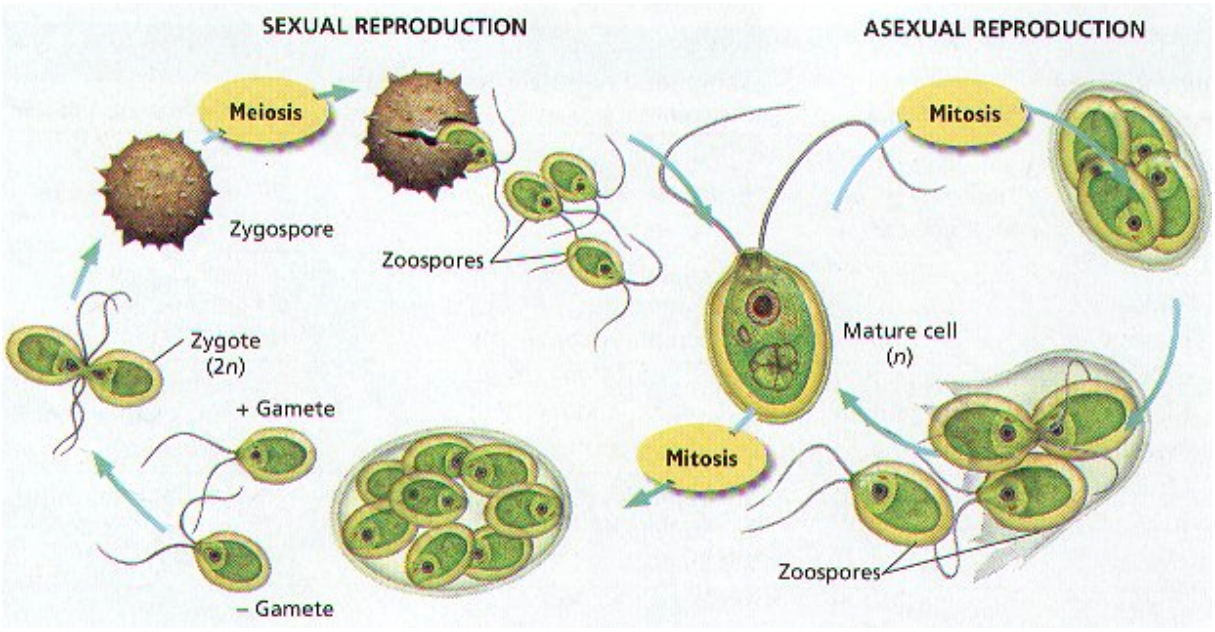

Fig 1.2.1. Life cycle of *Chlamydomonas reinhardtii*.
Source : http://sps.k12.ar.us/massengale/images/chlamydomonasrepro.jpg

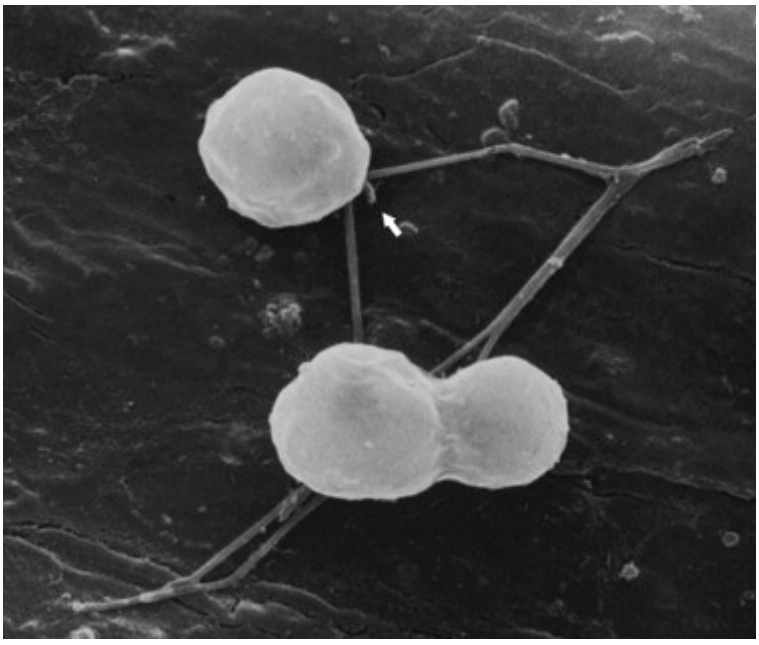

Fig 1.2.2 Shows the mating of three *Chlamydomonas* cells. Adhered by flagella, the lower two just fused to form a zygote, the upper displaying an activated mating structure (arrow) (Goodenough et al, 2007).

1.3 *Chlamydomonas reinhardtii* as a model organism

Chlamydomonas harbors three separate genetic systems, the nucleus, chloroplast and mitochondria. These systems do interact (Rochaix., 1995). All three genomes of *C.reinhardtii* are sequenced and transformation techniques are available for all three of them (Lefebvre and Silflow, 1999).

C.reinhardtii is facultative heterotroph. It can utilize organic carbon sources or light as energy source. There are defined three potential growth conditions for algae that can utilize organic carbon sources (Harris, 1989).

1.Photoautotrophic growth: light is only energy source.

2.Heterotrophic: Acetate is only energy source and culture is grown in the dark.

3.Photo-heterotrophic growth: algae are grown in the light in the presence of acetate.

Thanks to the different growth conditions possible in *C.reinhardtii* several photosynthetis- deficient mutants have been isolated and maintained (Davies and Grossman, 1998). By subjecting *C.reinhardtii* to alternate light and dark cycles cell division can be synchronized (Rochaix, 2001). Generation time is about 5 hours. The chloroplast genome of *Chlamydomonas* can best be transformed by the use of microprojectile particle bombardment (Boynton et al., 1988). In addition to yeast, *Chlamydomonas* gives additional insight into eukaryotic cell (Rochaix, 1995) and *Chlamydomonas* is also used in research into other areas such as: phototaxis, cell wall synthesis, mating reactions, gametogenesis and the study of metabolism of carbon, nitrogen and sulphur (Harris, 2001).

1.4 Some methods used for identifying nuclear genes that function in plastid gene expression

Nuclear gene products have been implicated in chloroplast gene expression through genetic studies and biochemical assays. Chloroplast proteins with relevant activities like nucleic acid binding activity have been detected by the use of these methods. With the recent increase however of genome sequence data, it is now more common that a role in chloroplast gene expression is inferred solely from a protein's amino acid sequence. Many proteins that are predicted to have chloroplast targeting sequences have nucleic acid binding motifs or resemble bacterial proteins with well-defined roles in gene expression (Barkan, 2000).

1. Biochemical approaches

In the chloroplast there are very few open reading frames (OFR`s) whom function is unknown. Therefore new chloroplast proteins that are identified biochemically are likely nucleus-encoded. Proteins purified from chloroplasts have been found to function in a variety of activities, including nucleic acid binding, RNA maturation, transcription, and polyadenylation. Antibodies have been raised to immunoprecipitate some of these regulatory proteins (Barkan, 2000). Proteins belonging to the plastid translation machinery have been found by the use of biochemical methods. These include nucleus-encoded plastid ribosomal proteins, initiation factors and elongation factors (Harris et al., 1994).

2. Genetic approaches

Nuclear mutations can be done in genetic studies to see if they disrupt chloroplast gene expression. In this way, genes can be defined that participate in chloroplast gene expression. This type of studies has been done with *Zea mays* (maize), *Arabidopsis thaliana* (*Arabidopsis*), and *C. reinhardtii* (Barkan, 2000).

3. Genetic screens

An example of a screening methodology is lack of growth of a mutant non-photosynthetic cell on minimum medium but growth if the medium also contains acetate. For example, genetic screens have revealed a nuclear mutant, 76-5EN, in *C. reinhardtii* that has a defect in plastid transcription (Hong and Spreitzer, 1998). This mutant does not grow photoautotrophically because it fails to accumulate *rbcL* mRNA (Hong and Spreitzer, 1994).

1.5 The *Chlamydomonas* chloroplast genome

The *Chlamydomonas* chloroplast has about 80 copies of its genome (Chiang and Sueoka, 1967; Gilham, 1978) in the form of double stranded circular molecules. A molecule may also exist in a linear form or in the form of dimmers. The genome holds around one hundred genes (Maul et al., 2002). The informational content of DNA is relatively small compared to the DNA content of the nucleus which is about 120 Mb contained on 17 chromosomes (Jain et al., 2007). The mitochondrial genome is a linear 15.8 kb molecule (Harris, 2001). The sequence of the *Chlamydomonas* chloroplast genome is available, and the circular map of the *C.reinhardtii* genome is shown in figure 1.5.1 (Maul et al., 2002).

