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Mutagenesis and Suppression of a Light-Regulated Group I Intron in the Chloroplast *psbA* Gene of *Chlamydomonas reinhardtii*

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Mutagenesis and Suppression of a Light-Regulated Group I Intron in the Chloroplast *psbA* Gene of *Chlamydomonas reinhardtii*

by

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Dedication

To Min-su Lee, Min-young Lee, and Yunjeong Park.

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Mutagenesis and Suppression of a Light-Regulated Group I Intron in the Chloroplast *psbA* Gene of *Chlamydomonas reinhardtii*

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Four chloroplast *psbA* introns in the green alga *Chlamydomonas reinhardtii* (*Cr.psbA1*-*Cr.psbA4*) are self-splicing group I introns and their splicing is light-promoted. Because evidence indicates that some chloroplast mRNAs are in great excess over what is needed to sustain translation rates, the physiological significance of light-promoted *psbA* splicing has not been clear. Also, splicing factor(s) for these introns have not been identified.

I have generated several point mutations in the core helices of the *Cr.psbA4* intron, and tested their effects on self-splicing *in vitro*. I also replaced the wild-type intron *in vivo* with each mutant, and compared the *in vitro* self-splicing data to the

effects of these mutations *in vivo*. The results indicate that destabilizing mutations in the intron core have less effect *in vivo* than *in vitro*, suggesting that there is considerable stabilization of the intron's active structure *in vivo*. Interestingly, however, there were approximate correlations between *in vivo Cr.psbA4* splicing efficiency, synthesis of full-length D1, and autotrophic growth rates. For example, the P4′-3,4 mutant, which showed a 45 % reduction in *psbA* mRNA, had a 28 % decline in synthesis of full–length D1, and a 18 % decline in photoautotrophic growth rate. These results indicate that *psbA* mRNA is not in great excess, and that efficient splicing of the *psbA* introns, which is light-dependent, is essential for maintaining robust autotrophic growth.

I have also found that three nuclear suppressors of a mutated 23S rRNA intron (7120, 7151, and 71N1) also suppressed the P4′-3,4 mutation in the *Cr.psbA4* intron. This was accomplished by replacing the wild-type *Cr.psbA4* intron in the suppressors with the P4′-3,4 mutant; higher steady-state levels of spliced *psbA* mRNA and less precursor was observed in the suppressors. This result indicates that these genes play a role in splicing of multiple group I introns in the chloroplast.

In addition, further genetic analysis was performed with the 7120 suppressor, which had been only partially characterized previously. Tetrad analysis of a cross of 7120 (mt+) with wild-type (mt-) indicated that it is a single nuclear gene mutation. Complementation analysis in diploids demonstrated that the suppressor mutation is dominant. We propose to call this gene *css2* (for chloroplast splicing suppressor).

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1.1 Introns and RNA splicing

1.1.1 Discoveries of introns and RNA splicing

Introns are intervening sequences, which are parts of a primary transcript that are removed by splicing and are not included in the mature, functional mRNA, rRNA, or tRNA. Introns and RNA splicing were first found in the 5′ terminal sequence of several adenovirus mRNAs by observing R-loops generated by hybridization of mRNA from infected cells to Adenovirus genomic DNA under the electron microscope (Berget et al., 1977; Chow et al., 1977).

1.1.2 Discovery of self-splicing RNA

Thomas Cech and his colleagues first discovered that RNA could self-splice *in vitro* while studying the large ribosomal RNA gene from *Tetrahymena thermophila* (Cech et al., 1981). Shortly thereafter, Altman's group at Yale University found that the RNA moiety of ribonuclease P, M1 RNA, from *Escherichia coli* is the catalytic subunit of the enzyme (Guerrier-Takada et al., 1983). The term "ribozyme" was given for these catalytic RNAs. Since then, a number of naturally occurring catalytic RNAs have been found, including the hammerhead, hairpin, hepatitis delta virus (HDV), and Varkud Satellite (VS) ribozyme, and the ribosome (Table 1.1) (Symons, 1992; Cech,

Table 1.1 Naturally occurring ribozymes and ribonucleoprotein enzymes (adapted from Doudna and Cech, 2002).

Ribozyme	Sequenced examples	Size (nt)	Activity (reaction product)
Hammerhead	11	40	Self-cleavage via transesterification (2′,3′ cyclic phosphate)
Hepatitis delta virus	2	90	
Hairpin	1	70	
Varkud satellite	1	160	
Group I intron	>1,500	210	Self-splicing via transesterification (3′-OH)
Group II intron	>700	500	
RNase P	>500	300	Pre-tRNA processing via hydrolysis (3′-OH)
*Spliceosome (U2 + U6 snRNAs)	70,500	180,100	RNA splicing via transesterification (3′-OH)
*Ribosome (23S rRNA)	>900	2,600	Peptidyl transfer (amide)

Number of sequenced examples is a snapshot as of 2002 and is influenced by DNA sequencing strategies and database upkeep; it may provide a rough indication of relative abundance. RNAs in any group vary in size; the size provided here indicates the lower end of the length distribution for the natural examples. See ww.rna.icmb.utexas.edu and www.jwbrown.mbio.ncsu.edu/RnaseP/.

*Ribonucleoprotein enzymes. RNase P: bacterial and archaebacterial RNAs have the relevant activity in the absence of protein. Spliceosome: U2 and U6 small nuclear RNAs (snRNAs) alone show an activity related to the natural activity. Ribosome: no activity has yet been observed with protein-free, large-subunit rRNA.

1993; Kenell et al., 1995; Muth et al., 2000; Nissen et al., 2000).

1.1.3 Evolutionary implications of ribozymes

In the old model for the flow of genetic information, RNA was just an intermediate between DNA and protein. The "RNA world" hypothesis, however, which posits that RNA dominated organisms represented an important stage in the evolution of life on earth, has changed our concept of RNA into a central macromolecule (reviewed in Sharp, 1985; Orgel L. E., 1986). This hypothesis has been strengthened in recent years by the demonstration of an RNA molecules capable of catalyzing the synthesis of a pyrimidine nucleotide, and RNA polymerization *in vitro* (Unrau and Bartel, 1998; Johnston et al., 2001), and by the confirmation that protein synthesis in cells is catalyzed by ribosomal RNA (Muth et al., 2000; Nissen et al., 2000). Recently, Valadkhan and Manley (2001) provided direct evidence that RNAs U2 and U6 of the spliceosome have catalytic potential without proteins *in vitro*. They showed that a protein-free complex of U2 and U6 can bind and position a small RNA containing the intron branch site to attack a catalytically critical domain of U6 in a reaction that is related to the first step of splicing. This result provides further evidence that spliceosomal-based splicing is catalyzed by RNA.

1.1.4 Classification of introns

Introns have been categorized into five groups based on the presence of

conserved nucleotide sequences and /or RNA secondary structures; Groups I-III, nuclear pre-mRNA, and tRNA introns. A brief description of these groups is given below.

A. Group I introns

Group I introns are ribozymes which need an exogenous guanosine nucleotide (GTP, GDP, GMP), or guanosine, to catalyze the self-splicing reactions. They have distinctive secondary and tertiary structures, and contain a conserved catalytic center. The boundaries of group I introns are marked by a U residue at the 3′ end of the 5′exon and a G residue at the 3′ end of the intron (reviewed in Cech, 1990). They have been classified into five major subgroups, designated IA to IE (Michel and Westhof, 1990; Suh et al., 1999). Group I introns are present in rRNA, tRNA, and protein-coding genes. They are common in fungal and plant mitochondrial DNAs, and in chloroplast DNA of *Chlamydomonas spp.*, but are also found in the nuclear rRNA genes of lower eukaryotes, in bacteriophage, and in several tRNA genes in eubacteria. They have not been found in higher animals (i.e., vertebrates) (reviewed in Perlman and Lambowitz, 1990; Saldanha et al., 1993; Tanner, 1999).

B. Group II introns

Group II introns are ribozymes with a distinctive secondary structure and a

catalytic center. They are found in rRNA, tRNA, and mRNA genes of organelles in fungi, plants, and protists; in bacteria (reviewed in Saldanha et al., 1993; Michel and Ferat 1995; Bonen and Vogel, 2001; Toor et al., 2001), and recently in archae (Dai and Zimmerly, 2003). Group II introns have conserved 5'- and 3'-boundary sequences (GUGYG and AY respectively), and are organized into a set of six helical domains (D1-D6) (reviewed in Pyle, 1996). Two major subclasses of group II introns (IIA and IIB) were initially distinguished based on structural features of the intron (reviewed in Michel et al., 1989b; Qin and Pyle, 1998). Recently, group II introns were defined as having at least 8 classes: two chloroplast-like Classes (I and II), one mitochondrial class, and five bacterial classes (A-E) (Toor et al., 2001; Toro et al., 2002). As in nuclear mRNA introns, splicing is initiated by the formation of an intron lariat in which the 5′ end of the intron becomes linked by a 2′-5′ phosphodiester bond to an internal nucleotide residue, usually an A near the 3′ end of the intron.

C. Group III introns

Group III introns are abbreviated versions of group II introns, and are common in chloroplast genes of *Euglena gracilis* and other Euglenoids. They don't contain domains I-III or Domain V of group II introns, but are excised as a lariat (Christopher and Hallick, 1989; Hallick et al., 1993).

D. Nuclear pre-mRNA introns

Nuclear pre-mRNA introns are found in nuclear mRNA genes of eukaryotes. They typically contain GU and AG nucleotides at the 5' and 3' boundaries, respectively, and are spliced by small ribonucleoprotein particles (snRNPs) that assemble into a spliceosome. The mechanism involves a 'lariat' structure for the intron similar to group II splicing (Ruskin et al., 1984).

Group I, group II and nuclear mRNA introns may be evolutionarily related because of their similar splicing chemistry, which involves transesterification reactions (Cech, 1986; Jacquier, 1990). Group II and nuclear mRNA introns appear to have a more direct relationship since both form a lariat linked by a 2'-5' phosphodiester bond to an internal A (Figure 1.1). There are also structural similarities among these groups, particularly between group II and nuclear mRNA introns (reviewed in Bonen and Vogel, 2001).

1.1.5 Splicing factors for group I and group II introns

Some group I and group II introns perform self-splicing, but most all probably require proteins for efficient splicing *in vivo* (reviewed in Lambowitz et al., 1999). Some splicing factors bind specifically to the intron RNAs, and either induce the formation of the catalytically active RNA structure, stabilize the folded structure, or use some combination of the two mechanisms. These protein factors include cellular or "host" proteins as well as intron- encoded proteins, called maturases (Mohr et

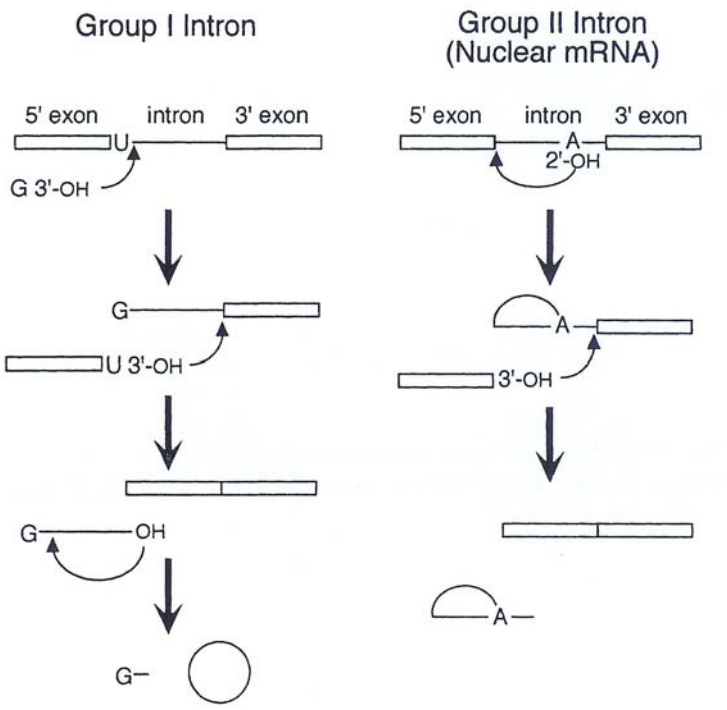


Figure 1.1 Splicing mechanisms of group I, group II, and nuclear mRNA introns (adapted from Tanner, 1999). A series of trans-esterification reactions are used to excise the intron and ligate the exons. The net number of phosphodiester bonds remains the same throughout. The reaction is initiated by a guanosine cofactor in group I introns, and by an internal adenosine in group II. The splicing reaction of nuclear pre-mRNAs follows the same general pathway as group II introns, but it occurs on a large transient ribonucleoprotein complex, the spliceosome.

al., 2002).

In addition to proteins that bind specifically to the intron, it has been suggested that nonspecific RNA-binding proteins may facilitate splicing of group I introns by functioning as RNA chaperones. The chaperones function by alleviating "kinetic traps" in RNA folding (Herschlag, 1995; Woodson, 2000). So far, all of the group I splicing factors that are known are from fungi, and they promote splicing of mitochondrial introns (reviewed in Lambowitz et al., 1999). Further discussion of group I splicing factors is in section 1.2.3.

For group II introns, several splicing factors have been identified. A nuclear gene in yeast, *MRS2*, promotes splicing of mitochondrial group II introns indirectly by maintaining Mg^{2+} homeostasis (Gregan et al., 2001). The *MSS116* gene encodes a DEAD box protein and functions in yeast mitochondrial splicing. However, the latter protein is not restricted to group II introns (Seraphin et al., 1989; Niemer et al., 1995).

In maize, the *crs2* gene is required for splicing of many chloroplast group II introns, and it encodes a homolog of peptidyl-tRNA hydrolase (Jenkins et al., 1997; Jenkins et al, 2001). The *crs1* gene, which is a very specific splicing factor for the *atpF* intron in maize, encodes a novel protein that is involved both in splicing and translation (Till et al., 2001).

A putative higher plant splicing factor, which is specific for chloroplast group II introns, is *matK*. This gene is encoded as an ORF within the lysyl tRNA (*trnK*) intron, and is possibly involved in splicing of the *trnK-matK* transcript *in vivo*

(Vogel et al., 1997, 1999).

In *Chlamydomonas reinhardtii*, the *maa2* gene, which is required for trans-splicing of the group II *trans*-spliced *psaA* mRNA, encodes a homolog of pseudouridine synthases, but doesn't seem to have the synthase activity (Perron et al., 1999).

Group I and group II introns have strong similarities in the evolution of their protein-assisted splicing factors which appear to have evolved from pre-existing proteins that had other functions (reviewed in Lambowitz et al., 1999).

1.1.6 Intron homing

Some group I and group II introns have been shown to be mobile genetic elements: they invade intronless alleles, and convert them into intron-plus in a process called "intron homing" (reviewed in Dujon, 1989; Perlman and Butow, 1989; Belfort and Roberts, 1997; Chevalier and Stoddard, 2001).

The homing mechanisms for these two groups are different: the homing for group I introns proceeds by a double-strand break model (Figure 1.2) (reviewed in Lambowitz and Belfort, 1993), whereas, for group II introns, RNP particles function in intron mobility by retrohoming or retrotransposition (Eskes et al., 1997; Eskes et al., 2000; Cousineau et al., 2000; Dickson et al., 2001). They, however, both need intron-encoded proteins for this process. The success of these introns as mobile elements is almost certainly linked to their self-splicing capability, which enables

them to spread to new genes and cellular compartments while only minimally impairing gene expression (reviewed in Lambowitz et al., 1999). Group I intron homing is initiated by a highly specific endonuclease encoded within the intron itself. The intron-encoded open reading frames (ORFs), which are either free-standing or in-frame with the upstream exon, specifically encode "homing endonucleases" that cleave the intronless allele near the site of intron insertion (reviewed in Mueller et al., 1993). Subsequent steps involve homologous recombination with the products being two intron-containing alleles (Figure 1.2). Four different types of endonucleases have been found encoded in group I introns, and these are distinguished by the presence of a conserved amino acid motif: LAGLIDADG, GIY-YIG, H-N-H, or His-Cys. Also, several of the homing endonucleases, all of which are LAGLIDADG proteins, have been found to promote splicing of the pre-mRNAs that encode them, and hence are also called maturases. A maturase has yet to be identified in a chloroplast group I intron. However, mobile group I introns have been discovered in *Chlamydomonas* chloroplast genes (Odom et al., 2001).

1.2 Group I introns

1.2.1 Structure of group I introns

Different Group I introns have relatively little sequence similarity, but their

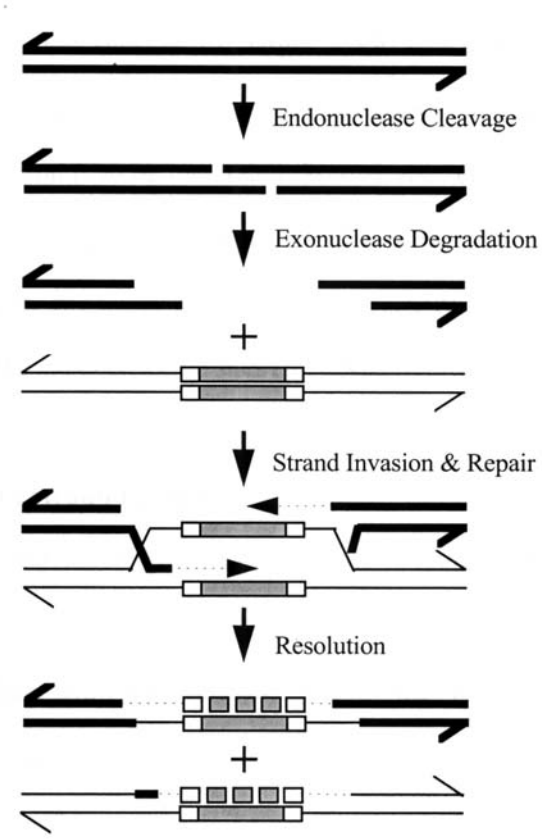


Figure 1.2 The double-strand-break repair pathway for group I intron mobility (adapted from Mueller et al., 1993). Following cleavage by the homing endonuclease, the recipient allele (heavy lines) is subjected to exonucleolytic degradation and aligned with homologous sequences of an intron –containing donor (thin lines with open and stippled boxes). Once aligned, a 3′ tail of the recipient invades the donor, which acts as the template for DNA-repair synthesis (dashed lines plus arrows). The Holliday junctions formed during this process are resolved, resulting in two intron-containing duplexes. Half-arrows, 3′ end of the DNA strands; open boxes, intron sequences; stippled boxes, endonuclease coding sequences.

secondary structures are conserved (Michel et al., 1982; Michel and Dujon, 1983). The high degree of similarity in the secondary structure implies that the conserved helices must be functionally important to group I introns (reviewed in Burke, 1988; Cech, 1988; Michel and Westhof, 1990).

The conserved group I intron secondary structure was first proposed by Michel and Dujon from comparative sequence analysis (Michel et al., 1982). The structure consists of a series of paired regions, denoted P1-P10, separated by single-stranded regions (denoted by J) or capped by loops (denoted by L) (Figure 1.3A). Specific features of the proposed secondary structures have been confirmed mostly by *in vitro* mutations and by structure mapping (reviewed in Burke, 1988; Cech, 1988; Michel and Westhof, 1990). Group I introns have been classified into five major subgroups, designated IA to IE (Michel and Westhof, 1990; Suh et al., 1999), based mostly on the presence or absence of optional substructures and peripheral domains (Gutell, 1995; Kuo, 1998; reviewed in Herrin et al., 1998). All self-splicing group I introns have fundamentally similar core structures, the region required for enzymatic activity; however, subgroup-specific structures appear to participate in additional interactions that stabilize the core structure in different ways.

A computer-based three-dimensional model of group I introns has been proposed (Figure1-3B) (Michel and Westhof, 1990). This model suggests that the intron folds into a compact structure with some regions exposed on the surface and others buried inside the RNA. The most prominent feature in this model is the

two major domains of coaxially stacked helices, the P4-P5-P6 (or P4-P6) domain, and the P8-P3-P7 domain. These domains form a cleft containing the intron's active site. The active site includes binding sites for the guanosine cofactor (in P7), and P1 and P10, which contain the 5′ and 3′ splice sites, respectively. Basic features of the predicted three-dimensional structure have been supported by the results of structural studies of the *Tetrahymena*, yeast, and phage group I introns (reviewed in Cech, 1993; Jaeger et al., 1996). Recently, a crystal structure of the *Tetrahymena* group I intron core was solved at 5-Å resolution; the overall architecture of the ribozyme is similar to the Michel-Westhof model (Golden et al., 1998).

1.2.2 The mechanism of self-splicing of Group I introns

Group I introns splice *in vitro* by a mechanism involving two trans-esterification reactions. In the first step, a guanosine nucleotide binds to the G binding site in the P7 helix (Wang and Cech, 1992), and its 3′-OH attacks the 5′ splice- site. This first step generates a 5′-exon molecule, which is still held via the IGS (internal guiding sequence) of the intron, and an intron-3′ exon molecule, which has the exogenous guanosine now covalently attached to its 5′-end. In the second step, the G^{ω} at the 3′-end of the intron is bound to the G binding site, and then the 3′-OH of the 5′-exon attacks the 3′ splice-site, producing ligated exons and a free intron molecule. The released intron can be circularized by the 3′- terminal G attacking the intron at or near the 5′-end (Figure 1.4). The self- splicing mechanism of group I introns was

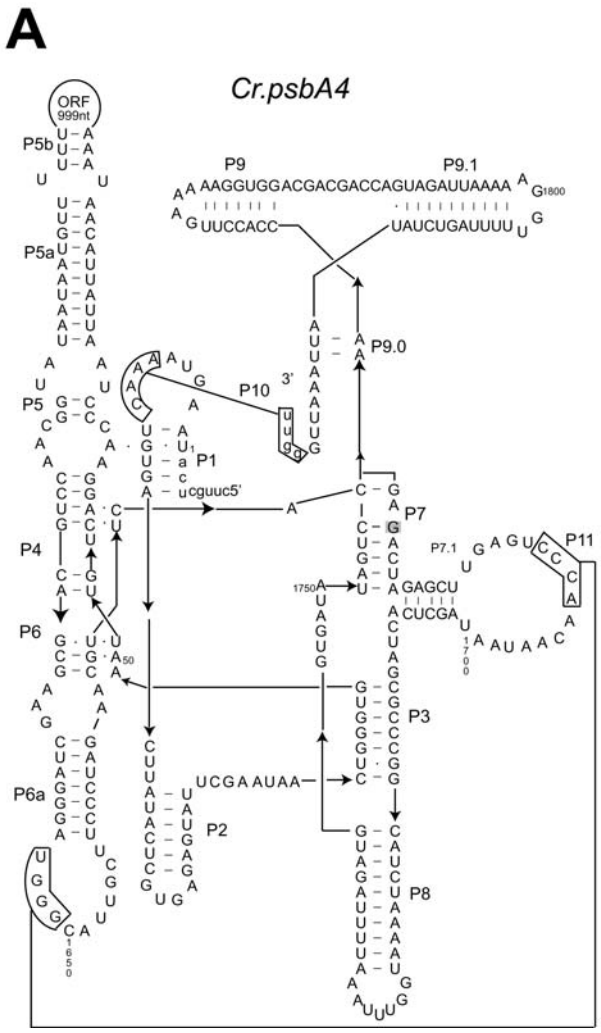
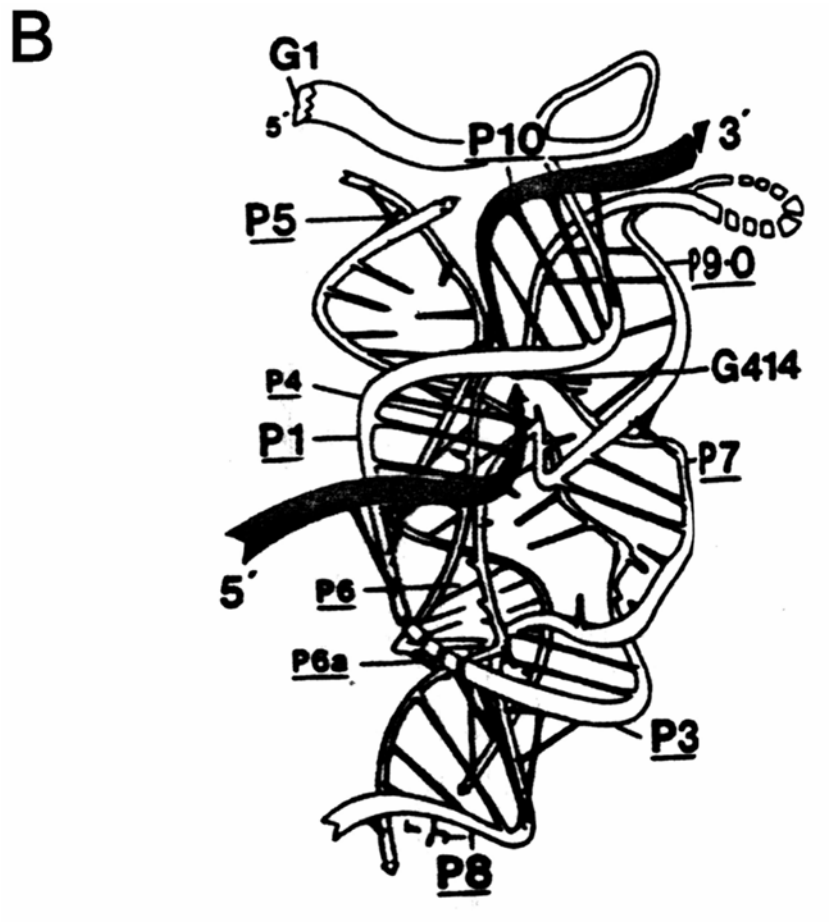