1.6 Endosymbiosis

Its postulated that plastids (e.g., chloroplasts) are the results of endosymbiosis between a eukaryotic host and cyanobacterium (Mereschkowsky, 1905). Mereschkowskys hypothesis has since been supported from electron-microscopical and biochemical studies (Margulis, 1981). Chloroplast structure and genome analysis support a hypothesis for a single primary endosymbiosis giving three groups of organisms: green plants (green algae + land plants), red algae and glaucophytes (Moreira, 2000). By engulfing a photosynthetic cyanobacterium the non-photosynthetic organism gains photosynthetic ability. There are genetic similarities between chloroplasts and cyanobacteria, however the chloroplast genome is substantially smaller than the cyanobacteriaie which may be accounted for by gene loss from the chloroplast to other organelles (e.g: the nucleus) throughout evolution (Maul et al., 2002).

The endosymbiosis theory is also supported by looking at structures on plastid RNA polymerases, plastid gene promoters, prokaryotic ribosome-binding sites and structures on other elements like the stem-loop at the 3` ends of plastid transcripts. This kinds of studies reflects plastids bacterial, hence its prokaryotic ancestry (Salvador et al., 2003; Hirose et al., 1998; Maliga 1998; Iglo and Kössel 1992).

Furthermore, in contrary to eukaryotic mRNA the chloroplast mRNA is not capped at its 5` end nor is it polyadenylated with a poly(a) tail at its 3` end (Hale et al., 2005).

Symbiosis have in general been important in evolution by giving new organisms, species but also organs and tissues (Witzany, 2005).

Primary endosymbiosis together with a secondary endocytobiosis where a heterotrophic host cell takes in a eukaryotic alga several new organisms have evolved (Kroth, 2002).

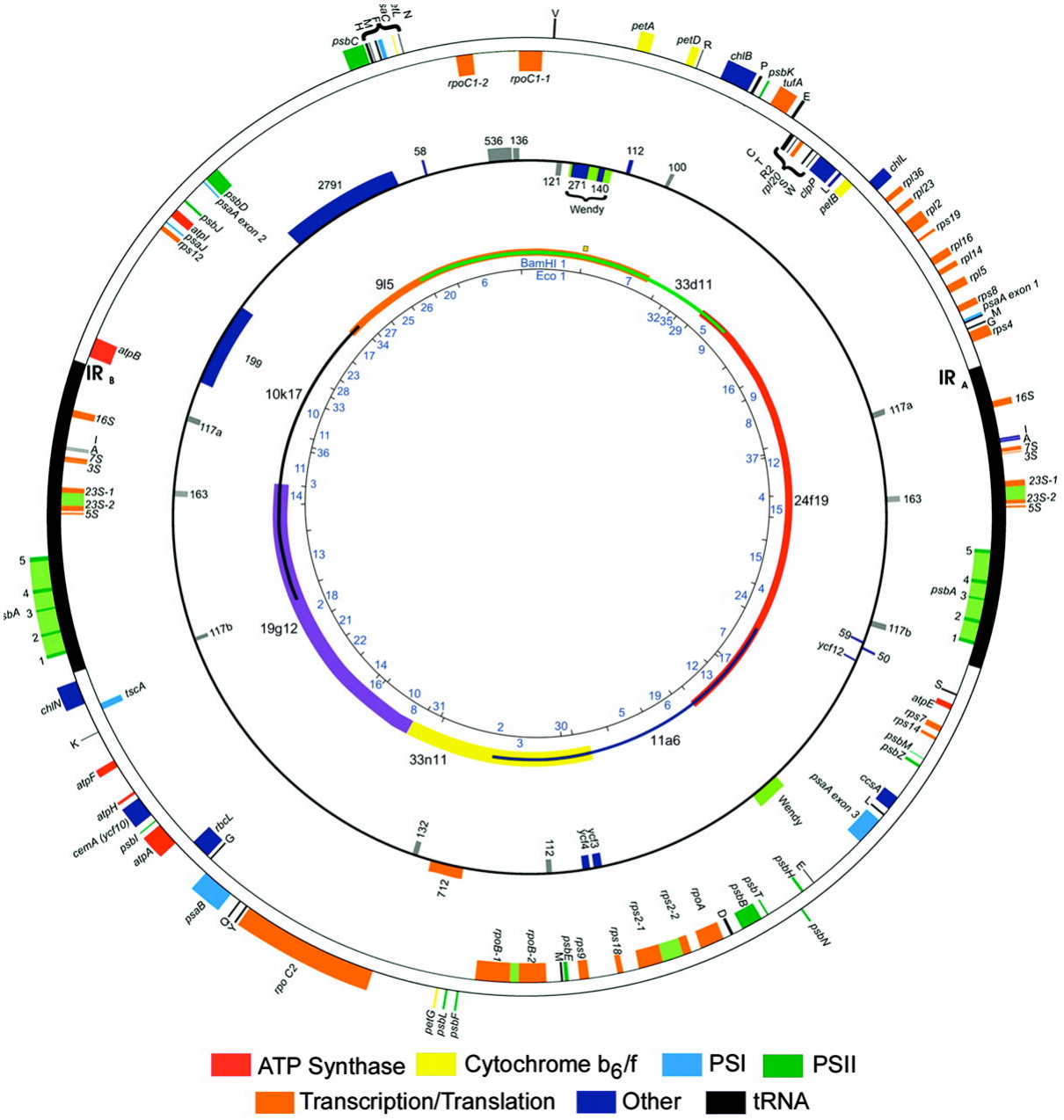

Figure 1.5.1 "The Plastid Chromosome of *C. reinhardtii*."

The inner circle shows BamHI and EcoRI restriction fragments mapped according to Rochaix (1980)✦ and numbered according to Grant et al. (1980)✦. Position 0 is shown by an orange square near the 12 o'clock position. The second concentric circle indicates seven overlapping BAC clones that span the genome. The third circle shows genes and ORFs of unknown function, including those for which disruption experiments were unsuccessful. Gray boxes represent newly identified ORFs. The outer circle shows genes of known or presumed function, with sequenced or hypothesized introns shown in olive green. Genes are color coded by function, as shown at bottom (Maul et al., 2002).

1.7 Synthesis of the photosynthetic apparatus

The biosynthesis of the photosynthetic apparatus depends on the concerted action of the nuclear and chloroplast genetic systems (Rochaix JD, 1996).

During photosynthesis CO2 fixation is carried out by an enzyme called Rubisco. In green algae rubisco is composed of eight large (55 kD) and eight small subunits (15-18 kD) coded by the chloroplast gene *rbcL* and the nuclear gene *rbcS* respectively (Spreitzer, 1993). Combined the subunits make up a 600 kD holoenzyme (Cohen et al., 2006).

Coordination of expression of these subunits happens in a process called: Controlled by Epistasy of Synthesis (CES), meaning that if one subunit in the complex is absent, synthesis of another subunit in the complex is decreased (Cohen et al., 2006).

1.8 Gene expression and its regulation

1.8.1 Overview of regulatory factors

In *Chlamydomonas reinhardtii* chloroplast gene expression is regulated at transcription and several post-transcriptional steps, like RNA stabilization, RNA processing, RNA splicing and translation. These processes are controlled by factors encoded in the nucleus. After being synthesized in the cytosol the factors are imported into the chloroplast and act on target sites here (Rochaix, 1996; Sugita and Sugiura, 1996; Goldschmidt-Clermont, 1998).

In *C.reinhardtii* these factors interact with cis elements on both the 5` and the 3` ends of mRNAs (Herrin and Nickelson, 2004). One factor has been found to act on a cis element in the coding region (Drapier et al., 2002). The interaction between factor and mRNA are in general very specific (Jenkins et al., 1997, Barkan et al., 1994).

In a cell the amount of RNA depends on relative rates of transcription and degradation. In chloroplasts the half life of an RNA may be of several hours and be influenced by factors like developmental stage, circardian rhythms and dark/light and environmental conditions (Monde at al., 2000: Salvador et al., 1993; Lilly et al., 2002).

There are two chloroplast RNA degradation pathways in *C.reinhardtii* of which one is light-dependent. The *rbcL* transcript is protected from degradation in the light dependent pathway with the help of element +329 to +334 (Singh et al., 2001). *rbcL* transcripts are protected from degradation by the light-independent pathway with help of element +38 to +47 (Salvador et al., 1993).

In *C.reinhardtii*, most chloroplast genes appear to possess their own promoters, rather than being transcribed as part of multicistronic operons (Sakamoto et al., 1994).

1.8.2 Promoters and regulation of transcription

Plastid genes in higher plants can be transcribed either by polymerases that are encoded in the plastid or by polymerases that are encoded in the nucleus. Nucleus encoded polymerase and plastid encoded polymerase are abbreviated NEP and PEP respectively. The products of transcription by both types of polymerases are typically polycistronic (Barkan, 2000).
The NEP are phage-like polymerases. Moreover, sigma-like factors coded in the nucleus give specificity to the PEP (Maul et al., 2002).