Figure 1.3 The structure of group I introns.

A. Representation of the secondary structure of a group I intron, using the *psbA* intron 4 from *Chlamydomonas reinhardtii* (*Cr.psbA4*) as an example. The secondary structure of group I introns is made up of paired regions or helices (P1, etc.), loops (e.g., L1) and joining regions (e.g., J4/5); however, only the paired regions are labeled in the figure. Two long-range paired regions, P10 and P11, are indicated as boxes and lines. The intron nucleotides are in upper case letters, and the exon nucleotides in lower case. The shadowed nucleotide in P7 indicates the guanosine binding site (^{1675} G). The ORF is located in the loop of P5b.



B. Three-dimensional structural model of the *Tetrahymena* large rRNA intron. The filled ribbon represents exons. The model is after the first step of splicing, G-dependent cleavage at the 5' splice-site in P1, has occurred. G414 is the G residue at the 3' end of the intron, which binds to the guanosine-binding site for step two of splicing.

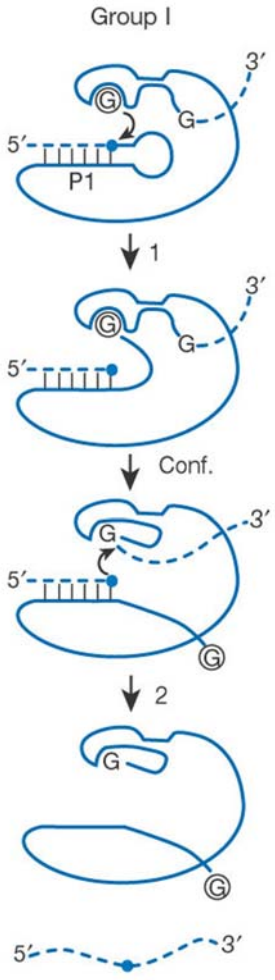


Figure 1.4 Splicing mechanism of group I introns (adapted from Doudna and Cech, 2002). Step 1 shows how an intron-bound guanosine or GTP (circled) cleaves the 5'-splice site while becoming covalently attached to the 5′ end of the intron. "Conf" indicates a conformational change whereby the G at the 3′ end of the intron replaces the free G in the G-binding site. In step 2, the cleaved 5′-exon, still held to the intron by base paring (P1), attacks the 3'-splice site; as a result, the exons are ligated and the intron excised.

first shown for the *Tetrahymena* nuclear large rRNA intron (Cech, 1981), but later was extended to studies to group I introns from *Neurospora* mitochondria (Garriga and Lambowitz, 1984), T4 phage (Belfort, 1990), and *Chlamydomonas* chloroplasts (Herrin et al., 1990; Herrin et al., 199; Thompson and Herrin, 1991).

Metals (presumably Mg^{2+} *in vivo*) play an important role in group I intron splicing. In fact, self-splicing of group I introns requires not only guanosine, but also Mg^{2+}. Mg^{2+} ions are believed to participate in the catalytic mechanism by activating the guanosine nucleophile and stabilizing the incipient transition state (Kuimelis and McLaughlin, 1998; Doherty and Doudna, 2001).

Mg^{2+} ions are also critical for the correct folding of the ribozyme to a catalytically active state: For the *Tetrahymena* intron, individual RNA domains fold in a specific order as Mg^{2+} is increased (reviewed in Cech, 1990; Celander and Cech, 1991). Five Mg^{2+} binding sites in a three helix-junction of the P5abc region have been localized in the crystal structure of the P4-P6 domain of the *Tetrahymena* group I intron (Cate et al., 1997). This magnesium ion core may be the RNA counterpart to the protein hydrophobic core, burying parts of the RNA molecule in the native structure.

1.2.3 Splicing factors for group I introns

Although some group I introns are able to self-splice *in vitro*, evidence suggest that these introns need *trans*- acting factors for splicing *in vivo*. First,

efficient *in vitro* self-splicing requires non-physiological conditions, typically higher concentrations of Mg^{2+} and/or elevated temperature (Thompson and Herrin, 1991). Also, in fungi and yeast, nuclear mutations were found to affect splicing of mitochondrial group I introns *in vivo* (McGrow and Tzagoloff, 1983; Garriga and Lambowitz, 1986). Subsequently, several splicing factors from fungi were cloned and characterized. Proteins that promote splicing of group I introns are classified into three major groups: Maturases, specific nuclear-encoded splicing factors, and RNA chaperones.

1.2.3.1 Maturases

Some Group I introns encode site-specific homing endonucleases that function to promote their mobility by specifically cutting the intronless allele near the point of intron insertion. Four different types of endonucleases have been found encoded in group I introns, and these are distinguished by the presence of a conserved amino acid motif, either LAGLIDADG, GIY-YIG, H-N-H, or His-Cys (reviewed in Belfort and Roberts, 1997). Interestingly, several of the homing endonucleases, all of which are LAGLIDADG proteins, have also been found to promote splicing of the pre-mRNAs that encode them. This splicing function was demonstrated genetically for the proteins encoded by introns bI2, bI3, and bI4 of the yeast mitochondrial (mt) cytochrome *b* gene, and aI1, and aI2 of the yeast mt cytochrome oxidase I (*COX1*) gene (reviewed in Lambowitz and Perlman, 1990; Saldanha, 1993). In each

case, mutations in the ORF resulted in defective splicing of the intron, which could then be rescued by providing the intron-encoded protein *in trans*. Such intron-encoded splicing factors have been termed "maturases". However, experiments with the activated maturase showed that mutations in the first LAGLIDADG motif (P1) of the aIα4 intron of yeast gene inhibited endonuclease but not maturase activity, whereas identical mutations in the second LAGLIDADG motif have the opposite effect, suggesting that the two motifs function in splicing or endonuclease activity (Henke et al., 1995).

In contrast, none of the phage or chloroplast group I intron encoded proteins have been reported to be maturases, and at least some have been tested (reviewed in Belfort, 1990), including the LAGLIDADG protein encoded by the *Cr. LSU* intron, I-*Cre*I (Thompson and Herrin, 1991).

An *in vitro* splicing system was developed for the maturase encoded by the *Aspergillus nidulans* mt cytochrome *b* (*Ancob*) intron, which is homologous to the yeast bI3 maturase. The *Ancob* protein (I-*Ani*I) was expressed in *Escherichia coli*, and both the splicing and endonuclease activities were demonstrated biochemically (Ho et al., 1997) . Kinetic measurements of splicing suggested that I-*Ani*I binds to the COB intron tightly with an equilibrium constant (K_d) in the picomolar range (Ho et al., 1997; Ho and Waring, 1999). RNAse T1 footprinting experiments provided evidence that I-*Ani*I facilitated folding of the *Ancob* intron, which has limited, if any, tertiary structure in the absence of protein (Ho and Waring, 1999). *Ancob* peripheral

domains that function to stabilize intron tertiary structure could not be deleted or truncated without significant decreases in protein-assisted splicing activity, suggesting that extensive RNA folding is required for protein recognition (Geese and Waring, 2001). The AnCOB intron lacks significant stable tertiary structure in the absence of protein, supporting a model in which the maturase preassociates with an unfolded COB intron via a "labile" interaction that facilitates correct folding of the intron catalytic core (Solem et al., 2002).

1.2.3.2 Specific nuclear-encoded splicing factors for group I introns

The best studied example of a nuclear-encoded splicing factor is the bifunctional mitochondrial tyrosyl-tRNA synthetase (mt TyrRS) encoded by the *cyt-18* gene from *Neurospora crassa*. This protein functions in both $tRNA^{Tyr}$ aminoacylation and group I intron splicing. The CYT-18 protein promotes splicing of three of the ten *N. crassa* mtDNA group I introns *in vivo* (LSU, *cob*-I2, and *ND1*) (Mannella et al., 1979; Collins and Lambowitz, 1985; Wallweber et al., 1997). The three CYT-18-dependent introns show no detectable self-splicing *in vitro*, but all splice efficiently at physiological Mg^{2+} concentrations in the presence of purified CYT-18 protein (Garriga and Lambowitz, 1986; Wallweber et al., 1997). In addition, *in vitro*, CYT-18 binds to and facilities the splicing of group I introns from a variety of other organisms, but only those that lack the extended P5abc domain; apparently that interferes with CYT-18 binding.

The introns spliced by CYT-18 have relatively little sequence similarity, indicating that the protein primarily recognizes conserved secondary and tertiary structural features of the intron RNAs (Guo and Lambowitz, 1992; Mohr et al., 1992, 1994, 2001).

The regions of CYT-18 required for promoting splicing include an idiosyncratic N-terminal extension, the nucleotide-binding fold domain, and the C-terminal RNA-binding domain, which is required for both tyrRS and splicing activity. The idiosyncratic N-terminal region is in fact comprised of two functionally distinct parts: an upstream region consisting predominantly of a predicted amphipathic α-helix (H0), which is absent from bacterial tyrosyl-tRNA synthetases (TyrRSs), and a downstream region, which contains predicted helices H1 and H2 that correspond to features in the X-ray crystal structure of the *Bacillus stearothermophilus* TyrRS. The N-terminal extension is required only for splicing activity, and the H1/H2 region is required for splicing activity and TyrRS activity with the *N. crassa* mt $tRNA^{tyr}$, but not for TyrRS activity with *Escherichia coli* $tRNA^{tyr}$, implying a somewhat different mode of recognition of the two tyrosyl-tRNAs (Kittle et al., 1991; Mohr et al., 2001).

CYT-18 promotes the splicing of group I introns by stabilizing the catalytically active structure of the intron core (Guo and Lambowitz, 1992; Mohr et al., 1992). The catalytic core consists of two extended helical domains, P4-P6 and P3-P9, which form a cleft containing the intron's active site (Michel and Westhof, 1990). CYT-18 protection sites on the phosphodiester backbone are located

primarily on the side of the core opposite the active-site cleft (Caprara et al., 1996a, b). Interestingly, comparison of the CYT-18 binding sites in the *N. crassa* mt LSU intron with that in the *N. crassa* mt $tRNA^{Tyr}$ by graphic modeling indicated an extended three-dimensional overlap between the tRNA and highly conserved regions of the group I intron catalytic core (Caprara et al., 1996b). Directed hydroxyl-radical cleavage assays showed that the nucleotide-binding fold and C-terminal domains of CYT-18 interact with the expected group I intron cognates of the aminoacyl-acceptor stem and D-anticodon arms, respectively. Further, three-dimensional graphic modeling, supported by biochemical data, shows that conserved regions of group I introns can be superimposed over the protein interacting regions of the tRNA in the *Thermus thermophilus* TyrRS/$tRNA^{tyr}$ co-crystal structure (Myers et al., 2002).

Another nuclear splicing factor for group I introns is the yeast CBP2 gene, which is required for splicing of the yeast mt cytochrome *b* intron, bI5. The CBP2 gene encodes a 630-amino acid protein lacking clear homology with other proteins (Hill et al., 1985; Li et al., 1996b; McGraw and Tzagoloff, 1983). Its splicing function was demonstrated directly by an experiment in which purified CBP2p (that was expressed in *E. coli*) stimulated the splicing of bI5 at low (5 mM) Mg^{2+}, an inefficient condition for self-splicing (Gampel et al., 1989). CBP2p binds specifically to bI5 RNA with a K_d of ~50 pM, but also has moderate affinity for the *Tetrahymena* LSU and phage T4 *nrdB* Group I introns (K_d values = 10-100 nM). Kinetic studies indicate that CBP2 promotes splicing by a mechanism termed tertiary-structure capture.

In this mechanism, CBP2 does not induce folding of the intron, but rather binds to and stabilizes the active structure after it is formed. Once associated with the core, the protein makes additional contacts with the P1 helix, thereby promoting the association of the 5′ splice site with the core (Weeks and Cech, 1995a, b, 1996).

Table 1.2 summarizes a comparison of protein-dependent transition states for ribonucleoprotein assembly between CYT-18 and CBP2 for the bI5 intron (Webb et al., 2001a). The yeast mt leucyl-tRNA synthetase (mt LeuRS), encoded by the nuclear gene *NAM2*, also functions in splicing group I introns. Genetic evidence indicates that protein (NAM2p) assists the splicing of the closely related introns bI4 and aI4α by acting in concert with an intron-encoded maturase (Dujardin et al., 1983; Labouesse et al., 1987; Herbert et al., 1988; Labouesse, 1990; Li et al., 1996a). *Nam2* was identified by dominant nuclear mutations that suppressed the splicing defects of a strain carrying a defective bI4 maturase (NAM = nuclear accommodation of mitochondria) (Dujardin et al., 1980; Groudinsky et al., 1981). Recent two-hybrid and three-hybrid assays showed that the NAM2p and the maturase can bind directly and independently to the bI4 intron, and that the intron bridges the interactions between the two proteins (Rho et al., 2000).

Bassi et al. (2002) recently showed that the splicing of the yeast mt bI3 group I intron *in vitro* requires both an intron-encoded protein, the bI3 maturase, and the nuclear-encoded protein, *Mrs1*. The bI3 maturase is a member of the LAGLIDADG family of DNA endonucleases, but appears to have lost DNA cleavage activity.

Table 1.2 Comparison of CBP2 and CYT-18 group I intron splicing cofactor proteins (modified from Webb et al., 2001a).

	CBP2	**CYT-18**
Organism	*Saccharomyces cerevisiae*[a]	*Neurospora crassa*[b]
Functional unit	Monomer [c]	Dimer [d]
Facilitates splicing of	bI5 group I intron, other group IA1 introns (weakly)[e]	Most group I introns with accessible P5-P4-P6 domain[b,f]
k_c (reaction of native RNP complex, 35°C	1-2 min^{-1} [c,i]	0.1-0.9 min^{-1} [d,i]
Binds at	P3-P8 and P7.1-P7.1a [g,h,i]	P5-P4-P6 [i,j,k]
Multi-step assembly	Yes [i,l,m]	Yes [d,i]
Stable protein binding limited by	Unimolecular RNA folding [i,l]	Diffusion limit [i,d,k]
Kinetic signature for assembly	Biomolecular assembly limited by unimolecular (first order) step [l]	$k_{off}/k_{on} \neq k_d$ [i,n]
Complex dissociation	Slow [l]	Slow [d,k]
$\Delta H^{\ddagger}_{overall}$ for assembly	Negligible (0.5 kcal.mol)[h]	Large (18 kcal/mol) [i,n]
Catalyze RNA folding	No [i,l]	Yes [i]
Mechanism of structure recognition	Structure capture[o]	Structure induction[o]

References are: **a.** McGraw and Tzagoloff, 1983; Bonitz et al., 1980; **b.** Akins and Lambowitz, 1987; Majumder et al., 1989; **c.** Weeks and Cech, 1995a; **d.** Saldanha et al., 1995; **e.** Gampel and Tzagoloff, 1987; Partono and Lewin, 1988; Gampel et al., 1989; Shaw and Lewin, 1997; **f.** Guo and Lambowitz, 1992; Mohr et al., 1994; **g.** Weeks and Cech, 1995b; **h.** Buchmueller et al., 2000; **i.** Webb et al, 2001a; **j.** Caprara et al., 1996a; **k.** Saldanha et al., 1996; **l.** Weeks and Cech, 1996; **m.** Webb and Weeks, 2001b; **n.** as demonstrated for assembly with the bI5 intron, these properties might differ for other introns; **o.** Lorsch, 2002.

Mrs1 is a divergent member of the RNase H-fold superfamily of dimeric DNA junction-resolving enzymes that also appears to have lost its nuclease activity. Both proteins bind independently to the bI3 intron RNA, and whereas the bI3 maturase binds as a monomer, *Mrs1* is a dimer in solution that assembles cooperatively as two dimers on the RNA. The active six-subunit complex has a molecular mass of 420 kDa, and binds the guanosine nucleophile with an affinity comparable to other group I introns. Thus, the bI3 ribonucleoprotein is the product of a process in which a once-catalycally active RNA now obligatorily requires two facilitating protein cofactors, both of which are compromised in their original functions. However, *in vitro* splicing of the bI3 intron still requires non-physiological temperature (36^{o}C), suggesting other splicing factors are needed for this process *in vivo* (Bassi et al., 2002).

1.2.3.3 RNA Chaperones for group I introns

Some splicing factors function as RNA chaperones, which are defined as proteins that facilitate RNA folding by preventing or reversing misfolded states, but do not have to remain associated with the folded RNA for catalysis to occur (reviewed in Herschlag, 1995; Lorsch, 2002). Two *E. coli* proteins, ribosomal protein S12 and StpA, stimulate the splicing of the phage T4 *td* intron *in vitro* via RNA chaperone activity (Coetzee et al., 1994; Zhang et al., 1995). They are relatively non-specific RNA-binding proteins and prefer to bind to unstructured regions, such as exons, and intron-ORF regions.

StpA was identified initially by its ability to suppress a mutation in the P7 region of the *td* intron, suggesting that it also stimulates splicing *in vivo* (Zhang et al., 1995). A recent study showed that StpA loosens the tertiary structure of the *td* intron, thereby helping the RNA to continue along its folding pathway (Waldsich et al., 2002).

Semrad and Schroeder (1998) proposed that the ribosome acts as an RNA chaperone to facilitate proper folding of the phage T4 *td* intron *in vivo* by resolving exon-intron interactions that interfere with intron foloding. Another RNA chaperone, *Escherichia coli* host factor I (Hfq), was reported recently. The Hfq was able to rescue an RNA 'folding trap' in a splicing defective T4 bacteriophage *td* gene *in vivo* like StpA (Moll et al., 2003)

In yeast mitochondria, a putative RNA helicase, MSS116, appears to function in promoting splicing of both group I and group II introns, perhaps by acting as an RNA chaperone (Seraphin et al., 1987, 1989; Staley and Guthrie, 1998). A recent study showed that a DEAD box protein (CYT-19), which presumably might have an RNA helicase activity, turned out to be another RNA chaperone. This protein functions in concert with CYT-18 to promote group I intron splicing *in vivo* and *in vitro*. However, in contrast to CYT-18, CYT-19 does not bind to the intron, and alleviates the kinetic traps by using the energy of ATP hydrolysis to disrupt nonnative structures, enabling iterative folding to the final native structure, which is stabilized by CYT-18 (Mohr et al., 2002).

1.3 *Chlamydomonas reinhardtii*

1.3.1 *Chlamydomonas reinhardtii* as a model system

Chlamydomonas reinhardtii (*C. reinhardtii*), sometimes called the "green yeast", is a unicellular green alga that has been used as a model system for studying a large set of biological processes, including splicing of group I and group II introns, photosynthesis, chloroplast and mitochondria biogenesis, flagellar assembly and motility, phototaxis, circadian rhythms, gametogenesis and mating, molecular interactions between nucleus and chloroplast, and cellular metabolism (reviewed in Harris, 1989, 1998, 2001; Goodenough, 1992; Rochaix, 1995, 2001, 2002; Herrin et al., 1998; Hippler et al., 1998; Grossman, 2000; Dent et al., 2001). There are a number of reasons why scientists use this organism as a model including, its ability to grow in the absence of light when provided with acetate as a carbon source; its short doubling time (<10 h); it is the only known eukaryote in which the nuclear, chloroplast, and mitochondrial genomes can all be transformed; it is an excellent genetic system because it is normally haploid and has a controlled sex cycle with the possibility of tetrad analysis; and a growing array of tools and techniques for molecular genetic studies (reviewed in de Vitry and Vallon, 1999; Dent et al., 2001; Harris, 2002; Rochaix, 2002). Table 1.3 compares four model photosynthetic organisms: *Synechocystis*, *Chlamydomonas*, *Arabidopsis*, and maize (reviewed in Dent et al., 2001).

Table 1.3 Comparison of model photosynthetic organisms (modified from Dent et al., 2001).

SynechocystisPCC6803	***Chlamydomonas***	***Arabidopsis***	**Maize**
Unicellular prokaryote	Unicellular eukaryote	Multicellular eukaryote	Multicellular eukaryote
Rapid growth rate (doubling time <10 h)	Rapid growth rate (doubling time <10 h) and life cycle	Complete life cycle takes 6 weeks	Complete life cycle takes at least 4 months
Oxygenic photosynthesis; heterotrophic growth possible	Oxygenic photosynthesis; heterotrophic growth possible with normal assembly of photosynthetic machinery in the dark	Oxygenic photosynthesis; heterotrophic growth possible at seedling stage	Oxygenic photosynthesis; heterotrophic growth possible at seedling stage
Transformation by homologous recombination	Transformation of nuclear, mitochondrial and chloroplast genomes; homologous recombination in chloroplast and mitochondrial genomes	Transformation of nuclear genome; transformation of chloroplast genome has low success rate	Transformation of nuclear genome only
Segregation of wild-type copies of the circular genome required for expression of loss-of-function mutant phenotypes	Haploid, therefore immediate expression of nuclear mutant phenotype	Diploid (possible ancestral tetraploid), therefore recessive mutations not expressed in heterozygotes	Ancestral tetraploid, therefore recessive mutations not expressed in heterozygotes
Replica plating for large-scale screening	Replica plating for large-scale screening	Replica plating for large-scale screening not possible	Field space required for large-scale screening
Tetrad analysis not applicable because no sexual reproduction	Tetrad analysis possible	Tetrad analysis possible, but only in specific genetic backgrounds (e.g. the *qrt* mutant)	Tetrad analysis not possible
Genome size = 3.5 Mbp	Genome size = 100–160 Mbp	Genome size = 120 Mbp	Genome size = 2500 Mbp
Genome sequenced	*DOE Joint Genome Institute (JGI) just finished draft at 9X coverage (~50 Mbp)	Genome sequenced	Limited sequence available, but sequencing projects have been initiated

* The web site is as follows: http://genome.jgipsf.org/chlre1/chlre1.home.html

1.3.2 Basic growth properties and life cycle of *Chlamydomonas reinhardtii*

C. reinhardtii cells are oval shaped, approximately 10 μm in length and 3 μm in width, with two flagella at their anterior end. The cells contain a single cup-shaped chloroplast occupying 40 % of the cell volume, and several mitochondria (Figure 1.5). There are two mating types, mt + and mt -, determined by two structurally distinct alleles of the mating type locus. The haploid vegetative cells multiply through mitotic divisions. However, upon nitrogen starvation, the vegetative cells differentiate into gametes, and gametes of opposite mating type fuse to give rise to a zygote. Under appropriate conditions, the zygote undergoes meiosis, and produces four or eight haploid daughter cells that can resume vegetative growth. It is also possible to recover stable diploids after the mating reaction, since as many as 1-5 % of mated gamete pairs may divide mitotically as vegetative diploids rather than forming meiotic zygotes (Figure 1.6). Such diploids can be selected deliberately through the use of complementing auxotrophic mutations, and confirmed by their mating type (-) and larger cell size. Vegetative diploids are useful for determining whether a mutation is recessive or dominant, and for testing whether mutations with the same phenotype belong to the same complementation group (reviewed in Harris, 1989; Rochaix, 2001).