In *Chlamydomonas reinhardtii* no polymerases that transcribe chloroplast genes are encoded in the nucleus (Lilly et al., 2002). The genes coding for PEP are *rpoA*, *rpoB*, *rpoC1* and *rpoC2* (Troxler, 1994). The genes transcribed by PEP have promoters that are bacterial E.coli sigma70-like, with promoter elements lying upstream of the transcription start site at -35 and -10. The consensus sequences of the promoter elements are TTGACA and TATAAT respectively (Salvador et al., 2004).

The fact that *C.reinhardtii* lacks NEP suggests that promoters in *C.reinhardtii* chloroplast would be of bacterial sigma70 type with -35 and -10 elements. Such promoters has been described, e.g. for the *16S rRNA* gene. However the promoter for the gene *atpB* lack a functional -35 element, while having an extended -10 sequence (TATAATAT). The latter type of promoter is the most common one for chloroplast protein genes in *C.reinhardtii*. Promoters with -35 element are found in front of the ribosomal RNA genes and are more active (Salvador et al.,2004). It may be added that in constructs were the distance between promoters are changed the promoter strengths is weakened (Stefano and Gralla 1982).

The promoter for *rbcL* has an extended -10 sequence (TATAATAT) but lacks the -35 sequence. Alone, this promoter element can only drive a basal level of transcription but together with a cis-acting enhancer-like sequence located in the 5`transcribed region between position +108 and +143 full rate of transcription is accomplished (Anthonisen et al., 2002).

The gene transcription of chloroplast in *C.reinhardtii* cells is seen to change as a result of changes in the supercoiling of the chloroplast DNA. This change in DNA supercoiling results in cells grown in diurnal rhythm 12-h dark-12-h light periods (Salvador et al., 1998).

At least one nuclear factor are seen to affect transcription in the *C.reinhardtii* chloroplast as shown with the chloroplast *rbcL* gene which is blocked in a *C.reinhardtii* nuclear mutant termed 76-5EN (Hong and Spreitzer, 1994).

1.8.3 RNA editing

RNA editing were single nucleotides in the transcript are changed does not take place in algae. These processes do take place in land plants though (Bock, 2000).

1.8.4 Intron splicing

Only three chloroplast genes in *C.reinhardtii* is known to have introns. These genes are *psbA*, *psaA* and *23S rRNA* genes (Maul et al., 2002). The *C.reinhardtii* chloroplast *psaA* mRNA is coded by three separate loci giving three separate transcripts which assembles with the help of trans-splicing. Fourteen nuclear loci or more together with one chloroplast gene, *tscA*, are needed in this trans-splicing process (Rivier et al., 2001).
In *C.reinhardtii* only some few nuclear genes are transcribed as part of polycistronic primary transcripts (Rochaix., 1996).

1.8.5 5`end and 3` end maturation

The *petD* chloroplast gene in *Chlamydomonas reinhardtii* is an example of the need for 5`end maturation to take place for the transcript to be translated. This maturation is thought to be accomplished by endonucleolytic processing (Sakamoto et al., 1994). Inverted repeat (IR) sequences are found at the 3`ends of most chloroplast protein coding regions in *C.reinhardtii* and exemplified by atpB mRNA these 3`ends are suggested to be processed in vivo. The atpB transcript are cleaved at a position 10 nucleotides downstream from the mature 3` terminus (Stern and Kindle, 1993). *In C.reinhardtii*, *atpB* transcripts that lack the 3`Inverted Repeats (IR) will be degraded by 3` → 5` exoribonuclease activity (Drager et al., 1996) and accumulation of *atpB* and *psaB* mRNAs are markedly reduced if a cis-acting sequence in a secondary structure at the 3`UTR is deleted (Stern et al., 1991; Lee et at., 1996).

1.8.6 RNA stability and regulation of translation

RNA levels are regulation points of gene expression in many organisms. The determinants for longevity of mRNAs are mostly located in the 3`UTR of mRNAs in eukaryotes and in the 5`UTR of bacteria and organelle mRNAs. The 5`untranslated regions (UTRs) of chloroplast transcripts include sequences that are critical for RNA stability (Salvador et al., 1993b; Ross, 1996; Gutierrez et al., 1999; Mitchell and Tollervey 2001).

Exemplified by the chloroplast *psbA* gene in *Chlamydomonas reinhardtii* some regulation regarding the 5`UTR and other sequences is illuminated. The *psbA* chloroplast gene codes for the D1 protein which is a core component of photosystem II (Fromm et al., 1985; Klein et al., 1988; Malno et al., 1988; Krupinska and Apel, 1989).

Processing of the *psbA* 5`UTR is correlated with association of the RNA with ribosomes (Bruick et al., 1998). A set of nuclear-encoded RNA binding proteins binds to the 5`UTR of the *psbA* transcripts in *Chlamydomonas reinhardtii* (Danon et al, 1991, Danon et al., 1994; Yohn et al., 1996). Four proteins with sizes of 38, 47, 55 and 60 kDa have been found to bind with high affinity to *psbA* transcripts (Barnes et al., 2004) in a light regulated fashion (Danon et al., 1991).

The *psbA* 5`UTR have several regulatory elements that are important for *psbA* translation. Amongst these are: An AU-box, a Shine-Dalgarno sequence (SD) which function as a ribosome binding site, a stem-loop structure immediately upstream of the SD sequence, a U-rich region which is immediately upstream of the stem-loop and a U-rich region which is positioned between the SD sequence and the initiation codon (Mayfield et al.,1994; Hirose et al., 1996; Barnes et al., 2004). For the *psbA* gene to be translated the stem-loop must be removed during mRNA maturation (Bruick et al., 1998).

In vivo most of the *psbA* mRNA do lack sequences upstream of the SD sequence, including any stem-loop (Erickson et al., 1984; Nickelsen et al., 1994; Shapira et al., 1997). This removal of the stem-loop is facilitated by binding of the RB38 protein to the U-rich region. This happens in conjunction with the early stages of ribosome assembly on the mRNA. It is also possible for the RB38 to bind a U-rich region between the Ribosome binding site and the initiation codon. This can help the ribosome to localize itself to the initiation codon of the mRNA (Barnes et al., 2004).

The importance of the SD sequence is shown by the fact that if the SD sequence is removed the *psbA* 5`UTR is not processed and the *psbA* mRNA can no longer associate with ribosomes for protein D1 translation (Mayfield et al., 1994), i.e. binding of the mRNA to the ribosome seems to be needed for mRNA processing (Bruick et al., 1998).

Processing of the *psbA* 5`UTR by a nuclease involves a shortening of the RNA from a 90 nucleotide sequence to a 36 nucleotide sequence upstream of the initiation codon (Bruick et al., 1998).

In vitro, removal of the stem-loop does not prevent nuclear-encoded proteins to bind to the mRNA nor does it prevent translational regulation mediated by the *psbA* Ribosome binding (RB) complex Thus, ribosome association with parts of the regulatory protein complex coded in the nucleus are together with processing of the 5`UTR acting to stabilize the message (Bruick et al., 1998).

In all the models made to explain the translational regulation of the *psbA* chloroplast gene some features are all the same: After changes in the photosynthetic activity the binding activity of binding proteins is modulated via redox events leading to reduction of RB60 protein. This in turn breaks disulfide bonds in the RB47 thereby reducing the RB47 protein (S-S to S-H H-S). RB47 can then recognize the A-rich 5`UTR of *psbA*, allowing *psbA* translation to occur (Fong et al., 2000).

A nuclease should be responsible for the processing of the *psbA* 5`UTR by cutting off nucleotides from the 5`UTR end. Such a nuclease has not been identified but seems to be closely associated with one of the ribosome subunits or with a protein complex that is required for mRNA association with the ribosome (Bruick et al., 1998).

In Escherichia coli ribonuclease R plays an important role in degradation and/or processing of RNA (Liou et al., 2000) and in cyanobacteria, dark-induced mRNA instability involves endoribonuclease cleavage at an AU-box and SD sequences by RNases of the E and G-type (Horie et al., 2007).

Another indicator for the existence of 5`-to-3` exoriboinucleases in *C.reinhardtii* is shown by the breakdown of mRNAs in chloroplast after insertion of a poly (G) cassette into the 5`UTR of *petD* and *psbB* genes. This cassette will hinder exoribonuclease movement along the mRNAs from these genes thereby protecting such mRNA from rapid degradation (Vaistj et al., 2000; Drager et al., 1999, 1998).

1.9 The *rbcL* transcripts

1.9.1 The *rbcL* 5`UTR and transcript stability

The *rbcL* and *atpB* chloroplast genes code for the large subunit of ribulosebisphosphate carboxylase and the beta-subunits of the ATP synthase complex, respectively.These are examples of transcripts that exist in high abundance in *C.reinhardtii* (Salvador et al., 1993).

At the beginning of the 5`UTR of mRNA from the *Chlamydomonas* chloroplast *rbcL* gene lies an essential RNA-stabilizing stem-loop.This stem-loop is very important for the mRNA stability and longevity (Suay et al., 2005). In many cases a reduction in mRNA accumulation from this gene has been shown to be the result of reduced RNA stability (Salvador et al., 2004).