In the presence of acetate, the photosynthetic function of *C. reinhardtii* cells is

dispensable. This feature has been exploited extensively for isolating and maintaining

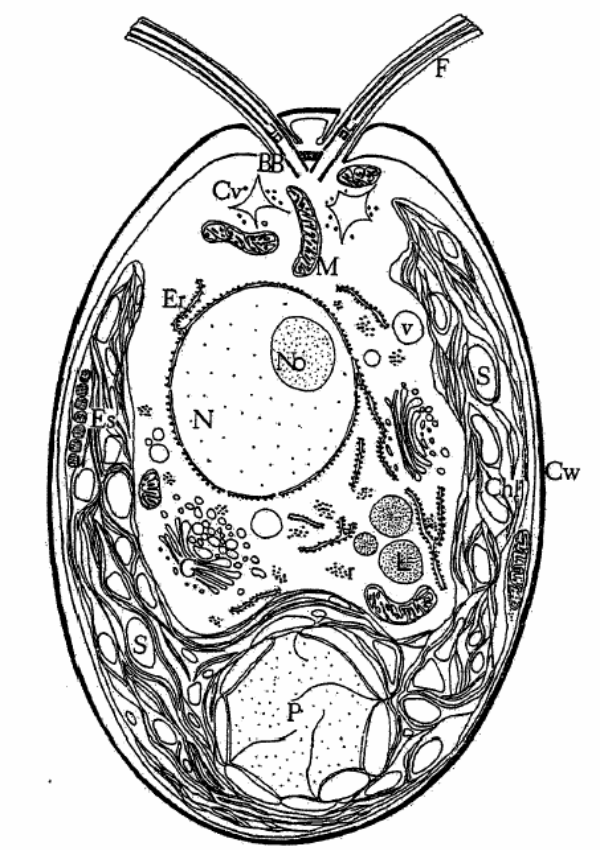


Figure 1.5 A semidiagrammatic representation of an interphase *Chlamydomonas* cell (adapted from Harper et al., 1999). Cell length, 10 μm; BB, basal bodies; Chl, chloroplast; Cv, contractile vacuole; Cw, cell wall; Er, endoplasmic reticulum; Es, eyespot; F, flagella; G, Golgi apparatus; L, lipid body; Mi, mitochondria; N, nucleus; No, nucleolus; P, pyrenoid; r, ribosomes; S, starch grain; v,vacuole.

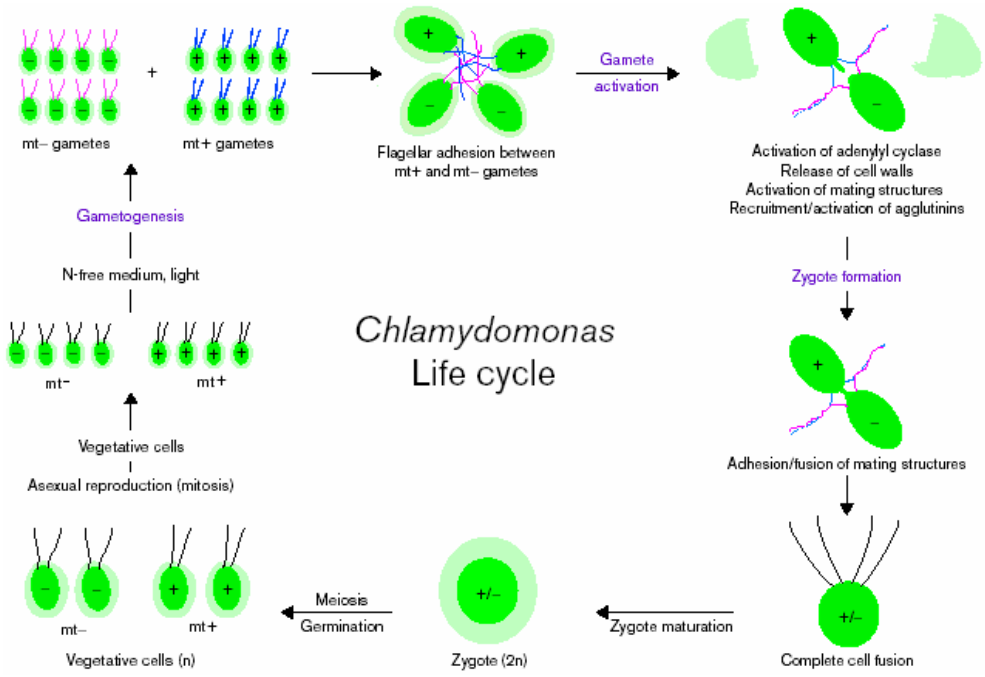


Figure 1.6 Sexual reproduction during the life cycle of *Chlamydomonas* (adapted from Pan and Snell, 2000). It comprises three central phases — gametogenesis, gamete activation and cell fusion/zygote formation. Gametogenesis is induced by loss of a nitrogen source and brings about the light-dependent differentiation of vegetative cells into gametes. When gametes of opposite mating types are mixed together, flagellar adhesion triggers gamete activation — a collection of cellular and molecular events that readies the interacting gametes for cell–cell fusion. Fusion itself generates signals that turn off gamete-specific functions and activate the zygote developmental pathway. The cycle begins again when the appropriate environmental conditions stimulate the dormant zygote to undergo germination to produce new, haploid mt+ and mt– vegetative cells.

mutants deficient in photosynthesis. The cells are commonly grown under one of the following three different conditions: in minimal medium with light and CO_2 as the sole carbon source (phototrophic or autographic growth), in acetate-containing medium plus light (mixotrophic growth), or without light (heterotrophic growth). In addition, the growth and division of the cells can be easily synchronized by light-dark cycles (12 h:12 h). In this condition, the cells divide during the mid to late dark period.

1.3.3 Three transformable genetic systems in *C. reinhardtii*

Like plants, *C. reinhardtii* contains three genetic systems, located in the nucleus, chloroplast, and mitochondria, respectively. Mutations in the genomes of these three compartments can be easily recognized by their unique segregation patterns during crosses. Whereas nuclear mutations segregate according to the classical Mendelian rules, chloroplast and mitochondrial mutations are normally transmitted uniparentally from the mt+ and mt- parents, respectively (Harris, 1989).

The complexity of the nuclear genome has been estimated at 1.2×10^8 bp (reviewed in Dent et al., 2001). Currently, the genetic map includes 148 loci distributed over 17 linkage groups. In addition, approximately 240 RFLP and short tagged sequence markers have been mapped to all linkage groups with an average

spacing of 4 to 5 centiMorgan or 4 to 5 $\times$ 10^5 bp (reviewed in Silflow, 1998). The nuclear transformation yield is sufficiently high enough, in at least some cases, to allow for genomic complementation of nuclear mutants (reviewed in Kindle, 1998b). In addition, because nuclear transformation of this alga occurs mainly through non-homologous recombination, the transforming DNA integrates randomly into the nuclear genome. Thus, transformation can be used to tag genes for subsequent cloning.

The chloroplast genome of *C. reinhardtii* has general features similar to higher plant chloroplast genomes with inverted repeat, large single-copy (LSC), and small single-copy (SSC) regions. Genes for the herbicide-binding protein of photosystem II (*psbA*), and for the ribosomal RNAs (*rrnL*) are located in the inverted repeat. The sequence of the chloroplast genome was recently completed, although most of the genes were already known from previous work (Harris, 1989; Maul et al., 2002). The genome consists of mainly circular molecules, 203,395 bp in length, and contains 34 genes involved in photosynthesis, 31 genes involved in chloroplast transcription and translation, one protease gene, 29 tRNA genes, and nine genes of unknown function (Maul et al., 2002).

The inheritance pattern of chloroplast DNA in *C. reinhardtii* is uniparental, and almost exclusively from the mating type (+) parent. The best transformation device for the chloroplast genome of *C. reinhardtii* is a biolistic gun (Boynton and

Gillham, 1993), whereas common nuclear transformation protocols for *C. reinhardtii* include glass bead and electroporation methods (Kindle, 1990, 1998a, 1998b; Shimogawara et al., 1998). In contrast to nuclear transformation, chloroplast transformation occurs exclusively through homologous recombination, and because foreign selectable marker genes are available, e.g. *aadA* (Goldschmidt-Clermont, 1991) and *aphA-6* (Bateman and Purton, 2000), that confer resistance to specific antibiotics, it is possible to inactivate specific genes or to perform site-directed mutagenesis on any plastid gene of interest that is not lethal. This reverse genetics approach has been rather successful, especially for elucidating the function of conserved open reading frames, also called *ycf*s, present in the plastid genomes of several plants, algae, and cyanobacteria (reviewed in Rochaix, 1997).

1.3.4 Molecular cross talk between the nucleus and the chloroplast

An area in which *C. reinhardtii* is especially powerful as a model system is the biosynthesis of the photosynthetic apparatus that occurs through the concerted interactions between the chloroplast and nuclear genomes. An extensive analysis of nuclear mutants deficient in photosynthesis has revealed that besides the mutations that directly affect the genes of the components of the photosynthetic apparatus, the vast majority of the mutations are in genes encoding factors that are required for several chloroplast post-transcriptional steps, including RNA stability, RNA processing, translation, and the assembly of photosynthetic complexes. The

number of these genes is surprisingly high, and their products act in a gene-specific manner. Several of these genes have recently been isolated through genomic complementation of the mutants with genomic cosmid libraries, or by gene tagging (Tam and Lefebvre, 1993; Purton and Rochaix, 1994). The phenotypes of some of these photosynthetic mutants resemble those of mutants of *Arabidopsis* and maize (*Zea mays*), indicating that a similar complex nuclear-chloroplast network may exist in higher plants (reviewed in Barkan and Goldschmidt-Clermont, 2000; Rochaix, 2001). The sequencing of the Arabidopsis nuclear genome has indeed revealed that as many as 3,000 nuclear genes encode chloroplast proteins (reviewed in Abdallah et al., 2000). Many of these genes may be involved in chloroplast gene expression.