To investigate the RNA-stabilizing function of the 5`-terminal stem-loop structure several different variants of the *rbcL* 5`UTR have been constructed. In these experiments the stem-loop has been both destroyed, retained or restored without changing the total number of nucleotides in the 5`UTR. Transcripts were only found in the chloroplast when the terminal stem-loop was formed. Transcripts also accumulated in cases were the stem-loop was either present but in an altered form or replaced by a perfect hairpin (Suay et al., 2005) that has been found to stabilize transcripts in *E.coli* also (Bouvet and Belasco, 1992).

This work was conducted by making chimeric *rbcL*:beta-glucuronidase (GUS) constructs (Salvador et al.,2004) that are inserted in front of the *rbcL* 5`UTR. This construct also contains some of the nucleotides in the beginning of the *rbcL* gene (Salvador et al., 2004).

Figure 1.9.1 shows mRNA from *rbcL*:GUS constructs where nucleotides from positions +5 to +37 have been altered. Construct (p6-36) has a different sequence here compared to wild-type while (p5-37) has the wild-type sequence in inverted form. In both cases stem-loop structures are assumed to form as shown in the figure. Northern blots show accumulation of transcripts from both constructs. Since the constructs have different sequences the accumulation of transcripts should not be due to increased number of hydrogen bonds stabilizing them. The first five nucleotides were not changed as they are believed to be important in base-pairing with the RNA-stabilizing sequence element at positions +38 to +47. This RNA element are boxed in figure 1.8.1 and is shown to function as stabilizing element by binding to trans-factors. Changes in the RNA conformation around the base of the stem-loop hinders binding to trans-factors followed by mRNA degredation by an assumed nuclease despite the presence of a secondary structure. The real structure protecting the mRNA from degradation is thus the folding of a sequence element around its base into a specific RNA conformation and not the base-loop structure itself. Changes in the first four basepair at the bottom of the stem will make the transcript unstable presumably due to disruption of the base in between the stem-loops. All in all, sequences between positions +1 and +36 of the 5`UTR of the *rbcL* gene in *C.reinhardtii* chloroplast are needed for transcripts to be stable and furthermore no promoter lays here (Suay et al., 2005; Salvador et al., 2004; Anthonisen et al., 2002; Anthonisen et al., 2001).

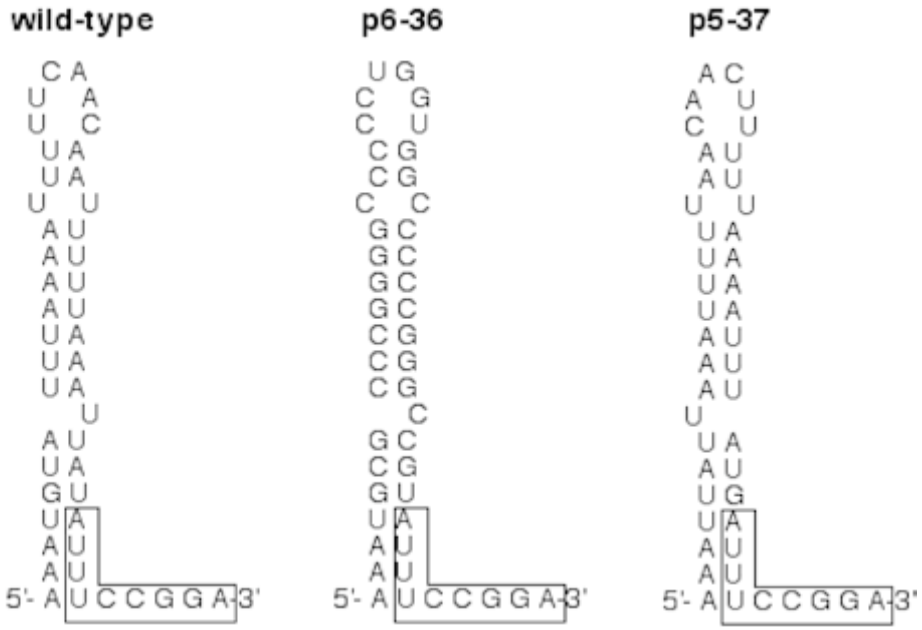

Fig 1.9.1: "Predicted RNA conformations at the 5` ends of transcripts from wild-type and mutated *rbcL*:GUS genes." "Secondary structures at the 5` ends of wild-type and mutated 5`UTRs, as predicted by the mfold program" (Zuker et al. 1999). "The sequence previously shown to function as an RNA stabilizing element is boxed" (Anthonisen et al. 2001).

As exemplified earlier with the *psaA* transcript a, a similar relationship between translation and transcript stability may not exist for the *rbcL* transcript.

1.9.2 The chimeric *rbcL*:beta-glucuronidase (GUS) construct

The rate of transcription of the chimeric GUS gene has been measured by determining the rate of incorporation of 32P-labeled inorganic phosphate into GUS transcripts in a 10 min interval. Correction for length of transcripts was done and signal intensities of transcripts were compared. Results from this work show that loss of accumulation of transcripts as described in 1.9.1 is due to reduced RNA stability and not to reduced transcription (Salvador et al., 2004).

1.10 My thesis

1.10.1 Earlier work

Earlier work has shown that transcripts from the *C.reinhardtii* chloroplast *rbcL* gene are destabilized by point mutations in the 5`UTR.

Other work from master students has shown that addition of nucleotides to the 5`UTR of the *rbcL* gene may render the transcripts unstable depending both on the length of the extra end and the actual nucleotides added. Fig 1.10.1 shows the predicted secondary structure in the beginning of the 5`UTR of the *rbcL* chloroplast transcript in *C.reinhardtii* (Suay et al., 2005). The added bases are predicted not to change the wild-type secondary structures.

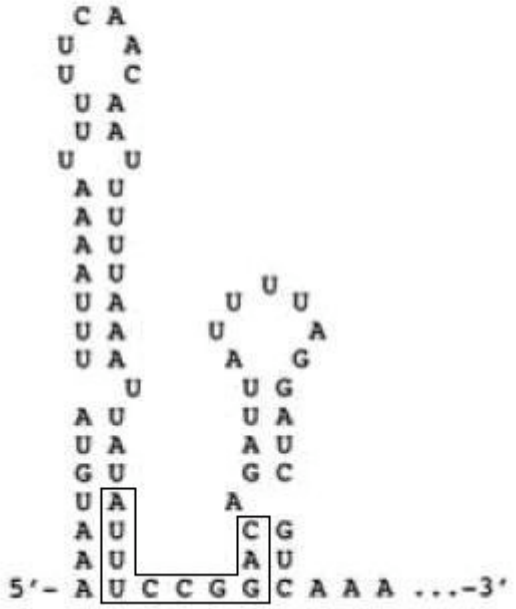

Fig 1.10.1 : Predicted secondary structure in the beginning of the 5`UTR of the rbcL chloroplast gene in *C.reinhardtii* (Suay et al., 2005). The boxed RNA element has previously been shown to function as stabilizing element. These are supposed to be the sequence to which a putative transacting factor binds (Anthonisen et al., 2002).

Adding more and more nucleotides to the 5`UTR less accumulation of transcripts occurs. The hypothesis is that an RNase may bind to the extra end created at the 5` end of mRNA and start to degrade nucleotides away from it. It is assumed that when the RNase later on reaches the trans-factor protein(s) that are bound to the stem-loop the RNase may push away the factor(s) and then continue to degrade the rest of the mRNA.

On the addition of 6 extra nucleotides to the 5`UTR, transcripts are stable (Suay et al., 2005). It is hypothesised that the extra bases at the 5`end of the mRNA do not give enough room for the RNase to bind, hence no degradation of mRNA is taking place. Also, with the addition of the two bases (GC) the transcript are stable (Personal communication with professor Uwe Klein).

The addition of 10 nucleotides to the 5`UTR renders the transcripts unstable. It is assumed that an RNase in this case binds to the transcript and starts degrading it.

The addition of 20 nucleotides to the 5`UTR seemed to destabilize the transcripts less than a 10 nucleotide extension (Anne Witsø, 2006). However, these 20 extra bases added seemed to produce extra secondary structures which are assumed to protect the transcript from beeing degraded by an RNase.

The addition of 2 or 5 extra nucleotides with bases (GG) and (GCGCG) respectively renders the transcripts unstable (Salvador et al., 2004). It is likely that these sequences pair with the sequences at the base in between the stem-loops thereby hindering factors from binding to the mRNA. RNase may then be able to bind to the mRNA instead and start degradation (Suay et al., 2005).

1.10.2 Goal of this work

Adding 20 and 10 bases to the 5` end makes the transcrips unstable while with 6 extra nucleotides the transcripts are stable.

In this work 19 extra nucleotides were added to the 5`terminus of the *rbcL* gene making transcripts with 19 extra nucleotides in the 5`UTR.

These 19 extra bases are not predicted to fold into secondary structures as was the case with the 20 extra nucleotides added in a previous master project (Anne Witsø, 2006). The goal of this work has been to determine whether the secondary structures in the 5` extensions of the +20 constructs are important for partial stability of the transcripts.