1.4 Group I introns in the chloroplast genome of *Chlamydomonas*

Among chloroplast genomes, group I introns are found in: the *trnL* gene of higher plants (Steinmetz et al., 1982); in rRNA and photosynthetic genes of a number of *Chlamydomonas* species (reviewed in Herrin et al., 1998); in *chlL*, *rrnl* and *trnL* genes of *Chlorella* (Kuhsel et al., 1990; Kapoor et al., 1996; Kapoor et al., 1997; Wakasugi et al., 1997); and also in *rbcL* genes in other Volvocales (Nozaki et al., 1998). Herrin et al. (1990) first reported a self-splicing group I intron from a chloroplast gene: *Cr.LSU*, in the chloroplast 23S *rrn* gene of *C. reinhardtii*. This result was confirmed by Dürrenberger and Rochaix (1991). Subsequently, it was shown that the four introns in the *psbA* gene of *C. reinhardtii*, *Cr.psbA1 – Cr.psbA4*,

five introns in the *rrnL* gene of *C. eugametos*, *Ce.LSU1*-*Ce.LSU4* and *Ce.LSU6*, two introns in the *psbC* gene of *C. eugametos*, an intron in the *psaB* gene of *C. moewusii*, and the introns in the *chlL* and *rrnL* genes of *Chlorella vulgaris* can also self-splice *in vitro* (Herrin et al., 1991; Turmel et al., 1991; Bao, 1993; Turmel et al., 1993; Côté and Turmel, 1995; Kapoor et al., 1996; Deshpande et al., 1997; Kapoor et al., 1997). Table 1.4 summarizes group I introns in the chloroplast genes of *Chlamydomonas* species (reviewed in Herrin et al., 1998).

1.4.1 The *Cr.LSU* intron

The *LSU* rRNA gene was the first intron-containing gene identified in *Chlamydomonas* (Rochaix and Maloë, 1978; Allet and Rochaix, 1979). The *Cr.LSU* intron is 888 bp, and located toward the 3′ end of the LSU gene. It can be folded into a secondary structure that belongs to subgroup IA3 (Michel and Westhof, 1990). A 489 bp free-standing ORF is located in the loop of P6 and encodes I-*CreI*, a homing endonuclease (Dürrenberger and Rochaix, 1991; Thompson et al., 1992). I-*CreI* is not an essential maturase (Thompson and Herrin, 1991) even though it contains the semi-conserved dodecapeptide motif, LAGLI-DADG, found in intron-encoded maturases of yeast. Efficient self-splicing of this intron *in vitro* requires non-physiological conditions, i.e., a high concentration of Mg^{2+} (~13-25 mM) and elevated temperature (35-50 ^{o}C) (Thompson and Herrin, 1991).

Nothing is known of group I splicing factors outside of a few fungi. In

addition, biochemical approaches to isolate group I splicing factors for *Cr.LSU*

Table 1.4 Group I introns in the chloroplast genes of *Chlamydomonas* spp (adapted from Herrin, et al., 1998).

Intron[a]	**Species**	**Gene**	**Subclass**[b]	**ORF**[c]	**Self-splicing**	**Ref**[d]
CeLSU1	*C. eugametos*	*rrnL*	IA3	no	yes	1,2
CeLSU2	*C. eugametos*	*rrnL*	IA1	no	yes	1,2
CeLSU3	*C. eugametos*	*rrnL*	IA1	no	yes	1,2
CeLSU4	*C. eugametos*	*rrnL*	IA1	no	yes	1,2
CeLSU5	*C. eugametos*	*rrnL*	IB4	I-*CeuI*	no	1-3
CeLSU6	*C. eugametos*	*rrnL*	IA3	yes	yes	1,2
CepsaB1	*C. eugametos*	*psaB*	IA1	no	?	4
CepsbC1	*C. eugametos*	*psbA*	IA2	no	yes	2,4
CepsbC2	*C. eugametos*	*psbC*	IA1	no	yes	2,4
ChLSU1	*C. humicola*	*psbC*	IB4	I-*ChuI*	?	5
CmpsaB1	*C. moewusii*	*psaB*	IA1	no	yes	2,4
CmpsbA1	*C. moewusii*	*psbA*	IA1	yes	?	6
CmpsbA2	*C. moewusii*	*psbA*	IB4	yes	?	6
CmpsbC1	*C. moewusii*	*psbC*	IA2	no	?	4
CmpsbC2	*C. moewusii*	*psbC*	IA1	no	?	4
CmSSU	*C. moewusii*	*rrnS*	IA3	no	?	7
CpLSU2	*C. pallidostigmatica*	*rrnL*	IB4	I-*CpaI*	?	8
CpSSU1	*C. pallidostigmatica*	*rrnS*	IA3	yes	?	9
CpSSU2	*C. pallidostigmatica*	*rrnS*	IA3	I-*CpaII*	?	9
CrLSU	*C. reinhardtii*	*rrnL*	IA3	I-*CpaI*	yes	10-13
CrpsbA1	*C. reinhardtii*	*psbA*	IA1	no	yes	14,15
CrpsbA2	*C. reinhardtii*	*psbA*	IA3	yes	yes	14-17
CrpsbA3	*C. reinhardii*	*psbA*	IA2	yes	yes	14,15
CrpsbA4	*C. reinhardtii*	*psbA*	IA1	I-*CreII*	yes	15,17

[a]Each intron was specified by the genus and species initials, followed by the gene name, and, where appropriate, the number of the intron (Michel and Westhof, 1990).
[b]Classification is based on the presence or absence of certain peripheral domains (Michel and Westhof, 1990).
[c]Some open reading frames (ORF) encode site-specific DNA endonuclease; for example, I-*CreI* is the first intron-encoded endonuclease from *C. reinhardtii*.
[d] [1]- Turmel et al., 1991; [2]- Côté and Turmel, 1995; [3]- Marshall et al., 1994; [4]- Turmel et al., 1993; [5]- Côté et al., 1993; [6]- Turmel et al., 1989; [7]- Durocher et al., 1989; [8]- Turmel et al., 1995a; [9]- Turmel et al., 1995b; [10]- Rochaix et al., 1985; [11]- Herrin et al., 1990; [12]- Dürrenberger and Rochaix, 1991; [13]- Thompson et al., 1991; [14]- Herrin et al., 1991; [15]-

Holloway et al., 1999; [16]- Bao and Herrin, 1993; [17]- Kim, 2000.
and *Cr.psbA2* were not successful (Bao, Y. and Herrin, D.L., personal communication), attributable in part to nucleases. However, a recent genetic approach with *C. reinhardtii* has yielded at least one nuclear gene that promotes splicing of the *Cr.LSU* intron. A splicing-deficient mutant was generated by transforming wild-type cells with *Cr. LSU* variants containing point mutations in the intron core (Holloway and Herrin, 1998). Heteroplasmic transformants (i.e., containing two different forms of the chloroplast genome) were obtained in most cases, except for P4 helix mutants; these strains grew slowly, were light sensitive, and had an RNA profile indicative of inefficient splicing (Holloway and Herrin, 1998). Li et al. (2002) used the *Cr.LSU* P4 mutants to isolate nuclear suppressors of the splicing-deficient mutant. The 7151 suppressor was characterized genetically and shown to contain a single dominant gene.

1.4.2 The *Cr.psbA* introns

The *psbA* gene encodes a 32-36 kDa polypeptide, known as the D1 protein of photosystem II (PSII) (Erickson et al., 1984). It is expressed at very high levels in the light (Herrin et al., 1986). It is also highly conserved. However, there are two features unique to the *C. reinhardtii psbA* gene: (1) it is located in the inverted repeat region of the chloroplast genome, and (2) it contains 4 large group I introns *Cr.psbA1*-*Cr.psbA4* (Erickson et al., 1984; Holloway et al., 1999). Figure 1.7 shows a map of the *psbA* gene of *C. reinhardtii*.

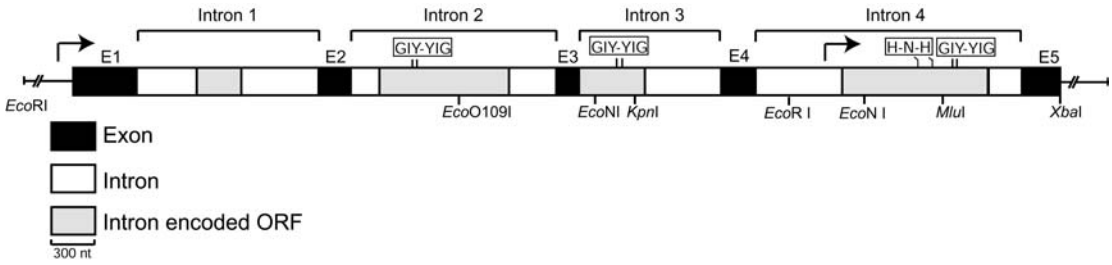


Figure 1.7 Map of the *C. reinhardtii psbA* gene.

The arrows indicate the direction of transcription, and the location of promoters. GIY-YIG motifs are located at nt 426-434 and nt 465-473 of *Cr.psbA2*, nt 239-247 and nt 278-286 of *Cr.psbA3*, and nt 1253-1261 and nt 1292-1300 of *Cr.psbA4*, and a H-N-H motif is located at nt 1025-1031 of *Cr.psbA4*.

The *Cr.psbA* introns are classified into three different structural subgroups: *Cr.psbA1* and *Cr.psbA4* are in subgroup IA1, and are closely related; *Cr.psbA2* is in subgroup IA3; and *Cr.psbA3* is in subgroup IA2. Interestingly, the intron to closest *Cr.psbA3*, at least structurally, is the *SunY* intron of phage T4. Table 1-5 summarizes some structural characteristics of the four *psbA* introns of *C. reinhardtii* (adapted from Holloway et al, 1999).

Holloway et al. (1999) found that the *Cr.psbA4* intron is also closely related to the first intron of the *C. moewusii psbA* gene (*Cm.psbA1*). First, *Cr.psbA4* and *Cm.psbA1* are located in the exact same position within the *psbA* gene, between amino acids His_{252} and Gly_{253}. Second, introns *Cr.psbA4* and *Cm.psbA1* show 75 % nucleotide identity in the intron core structure and contain conserved tertiary motifs, including P11. Third, both introns contain an ORF which is located in the same position in the intron, and with 58 % amino acid identity; both ORFs also contain the same two conserved motifs, H-N-H and GIY-YIG. These results suggest that this intron was present in the common ancestor of *C. reinhardtii* and *C. eugametos*, and therefore, is a fairly ancient intron (Holloway et al., 1999).

The large ORFs in introns *Cr.psbA2*-*Cr.psbA4* contain a variant of the GIY-YIG motif found in phage homing endonucleases, and it has been shown that intron *Cr.psbA4* can home (Odom et al., 2001). In order to test for intron mobility, constructs containing intron *Cr.psbA4* were introduced into a *C. reinhardtii* strain, IL, which contained an intronless *psbA* gene (Johanningmeier and Heiss, 1993). It

Table 1.5 Structural characteristics of the four *psbA* introns of *C. reinhardtii* (adapted from Holloway et al., 1999).

Intron	Insertion site[a]	Size (nt)	% (A+T)	Subgroup	ORF location	ORF size[b]	pI	Amino -acid motif
1	60	1249	62	IA1	L9.0	102	9.3	-
2	128-129	1412	67	IA3	L3.2-P4	298	9.2	GIY-YIG
3	175-176	1096	61	IA2	P1-L6a	160	10.6	GIY-YIG
4	253-254	1822	66	IA1	L5b	332	10.0	H-N-H[c]

[a] Amino-acid number

[b] Amino acids

[c] The ORF also contains a putative GIY-YIG motif.

was shown that *Cr.psbA4* integrated correctly into the intronless *psbA* gene, and that integration was dependent on its ORF (Odom et al., 2001).

All four of the *psbA* introns are self-splicing *in vitro* (Herrin et al., 1991). Table 1.6 shows the optimal conditions for self-splicing by the *Cr.psbA2*, *Cr.psbA4*, and *Cr.LSU* introns (reviewed in Herrin et al., 1998; this study). It has also been shown by this laboratory that the ORFs of *Cr.psbA3* and *Cr.psbA4* are not maturases. This was shown by replacing the wild-type introns with mutants lacking most of the ORF; there was no effect of these changes on splicing of these introns *in vivo* (O.W. Odom, unpublished results). The question of whether the *Cr.psbA2* intron ORF is a maturase has been addressed in this thesis (Chapter 5).