1.10.3 Hypothesis

We hypothesize that the 19 extra nucleotides will render the GUS transcripts highly unstable.

2 Materials and methods

2.1 Strains and media

2.1.1 *Escherichia coli*

E.coli bacteria strain TB1 was used for cloning.Bacteria were grown at 37°C on LB medium with 60µg/ml ampicillin.

2.1.2 *Chlamydomonas reinhardtii*

Chlamydomonas reinhardti strain CC-373 (ac-u-c-2-21) from the "Chlamydomonas Genetics Center at Duke University, North Carolina,U.S.A" was used for transformation of the *Chlamydomonas* chloroplast. This is a non-photosynthetic strain having a 2.5kb deletion in the chloroplast genome that comprises parts of the inverted repeats (IR) and parts of the *atpB* gene (Blowers et al., 1989). See figure 2.1.2 (Kindle et al., 1991). The *atpB* gene codes for the beta-subunit of the chloroplast ATP synthethase. Since part of the *atpB* gene is missing in the CC-373 strain photosynthesis is inhibited. This strain is heterotrophic and was grown in high salt (HS) medium (Sueoka, 1960) in the dark using acetate as the sole carbon source. If the deleted part of the *atpB* gene is restored due to a transformation, then the cabability of photosynthesis is also restored. We used this as a means of selection of transformed cells.

A strain termed MU7 has an unmodified version of the *rbcL* 5`UTR-GUS gene (Salvador et al., 1993b).This strain was used to compare the RNA abundance with my modified version of the construct. This strain was grown on high salt (HS) medium (Sueoka, 1960) in water bath at about 32°C and mixed by 2% CO2, enriched air.

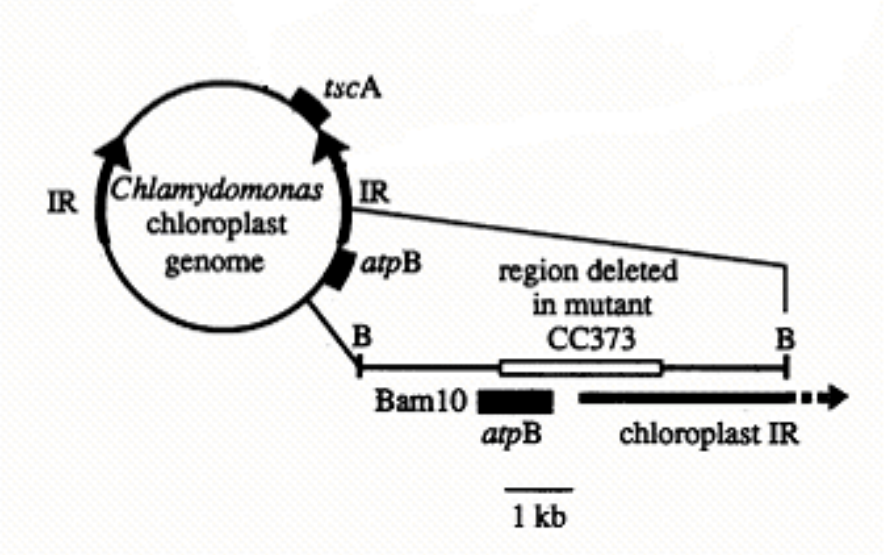

Figure 2.1.2 "The 2.5 kb deletion in the chloroplast genome of the mutant strain used as receiver for transformation. The inverted repeats are marked by arrows (Kindle et al., 1991)"(Lak, 2005).

2.2 Plasmids

2.2.1 Plasmid p+19/32SK+

Two plasmids were used in this experiment. The first is a 5kb pBluescript SK (Stratagene) based plasmid termed p+19/32SK. This plasmid is derived from p+157SK which has a 2.2 kb insert cloned in its polylinker between the cutting sites for enzymes *XhoI* and *XbaI*. This insert contains 227 basepairs originating from positions -70 to +157 of the *C.reinhardtii rbcL* gene. (Transcription start site being at +1). The 227 bp contain the promoter and the 5`UTR of the *rbcL* gene together with some of the *rbcL* gene coding sequence. The 227 bp are fused to the 5`end of the *E.coli uidA* gene coding for beta-glucuronidase (GUS). The GUS gene is used as a reporter gene. Plasmid also have a ampicillin resistance gene to select for transformed bacteria. The p+19/32SK also have a *SphI* site between *SwaI* and *BspEI* (See figure 2.4.1).

2.2.2 pCrc32 derived plasmid

The second plasmid used is the approximately 11 kb large pCrc32 plasmid. This plasmid also contains an ampicillin-resistance gene for selection in transformed *E.coli* together with an origin of replication for *E.coli* cells. It has a chimeric *atpB* promoter: GUS gene which is flanked by the *Chlamydomonas* wild-type *atpB* gene and some sequences from the inverted repeat on the other side. These flanking sequences are important for site-specific recombination and complementation of the *atpB* gene. The *atpB* gene in pCrc32 complements the 2.5 kb deletion in chloroplasts of the *Chlamydomonas* strain CC-373 (See figure 2.4.1).

2.3 DNA manipulation techniques

2.3.1 Annealing and phosporylation of oligonucleotides

Two complementary oligonucleotides were obtained from MWG Biotech AG, Ebersberg, Germany (See figure 2.3.1).

```
                              1
5`-TAA ACT TAA TCT ATA TAT AAA ATG TAT TTA AAA TTT TTC AAC AAT TTT TAA ATT ATA TTT-3`
3`-ATT TGA ATT AGA TAT ATA TTT TAC ATA AAT TTT AAA AAG TTG TTA AAA ATT TAA TAT AAA GGCC-5`
```

Figure 2.3.1: The two oligonucelotides used in this study ligated to each other. The 19 extra nucleotides are underlined.

The annealed oligos have one blunt end and one end with a 4nt overhang. Upper and lower strands shown in figure 2.3.1 contain 41 and 45 of the first nucleotides from the *Chlamydomonas rbcL* 5`UTR, respectively, in addition to the underlined 19 extra nucleotides. Note that the *SwaI* site (ATTTAAAT) located around position, +1 in wild-type plasmids is not present. An *SphI* site (GCATGC) which is present in plasmid p+19/32SK, the plasmid into which the oligonucleotides were cloned, is not present in the oligonucleotide sequence.

The oligonucleotides were resuspended in sterile water to a concentration of 100µM. We mixed 0.5 nanomoles of each strand together. The mixture was boiled for two minutes at 100°C followed by a slow cooling down to room temperature (Sambrook and Russel, 2001). Phosphorylation of the resulting double stranded DNA 5` terminals was carried out by adding T4-poynucleotide kinase (10 µl), polynucleotide kinase buffer (10X), ATP and allowing incubation at 37°C for 1h.

2.3.2 Restriction enzymes

Restriction enzymes from New England Biolabs were used according to the producer instructions.

2.3.3 Digestion of Plasmid p+19/32SK

Plasmid p+19/32SK was cut with restriction enzymes *SwaI* and *BspEI* (see figure 2.4.1). Restriction endonuclease *SwaI* cuts in the middle of the sequence 5`-ATTTAAAT-3` giving a blunt end at the very beginning of the transcription start site (+1). *BspEI* recognizes the sequence 5`-TCCGGA-3` and leaves a 4 nucleotide "sticky" overhang around position +40 in the 5`UTR. This 4 nt overhang complements the 4 nt overhang resulting from the oligo formation.

2.3.4 Agarose gel electrophoresis

Agarose was used at 1% concentration in 1X TAE buffer (Sambrook and Russel, 2001). Bands were visualized by ethidium bromide staining (0.25 ug/ml).
Electrophoresis was performed in 1X TAE buffer for 30-90 minutes at 80V and DNA samples were mixed with gel loading buffer (one tenth of total sample volume) before loading into the gel (Sambrook and Russel, 2001). A 1kb ladder from Invitrogen was used as a molecular weight marker to estimate sizes of DNA fragments. 1.3% agarose was used for isolating oligonucleotide fragments.

2.3.5 Isolation of DNA fragments using gel-electrophoresis

DNA fragments were run on a agarose gel followed by visualization under UV-light. A cut in the gel was made below each of the wanted fragments, giving wells where DNA could be collected. Into each well a dialysis membrane was placed to prevent DNA fragments from running out. The gel was then run "backwards" after first having filled each well with 1X TAE buffer and reduced the amount of buffer in the electrophoresis chamber. The DNA fragments were collected with a pipette as they entered the wells and further purified by phenol extraction and ethanol precipitation.

2.3.6 Ligation

Vector and oligonucleotides were mixed at a ratio of 1:5 in a 10 µl reaction mixture containing T4 ligase from "New England Biolabs". Ligation was done at 16°C for 4h (Sambrook and Russel, 2001). Ends of vectors and oligos ensured that insertion of oligo into vector was done in correct orientation.