1.5 Light regulation of *psbA* splicing and translation

Numerous studies have revealed the dynamics of *psbA* gene expression that occur in response to light; the early work was thoroughly reviewed in Erickson and Rochaix (1992). Light stimulation of *psbA* gene expression occurs at the transcriptional (e.g., Deshpande et al., 1997; Klein and Mullet, 1990; Tiller and Link, 1993) and several post-transcriptional levels, including RNA splicing (Deshpande et al., 1997), and translation (e.g., Klein et al., 1988; Staus and Maliga, 1994; Danon and Mayfield, 1994b; Zhang et al., 2000).

Table 1.6 The optimal conditions for self-splicing *in vitro* of three chloroplast group I introns in *C. reinhardtii*.

	[a]***Cr.LSU***	[b]***Cr.psbA2***	[c]***Cr.psbA4***
GTP (μM)	~100	~100	~100
Temperature (°C)	45-47	~45	~55
Mg^{2+}(mM)	~25	~25	~10

a. Thompson and Herrin, 1991; b. Bao, 1993; c. This study; for the monovalent ion, 100 mM KOAc was used instead of 100 mM $(NH_4)_2SO_4$.

Light promotes splicing of all four of the *psbA* introns by 6-10 fold, but not the *Cr.LSU* intron. This response to light is mediated by photosynthetic electron transport, but not by ATP synthesis (Deshpande et al., 1997). The developmental significance of light-promoted *psbA* splicing has not been clear, however, because *psbA* mRNA is highly abundant in *Chlamydomonas* (Herrin et al., 1986; Jensen et al., 1986; Yohn et al., 1998b), and some chloroplast mRNAs, including *psbA*, may be in considerable excess of what is needed to sustain translation rates (Eberhard et al., 2002). This evidence, as it concerns *psbA*, comes from reports that looked at the fraction of *psbA* mRNA found on polysomes (Klein et al., 1988; Yohn et al., 1998b), and in a recent drug-based study, where treatment of *Chlamydomonas* with a chloroplast transcription inhibitor for 6 h reduced the levels of *psbA* mRNA ~40 % while actually increasing D1 synthesis in phototrophic conditions (Eberhard et al., 2002). A major goal of this thesis was to assess the impact that a reduced splicing efficiency of one of the light-regulated *psbA* introns has on phototrophic growth.

The light regulation of *psbA* translation has been well studied and may serve as a model for light-promoted splicing. Translation appears to be activated by the interaction of four nuclear-encoded proteins; RB38, RB47, RB55, and RB60 (Figure 1.8). RB47, which is a member of the eukaryotic poly(A)-binding protein (PABP) family, interacts directly and specifically with the 5′ UTR of *psbA* and is required for the initiation of translation (Yohn et al., 1996; Yohn et al., 1998a; Yohn et al., 1998b). RB60 regulates the assembly and disassembly of the RNA-binding protein

complex. It is encoded in the nuclear genome and resembles an endoplasmic reticulum-located disulfide isomerase (Trebitsh, 2000). RB60 also partitions between the stroma and thylakoid fractions, where it may serve to localize translation (Trebitsh, 2000).

In the dark, the RNA-protein complex dissociates upon phosphorylation of RB60 by an ADP-dependent kinase (Figure 1.8). Assembly of the complex on the 5′ UTR of *psbA* in the light is promoted by the reduction of vicinal disulfide groups in RB60 and its dephosphorylation (Danon and Mayfield, 1994b; Trebitsh, 2000). Signals are transmitted to RB60 through components of photosystems I and II. One signal is initiated by the reduction of the plastoquinone pool, which is known to regulate the phosphorylation of photosystem II proteins and may regulate the phosphorylation of RB60 through a plastoquinone-activated kinase (Trebitsh and Danon, 2001). The second signal is a reductive signal from photosystem I, which is transduced by the ferredoxin–thioredoxin system (Trebitsh and Danon, 2001).

1.6 D1 protein

The D1 protein, which is encoded by the *psbA* gene, is the most highly conserved component of the photosystem II reaction center. D1, together with D2, were described more than 25 years ago by Chua and Gillham (1977) as two diffuse

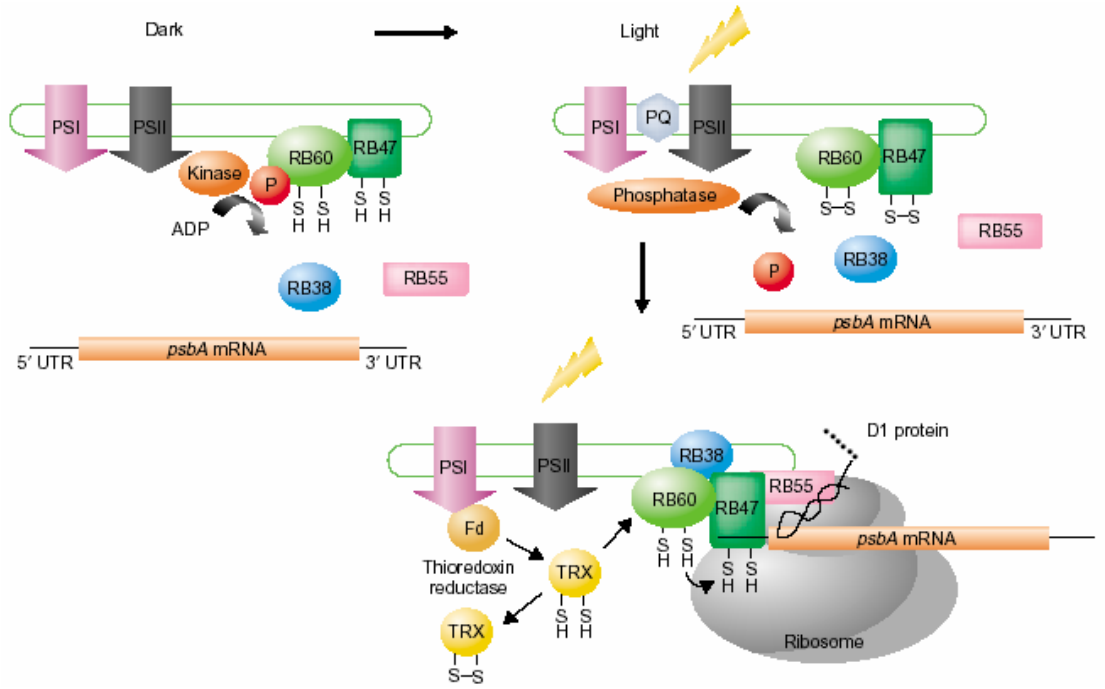


Figure 1.8 A model of molecular interactions in the light-regulated translation of *psbA* mRNA in chloroplasts (adapted from Fedoroff et al., 2002). See the text for an explanation. Fd, ferredoxin; PQ, plastoquinone; PSI, PSII, photosystem I and II; TRX, thioredoxin.

bands of 32 to 34 kDa from *Chlamydomonas reinhardtii* thylakoid membrane proteins labeled in the presence of cycloheximide. D1 was subsequently shown to be an integral part of the PSII reaction center and to bind plastoquinone and the atrazine herbicides. D1 is also homologous to the L- subunit of the purple bacterial reaction center (reviewed in Ruffle and Sayre, 1998; Ke, 2001; Yamamoto, 2001), and is believed to span the thylakoid membrane five times, with N-terminal and C-terminal tails being exposed to the stroma and and thylakoid lumen, respectively. It also binds photosynthetic pigments, and possibly a cluster of four manganese atoms, which appear to be ligated at the lumenal side. Tyrosine160, in the third α-helix of D1, is known to be involved in photosynthetic electron transport; it accepts two electrons from a cluster of four manganeses, and gives them to pheophytin.

D1 is synthesized on thylakoid membrane-bound ribosomes (Herrin and Michaels, 1985; Herrin et al., 1986; Klein et al., 1988) as a precursor with a C-terminal extension of 9-16 amino acid residues (Reisfeld et al, 1982; Marder, 1984) that must be cleaved after insertion of D1 into the membrane. The cleavage is accomplished by a lumenal serine protease called CtpA (Reisfeld et al., 1981; Fujita et al., 1995).

Another key characteristic of D1 is that it turns over rapidly in the light because it becomes damaged by singlet oxygen (Figure 1.9). Thus, the accelerated production of D1 during the daytime serves two functions: (1) it helps coordinate photosystem II production with other concurrent biogenetic processes (Lee and

Herrin, 2002, and references therein), and (2) it serves to replace damaged D1 (Figure 1.10). At very high, photoinhibitory light fluxes, the rate of D1 damage exceeds the rate of removal of damaged D1, and the result is loss of photosynthetic capacity (Schuster et al., 1988).

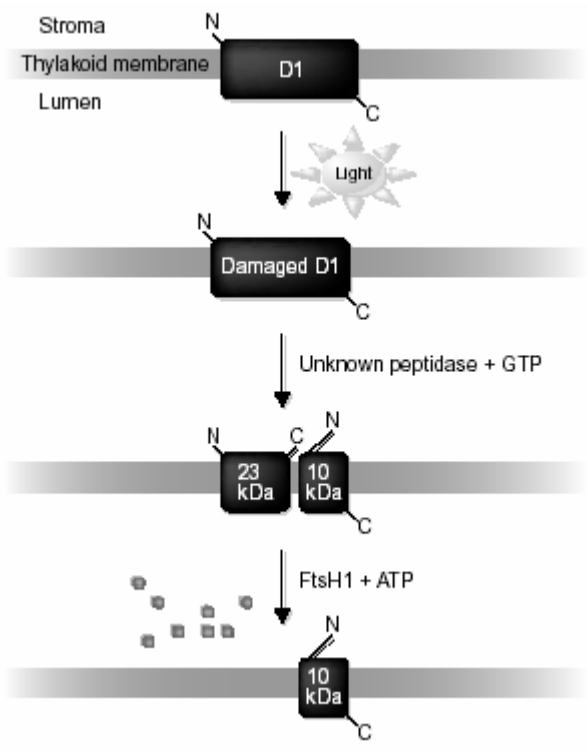


Figure 1.9 Model for degradation of the D1 protein after photooxidation (adapted from Estelle, 2001). After photodamage, the protein is first cleaved into 10- and 23-kDa fragments by an unknown GTP-dependent protease; N and C represent the amino and carboxyl termini of the protein, respectively. According to the model proposed by Lindahl et al. (2000), the new carboxyl terminus generated by this event serves as a recognition motif for the protease, FtsH1, resulting in degradation of the fragment.

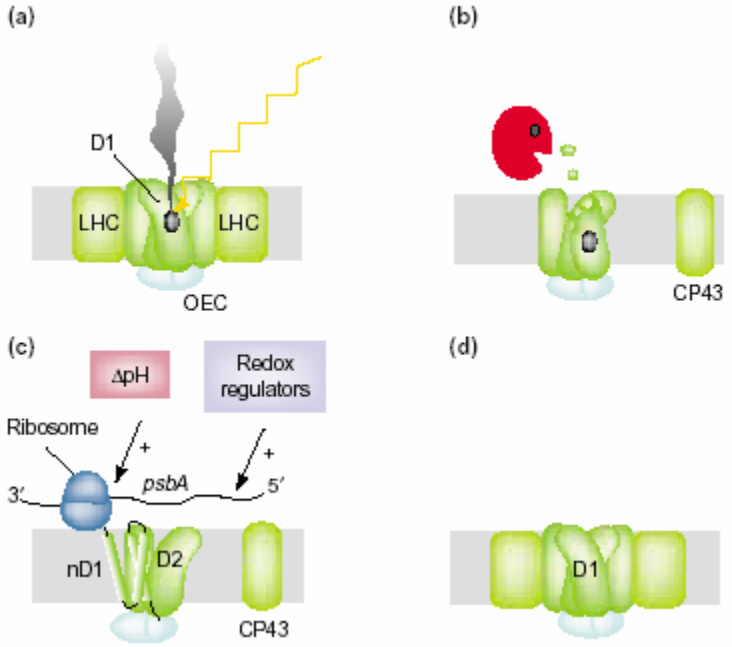


Figure 1.10 The photosystem II damage–repair cycle (adapted from Zerges, 2002). The thylakoid lipid bilayer is shown in gray.The photosystem II reaction center is shown in green with the relevant subunits (D1, D2, CP43) and associated light-harvesting (LHC) and oxygen evolving complexes (OEC). (a) The D1 protein subunit is damaged by photochemical reactions. The yellow zig-zag arrow represents a photon of light. (b) Damaged D1 is degraded by proteolysis. (c) Nascent D1 (nD1) is assembled into the photosystem II subcomplex during its translation by a chloroplast ribosome. (d) The repaired photosystem II is functional again.