2.3.7 Transformation

Plasmid DNA was transferred into $CaCl_2$ competent *E.coli* cells by the heat shock method (Sambrook and Russel, 2001). Cells were plated on agar containing ampicillin and grown over night. Transformed colonies were picked and moved to tubes containing 3 ml liquid LB medium. Medium also contained ampicillin. Cells were then incubated over night at 37°C on a rotating wheel.

2.3.8 Isolation of plasmid DNA from *E.coli*

Small-scale plasmid isolation from *E.coli* was carried out according to the miniprep protocol (Sambrook and Russel, 2001).

Large-scale isolation of plasmids was carried out as described in the protocol (Sambrook and Russel, 2001). By use of an ultracentrifuge, separation of RNA and DNA was conducted in a CsCl-Ethidium Bromide gradient. CsCl was then removed from the solution by dialysis followed by measurement of DNA concentration in a spectrophotometer at 260nm.

2.3.9 DNA/RNA quantification

The dot-spot method was used (Sambrook and Russel, 2001) to estimate concentration of both DNA that was isolated by gel-electrophoresis in 2.3.5 and DNA from *Chlamydomonas* transformants. DNA concentrations of maxi-prep and total RNA samples were more accurately determined by the use of spectrophotometric measurements at 260 nm (Sambrook and Russel, 2001).

2.4 Transformation of the *Chlamydomonas* chloroplast

Before transformation the mutant *C.reinhardtii* cells were brought into growth log-phase by growing on a shaker at 25°C and cells being diluted with fresh medium several times (Salvador et al., 1993). DNA was coated onto 0.6 µm gold particles and then "shot" into mutant CC-373 cells with the use of a helium-driven particle delivery system from "Bio-Rad" called "PDS-1000/He". Bio-Rad`s own protocol was followed together with bombardment parameters for algae at 1300 psi.

Once inside the cells the DNA construct is inserted into the cells chloroplast chromosome by several homologous recombinations. The construct ends up between the *atpB* gene and the inverted repeat of the cell`s chloroplast genome. See figure 2.4 (Gershuny, 2005).

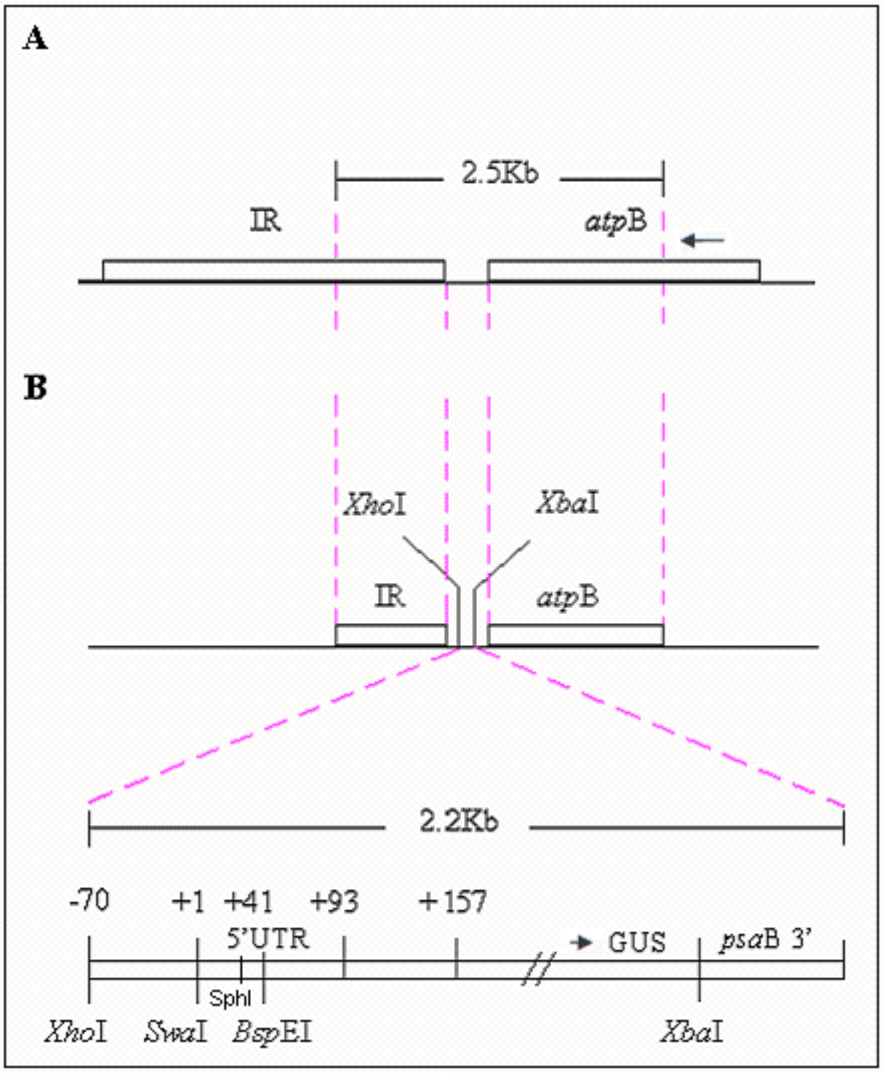

Fig. 2.4 "The 2.5kb deleted region in cc-373. **B** The transformation vector with homologous sequences complementing the deletion in cc-373 and carrying the 2.2kb construct made of rbcL 5'UTR, uidA (GUS) and psaB 3' region" (Gershuny, 2005). The SphI site is incorporated is also shown. SphI sequence is 5`-GCATGC-3`. Arrows indicate the direction of transcription.

2.5 Isolation of DNA and RNA from *Chlamydomonas*

MU7 and chloroplast transformants were grown on high salt (HS) medium (Sueoka, 1960) in water bath at 37°C and mixed by air supplemented with 2% CO2. For DNA isolation *C.reinhardtii* cells were illuminated under constant light during growth and DNA was extracted according to Blowers et al. (1989). In the case of RNA isolation, cells were grown in 12h light/ 12h dark cycles.

The mutant strain CC-373 was grown in high salt (HS) medium (Sueoka, 1960) containing 2.5 g/L potassium acetate (HSHA).

RNA isolation was done at 10 h into the dark period (Salvador et al., 1993) by the SDS/phenol method according to Merchant and Bogorad. (1986).

DNA isolation was conducted as described by Dellaporta et al. (1983).

RNA concentrations were determined by spectrophotometer at 260nm.

2.6 Hybridization analysis methods

2.6.1 Probes

The 1.9 kb coding region of the GUS gene was cut out from the vector pBI221 (Jefferson, 1987) with *SacI* and *BamHI*.This gene was used as a probe in both DNA slot-blot and Northern analysis. In the case of Southern analysis a 700bp restriction fragment was taken from the protein coding sequence of the *atpB* gene isolated from a different vector termed pCrcatpB (Blowers et al., 1990). This restriction fragment was cut out of the vector using enzymes *EcoRV* and *HpaI*. All probes were labelled with (alpha-32P) [dCTP] by random primer labelling using the Klenow fragment of *E.coli* DNA polymerase I (Feinberg and Vogelstein, 1983). Prior to labelling, probes were denatured in boiling water for about 5 minutes.

2.6.2 DNA slot-blot

In order to identify GUS-containing transformants, total DNA from transformants was screened for the presence of chimeric GUS gene construct. 1.5 µg DNA were denatured at 65°C in the presence of 0.3M NaOH and loaded into slots on the surface of a slot blot apparatus (PR600, Hoefer Scientific Instruments). The samples were sucked by vacuum through a nylon membrane (ZetaProbe; BioRad) leaving nucleic acids fixed/blotted to this membrane. The DNA was covalently linked to the membrane by shortwave UV-light for 2 min. Hybridization and washing were done according to the Zetaprobe manual. Blots were wrapped in plastic and placed target side up for exposure to X-ray film (Biomax MS, Kodak) at -80°C. The exposure time required depended both on the abundance of the target as well as the specific activity of the labelled probe.

After washing of the membrane the strength of the radioactive signal was measured using a Geiger counter before exposure of the blot to x-ray film (Kodak MS). With the use of an intensifying screen (Kodak) the typical exposure time was over night.

2.6.3 Southern blot

2 µg of genomic DNA was cut with enzymes *KpnI* and *HindIII* and the resulting restriction fragments were separated in a 1% agarose gel. The DNA were then transferred to a Zetaprobe nylon membrane by alkaline blotting and capillary pull procedure as described in the BioRad Zetaprobe manual. Blotting was done overnight. The membrane was afterwards hybridized to the radioactively labelled *atpB* probe. Washing was done according to ZetaProbe protocol. Radioactivity level was measured with a Geiger counter and exposure of the membrane to a Kodak Biomax MS film was done overnight at -80°C.

2.6.4 Northern blot

4 µg of total isolated RNA was denatured with formamide/formaldehyde followed by separation of RNA by electrophoresis on a 1.3% agarose-formaldehyde gel (Sambrook and Russel, 2001). RNA was then blotted onto a ZetaProbe nylon membrane for 5 hours by alkaline capillary blotting (BioradZetaProbe protocol). The membrane was then hybridized to the radioactive GUS probe over night. Washing was done as described in the ZetaProbe protocol. Using a Geiger counter, radioactivity was checked. The membrane was then exposed to a Kodak Biomax MS film at -80°C over night using a intensifying screen (Kodak).

2.6.5 Secondary structure prediction

All predictions of transcripts 5`end secondary structures were done at the Burnet Institute Mfold server (http://mfold.burnet.edu.au/), where folding is calculated from the maximum free energy change (Zuker, 2003).

3 Results

3.1 Cloning the oligonucleotide fragment into Plasmid p+19/SK

SK+19 plasmid was cut using restriction enzymes *SwaI* and *BspEI* releasing a 45bp fragment out of the 5kb large plasmid. The plasmid without the 45bp was then isolated on an agarose gel and used as vector in later steps. The annealed double stranded oligonucleotide with the 19 extra nucleotides was then annealed into the *SwaI*/*BspEI* site of the vector. The construct was then transformed into E.coli cells. DNA was isolated from several transformants, cut with *SwaI,* and then run on a gel. A uncut vector is a indicator for the presence of the oligonucleotide fragment. The reason for this is that a successfully inserted oligonucleotide will destroy the *SwaI* site in the vector (figure 3.1). The result was negative indicating that the oligonucleotide was actually incorporated into the vector. DNA was isolated from transformants with vectors carrying the inserted oligonucleotide by maxiprep.

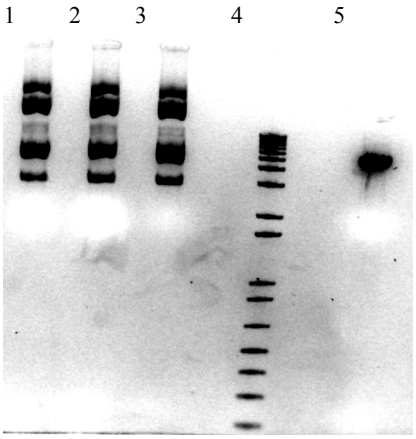

Figure 3.1
Miniprep plasmid DNA digested with *SwaI.*

Lane 1 to 3 : Miniprep DNA from three individual colonies all treated with *SwaI*

Lane 4 : 1 kb ladder
Lane 5 : Cut SK +157, as control

3.2 Cloning the *rbcL*-GUS construct into transformation vector pCrc32

XhoI and *XbaI* were used to cut the plasmid containing the new *rbcL* GUS construct releasing a 2.2kb fragment. Vector pCrc32/+10 was also cut with *XhoI* and *XbaI* to release a 2.2kb fragment. The 2.2kb fragment taken from the new plasmid was then ligated into the cut pCrc32/+10 vector creating a new vector termed pCrc32+19. All isolation of fragments was done on agarose-gels. DNA was isolated by miniprep and cut with *SwaI*. Since the new construct lacks one of the two *SwaI* sites present in pCrc32/+10 a vector that is only cut once is a indication of a successful cloning. Result was as expected indicating that cloning was successful (figure 3.2).

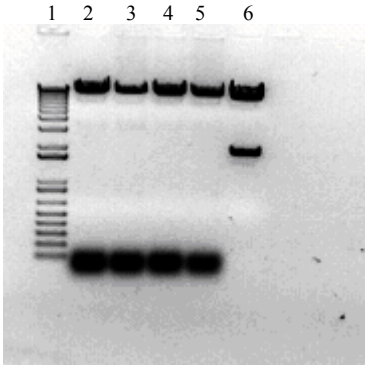

Figure 3.2
Miniprep plasmid DNA digested with *SwaI*

Lane 1 : 1 kb ladder
Lane 2 to 5 : DNA from four individual colonies
Lane 6 : DNA from plasmid pCrc32/+10 as control

3.3 Analysis of chloroplast transformants

3.3.1 Detecting the GUS gene by using a radioactive GUS-probe

The *Chlamydomonas* chloroplast genome was transformed by micro-projectile bombardment as described in "Material and Methods". Transformants were first grown on HS-agar in light and then transferred to HS-liquid media in a wather bath to be further grown under light illumination. Slot blot of DNA was done as described in "Materials and methods". The membrane was hybridized to a radio labelled GUS-probe and exposed to film. DNA from a MU7 transformant that is homoplasmic for the GUS gene was used as control (figure 3.3.1).

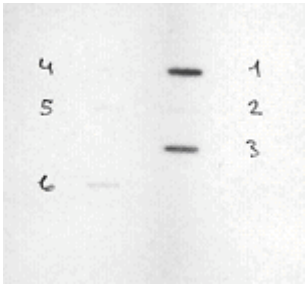

Figure 3.3.1
Slot-blot from different transformants.Transformants containing the highest numbers of genome copies of the oligonucleotide-rbcL/GUS construct with 19 extra nucleotides will give the strongest signal. Slot-blot 1 and 3 have most GUS incorporated.

Slot number 1 and 3 had most GUS incorporated and these transformants were used for further analysis.

3.3.2 Determining the percentage of homoplasmicity by Southern blotting

As mentioned, once inside the cell, DNA constructs are inserted into chloroplast chromosomes by homologous recombinations. The construct ends up between the *atpB* gene and the inverted repeat. Homologous recombination happens between several sites in the genome and all of the ~80 chloroplast genome copies may not get our construct.

Homoplasmicity was determined by cutting chloroplast DNA from chosen transformants with *HindIII* and *KpnI* (figure 3.3.2) and by DNA gel blot analysis.

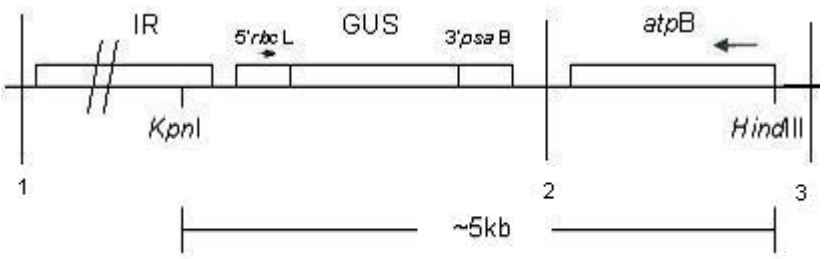

Fig 3.3.2 A ~5kb fragment is released from transformant DNA cut with *HindIII* and *KpnI* when the GUS gene is present in the genome. Vertical lines marked 1-3 represent possible sites between which recombination may occur. Arrows indicate the direction of transcription (Modified after Gershuny, 2005).

If the GUS gene is incorporated, a 5.4kb fragment is released by *KpnI* and *HindIII*, as shown in figure 3.3.2. If a genome copy receives the fragment between vertical lines 2 and 3 as shown in figure 3.4.2 a 3kb fragment will result from the cut by *HindIII* and *KpnI*. In the latter case only the *atpB* gene is incorporated. Figure 3.3.2b shows the transformants genomic DNA after digestion by *HindIII* and *KpnI*.

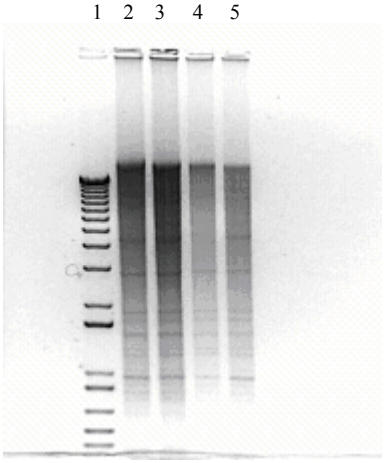

Figure 3.3.2b

HindIII and *KpnI* digested DNA from *Chlamydomonas transformants.*

Lane 1 : 1 kb ladder
Lane 2 . DNA from transformant 1
Lane 3 . DNA from transfromant 3
Lane 4 : DNA from Val27 (Not related to this work)
Lane 5 : DNA from +157 as control

The cut DNA from the chosen transformants was then blotted to a membrane as described in Materials and Methods (section 2.6.3) and hybridized to an *atpB* probe over night after which the membrane was washed and exposed to a Kodak Biomax MS film (figure 3.3.2c). As mentioned in 3.4.2, both types of homologous recombination inserts the *atpB* gene so they will both hybridize to the *atpB*-probe.

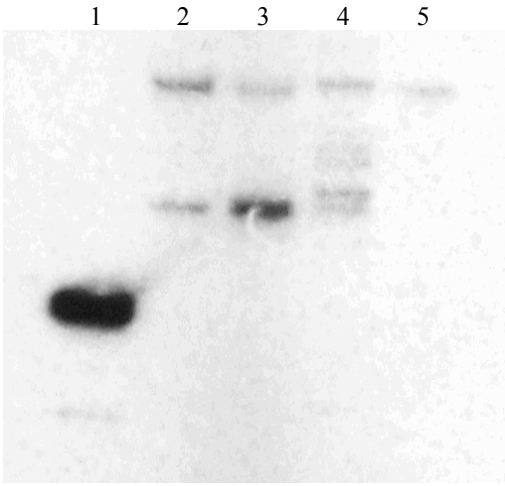

Figure 3.3.2c
Southern blot. Lanes 2 and 3 shows the degree of homoplasmicity of the two constructs 1 and 3.

Lane 1 : The 1.6 kb fragment from the 1kb ladder in figure 3.4.2b bound to the *atpB* probe. It is not known what content of the "ladder" that actually binds to the atpB probe.
Lane 2 : Chloroplast DNA from transformant 1
Lane 3 : Chloroplast DNA from transformant 3
Lane 4 : DNA from a construct from the University of Valencia, Spain, not further used in this experiment
Lane 5 : DNA from the homoplasmic +157 mutant as control

Homoplasmicity is estimated by comparing the intensity of the two bands. Transformants 1 and 3 are estimated to be about 60% and 30% homoplasmic, respectively.

3.3.3 Detecting levels of RNA by Northern blotting

Total RNA was isolated in the dark from cells growing in light/dark cycles. RNA was run on a denaturing gel (figure 3.3.3a).

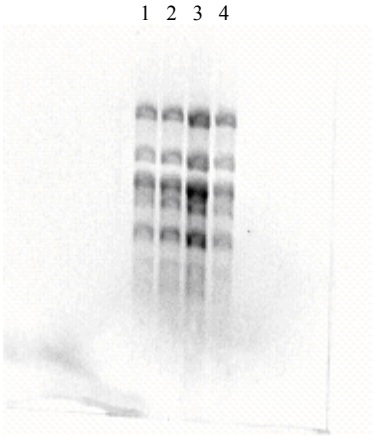

Figure 3.3.3a Northern blotting. Separation of Total RNA on a gel

Lane 1 : +157 as control
Lane 2 : Transformant 1
Lane 3 : Transformant 3
Lane 4 : MU7 as control

The RNA gel blot was then hybridized to a GUS probe followed by exposure to a Kodak Biomax MS film (figure 3.3.3b). RNA from MU7 and +157 were used as controls.

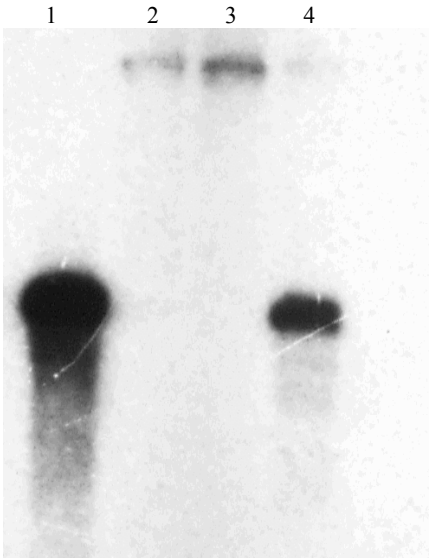

Figure 3.3.3b : Autoradiogram of the Northern blot

Lane 1 : +157 as control
Lane 2 : Transformant 1
Lane 3 : Transformant 3
Lane 4 : MU7 as control

There is no accumulation of GUS RNA in transformants 1 and 3 showing that the addition of 19 extra nucleotides to their 5`end completely destabilizes the *rbcL*:GUS transcripts in the chloroplast of *C.reinhardtii*.

4 Discussion

4.1 Construct

The construct tested in this work contains a 19 nucleotide 5` extension of the 5`UTR of the *Chlamydomonas reinhardtii* chloroplast *rbcL* gene. As mentioned, transcripts of this gene form two stem-loops in the beginning of the 5`UTR which are very important for mRNA stability and longevity. A large and small stem-loop are formed by nucleotides +1 to +38 and +42 to +47, respectively (Suay et al., 2005) (figure 4.1). Trans-acting factors may bind to an RNA element located between the two stem-loops (Anthonisen et al., 2002).

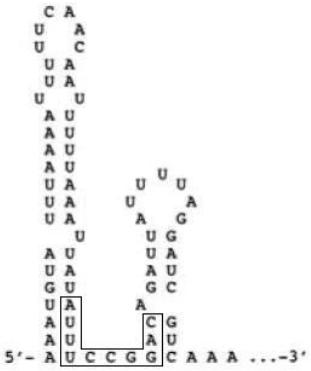

Fig.4.1: Predicted secondary structure in the beginning of the 5`UTR of the *rbcL* chloroplast gene in *C.reinhardtii* (Suay et al., 2005). The boxed RNA element has previously been shown to function as stabilizing element supposedly by binding to trans-factors (Anthonisen et al., 2002).

In this study 19 nucleotides were added to the beginning of the transcript to see whether this change renders the transcript unstable. The added nucleotides were designed not to produce an RNA secondary structure neither should they bind to any sequence in the 5`UTR.

Earlier experiments in which nucleotides have been added to the 5`UTR of the *rbcL* gene have shown that a change in transcript stability depended both on the number and the type of the nucleotides added. Extension up to 8 nucleotides did not significantly destabilize the GUS transcripts whereas longer extensions rendered transcripts unstable. A notable exception are extensions of 20 nucleotides which seemed to destabilize the transcripts less than a 10 nucleotide extension. Because the 20 nucleotide extensions were predicted to form secondary structures that could affect RNA stability we tested in this study a 19 nucleotide extensions that was not predicted to form a secondary structure (it was not possible to design a 20 nucleotide extension that does not fold into a secondary structure).

MU7 strain used as control lacks an enhancer situated around +126 in wild-type transcripts. The RNA at this point is also predicted to fold into a stem-loop (Singh et al., 2001) which may possibly interact with the stem-loop at position +1 to +38 through regulatory proteins. These data were obtained when the first 252 nucleotides of the *rbcL* gene coding sequence are included in the construct (Salvador et al., 1993). Even so, wild-type *rbcL* transcripts have a longer half-life than those from the chimeric gene construct. This may imply that part(s) of the wild-type *rbcL* transcript interact also with other region(s) in the same transcript (Singh et al., 2001). Some of the nucleotides in the +329 and + 17 regions are complementary to each other but transcripts of constructs with compensatory changes in the two regions are not stabilized (Singh et al., 2001).

As mentioned in 1.8.2, in constructs where the distance between promoters elements is changed the promoter is weakened. In this work the distance between the transcription start site at +1 and the promoter situated at -10 is getting longer due to the 19 extra nucleotides that we added. Because in our construct there is no change in distance between promoter elements we do assume that transcription is not reduced nor enhanced.

A final consideration is the possibility that trans-acting factors involved in stability and/or degradation of RNA may have bound to *rbcL*:GUS transcripts at the 19 extra nucleotides added. This could then alter the accumulation of reporter gene transcripts.

4.2 Omitted Methods

A primer extension study were not performed in this experiment to find out wether +19SK transcripts had been targeted by nucleases. Northern blotting showed no transcripts, hence there are no transcripts to do a primer extension study. We assume from other experiments where this type of study has been performed that nucleases do target +19SK transcripts.

A in vivo transcription assay were 32P-labeled inorganic phosphate where incorporated into GUS and signal intensity measured were not done either. However, it has been shown that reduced RNA stability rather than reduced transcription is the cause for reduced accumulation of GUS transcripts. Data has shown that the type of chimeric gene construct used in this experiment are transcribed (Salvador et al., 2004).

After slot-blot, DNA from MU7, a transformant that is homoplasmic for the GUS gene, was not used as a control. This has been done in several earlier experiments though. Transformant +157 was used as positive control. This transformant is homoplasmic for the GUS gene.

4.3 Perspectives

In future studies of *rbcL* gene expression it would be interesting to isolate the trans-acting factor(s) that are assumed to bind to the 5`UTR. Putative protein factors have been identified that are thought to stabilize *psbD* chloroplast transcripts by binding to sequence elements in the 5` untranslated regions (Nickelsen et al., 1994). This type of work should help in understanding the molecular mechanisms that underly regulation of transcripts stability. Understanding the trans-acting regulation of the *rbcL* gene may also help in understanding regulatory mechanisms of genes in higher organisms like animals and humans.

The study of the *rbcL* gene may also be of interest in an evolutionary perspective. The question in notion is how protein-encoding genes become parts of the transcriptional and translational regulating machinery.

Promoters together with other sequences can be mutated in such a way that abundance of both transcripts and translation could be changed. The mutations then giving changes in abundance of transcripts and translation might be the result of promoters becoming more or less active, change in stability of the transcripts, change in gene enhancers or other changes (Singh et al., 2001).

Pinpointing enzymes involved in homologous recombination may also be of interest.

Algae have been tested in anticancer drug screening due to the fact that algal cells require less complex and less expensive media than mammalian cells (Maucourt et al., 2002).

4.4 Conclusion

Results from northern and Southern blotting show that the 19 added nucleotides do render the *rbcL* transcripts unstable indicating that a 5`end extension without any secondary structures is less stable than extensions that form secondary structures.

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