1.7 Goals of the research described in this dissertation

One major goal of my research was to study the significance of light-promoted splicing of *psbA* introns by mutagenizing one of the introns and examining the effects of the mutations on splicing *in vitro* and *in vivo*. My main question was: why is *psbA* splicing promoted by light, given that the *psbA* mRNA is abundant and translationally regulated? Also, there have been no comparisons of the effects of mutations on splicing of a group I intron *in vitro* and *in vivo* in a eukaryote.

Li et al. (2002) used P4 mutants of the *Cr.LSU* intron to isolate three nuclear suppressors (7151, 71N1, and 7120) of this splicing-deficient intron. I wanted to determine if these suppressor mutations occur in genes that might encode general or specific splicing factors. Thus, another goal was to determine if these three suppressors would also suppress a splicing-deficient *Cr.psbA4* intron.

Also, the 71.20 and 71.N1 suppressors were incompletely characterized genetically, and thus I wanted to determine if the 71.20 suppressor carries a single-gene mutation, and if these suppressors were dominant or recessive. This information would be necessary for developing a cloning strategy for these genes.

Finally, I have asked the question of whether a selectable marker can be expressed from within one of the group I introns at sufficient levels to provide a phenotype. The rational for this was that this marker would be used to select mutants that overaccumulate the unspliced intron or the excised intron or both, since they are both potential mRNAs for the intron- encoded marker. The *Cr.psbA2* intron was

chosen for this experiment for two main reasons: (1) a starting construct, created by H. Han, was available that had the *Cr.psbA2* ORF replaced by the *aadA* gene, and (2) it was not known if the *Cr.psbA2* ORF was a maturase, and therefore replacing the wild-type intron with the *aadA*-containing version would answer that question.

Chapter 2 Materials and Methods

2.1 Strains and culture conditions

The *C. reinhardtii* strains used in this study are described in Table 2.1. The WT strain, 2137 (mt+) (CC-1021), and the mutants *cw15 arg2* (mt-) (CC-424), *cw10* (mt-) (CC-849) and *nit4* (mt-) (CC-2844) were obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC). The three suppressors strains (7120, 7151, and 71N1) that restore a splicing-deficient *Cr.LSU* intron were obtained from F. Li (Li et al., 2002). The intronless-*psbA* strain, IL (Johanningmeier and Heiss, 1993), was obtained from Udo Johanningmeier (Martin Luther University, Halle-Wittenberg, Germany).

The cultures were grown either mixotrophically (light plus acetate) in Tris/acetate/phosphate (TAP) medium (Harris, 1989), or autotrophically in TAP-minimal medium (the pH was adjusted to 6.8 with HCl instead of HOAc) at 23°C; liquid cultures were shaken at 125-150 rpm. Spectinomycin (100 μg/mL) for selection and growth of the spectinomycin-resistant transformants, erythromycin (200 μg/mL) for selection and growth of the erythromycin-resistant transformants, and/or 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; 3 μM) was added to the media for selection and growth of drug-resistant transformants. The light intensity was 80-100 μmoles/m^2/sec for bright light, 40-60 μmoles/m^2/sec for medium light, and 1-2 μmoles/m^2/sec for dim light. Cultures used for molecular analyses were always in

Table 2.1 Strains used in this study (Nuclear genotypes are followed by the chloroplast genotype in brackets).

Strain	Genotype	Phenotype	Reference
2137 (CC-1021)	*nit1*, *nit2*, mt+ [wt]	cannot grow on nitrate as sole N source	Harris, 1989
137c (CC-124)	*nit1*, *nit2*, mt- [wt]	cannot grow on nitrate as sole N source	Harris, 1989
IL	mt+ [Δ*Cr.psbA1-4*]	tends to form aggregates in liquid media	Johanningmeier, 1993
CC-424	*cw15*, *arg2*, mt-[wt]	cell wall-deficient (cells lyse in 1 % NP-40; forms flat, liquid colonies); requires arginine	Harris, 1989
CC-849	*cw10,* mt- [wt]	cell wall-deficient	Harris, 1989
Cr.LSU P4A	mt+ [*Cr.LSU* P4A*, spr-u-1-6-2, er-u-11*]	resistant to 100 μg/ml spectinomycin and 200 μg/ml erythromycin; slow growth in bright light and cold temperature	Li et al., 2002; this study
[a]*cw15 Cr.LSU* P4A	*cw15*, mt+ [*Cr.LSU* P4A*, spr-u-1-6-2, er-u-11*]	cell wall-deficient ; resistant to 100 μg/ml spectinomycin and 200 μg/ml erythromycin; slow growth in bright light	This study
7120	*nit1*, *nit2*, mt+ [*Cr.LSU* P4A*, spr-u-1-6-2, er-u-11*]	cannot grow on nitrate as sole N source ; resistant to 100 μg/ml spectinomycin and 200 μg/ml erythromycin	Li et al., 2002
[b]7120_2G	*nit1*, *nit2*, mt+ [*Cr.LSU* P4A, *spr-u-1-6-2, er-u-11*]	cannot grow on nitrate as sole N source ; resistant to 100 μg/ml spectinomycin and 200 μg/ml erythromycin	This study
7151	*css1, nit1*, *nit2*, mt+ [*Cr.LSU* P4A*, spr-u-1-6-2, er-u-11*]	cannot grow on nitrate as sole N source ; resistant to 100 μg/ml spectinomycin and 200 μg/ml erythromycin	Li et al., 2002
71N1	*nit1*, *nit2*, mt+ [*Cr.LSU* P4A, *spr-u-1-6-2, er-u-11*]	cannot grow on nitrate as sole N source ; resistant to 100 μg/ml spectinomycin and 200 μg/ml erythromycin	Li et al., 2002
nit4 (CC-2844)	*nit4,* mt- [wt]	cannot grow on nitrate as sole N source	Harris, 1989

[a]This strain was obtained from a cross of the *Cr.LSU* P4A (mt+) with CC424 (mt -).
[b]This strain was obtained from a cross of the 7120 (mt+) with WT137c (mt -).
The nomenclature of the strains starting with "CC-" is that of the *Chlamydomonas* Genetic Center (Duke University, Durham, NC).

the exponential phase of growth (0.3-3 $\times$ 10^6 cells/mL).

2.2 Measurements of growth rates in liquid and solid media

2.2.1 Phototrophic growth rate of *C. reinhardtii* in liquid minimal medium

About 6 mL of a stationary phase culture (~4 $\times$ 10^6 cells/mL), which had been grown in TAP medium in dim light (~2 μmole/m^2/sec), was diluted to 1 $\times$ 10^5 cells/mL in 250 mL of fresh TAP medium. The cultures were gently bubbled with 5 % CO_2 with shaking (125 rpm), or just shaken (125 rpm) in air; and in medium light intensity was used. Aliquots were removed at intervals, and cell number was determined with a hemacytometer after killing the cells with iodine (Harris, 1989).

2.2.2 Mixotrophic growth rate of *C. reinhardtii* on acetate-containing solid media using the spot test

For the growth rate experiments on solid media, ~1 $\times$ 10^4 cells in 100 μl of TAP were pipetted onto TAP plates (2 % agar), the liquid was allowed to absorb, and then the plates were either kept in bright light or dim light at 23°C. Growth was estimated by visual inspection.

2.2.3 Growth rate of *Escherichia coli* DH5α in liquid media

The growth rate experiments with *E. coli* were performed as follows. 50 μl of

stationary phase culture of *E. coli*. was inoculated into 5 mL of LB broth containing 100 μg/mL ampicillin, and with different concentrations of spectinomycin and/or streptomycin, respectively. The cultures were then incubated at 37°C with shaking (225 rpm), and the absorbance at 550 nm was measured at 1h intervals.

2.3 Recombinant plasmids

2.3.1 *Cr.psbA4* constructs

For mutating *Cr.psbA4*, the starting plasmid was pBX4.Δ which contains a modified *BstE*II-*Xba*I fragment of the *psbA* gene in pBluescript SK (Odom et al., 2001). The insert includes 50 bp of exon 4, wild type *Cr.psbA4* except for a deletion (544 bp) of most of the ORF from the non-conserved loop of P5b (Figure 1.3), and 263 bp of exon 5 (Figure 2.1). Exon 5 also contained the DCMU4 mutation (a T→G change of nt 34 of the exon), which provides resistance to DCMU (Erickson et al., 1984). The nucleotide substitutions were made using the Gene Editor *in vitro* site-directed mutagenesis system (Promega Inc.) with the following mutagenic deoxyoligonucleotides (the substitutions are underlined):

255, 5'-GCTTCGCTGCA<u>T</u>GTTGCCATATT-3' (P4'-2);

256; 5'-CCCATCCCT<u>T</u>GCTTCGCTGC-3' (P6a-2);

257, 5'-CTCAAGCTCTA<u>C</u>TCTCTGAACGT-3' (P7-4);

258, 5'-CTATTATTGTTGTGACTCAAGCTCT-3' (P11'-2);

259, 5'-TTTTAGATGCCTGGCGCTAGTGA-3' (P3'-3);

292, 5'-GGGACTCAAGCTCTACACTCTGAACGTTCTAG-3' (P7-4,5);

294, 5'-ATCCCTAGCTTCCCTGCAGGTTGCCA-3' (P6-2); and

299, 5'-TCCCTAGCTTCGCTGGTGGTTGCCATATTATT-3' (P4'-3,4).

The mutations were confirmed by sequencing the plasmid DNAs with oligodeoxynucleotides 99 and 100 (Odom et al., 2001). In some cases (i.e., the P7-4,5; P4'-3,4; P6-2; P6a-2; and P4'-2 mutant plasmids), the upstream 2.1-kb *EcoO*109I-*Bst*EII fragment of *psbA* was added to the plasmid insert (Figure 2.1). This was done by digesting the pBX4.Δ plasmids with *Eco*RI and *Dra*II, which is an isoschizomer of *EcoO*109I and cuts in the adjacent vector sequence, purifying the large fragment by gel electrophoresis, and ligating in the *Dra*II (*EcoO*109I)-*Eco*RI fragment of pER3. Plasmid pER3 (obtained from O.W. Odom) was derived from pEC23 (Herrin and Michaels, 1985), and contains the *EcoO*109I-*Eco*RI fragment of the *psbA* gene (Figure 2.1). This longer upstream sequence was added because it increased the frequency of transformants that had integrated the entire intron. This series of plasmids were called pEX4.Δ (Figure 2.1).

2.3.2 *Cr.psbA2* constructs

The atpX-i2S (sense) and atpX-i2A (antisense) plasmids were created by N. Deshpande (1996). The *Xba*I fragment of the *psbA* gene (Odom et al., 2001) from

plasmid pGEMR14.2 (Herrin et al., 1991) was cloned into the *Xba*I-digested atpX plasmid in both directions; atpX contains the promoter and 5′ untranslated region (UTR) of the *atpA* gene to drive expression in the chloroplast (Goldscmidt-Clermont, 1991).

The pGEMI2AAD-ORF plasmid was created by H. Han, and has not been described previously. The insert contains 75 bp of *psbA* exon 2, 759 bp of *Cr.psbA2* with the 553-bp ORF replaced by the 794-bp bacterial *aadA* gene, 142 bp of *psbA* exon 3, and 198 bp of *Cr.psbA3* (The details of construction of this plasmid and a map are given in Appendix I).

Plasmid I2-aadA (8.1 kb) was created from pGEMI2AAD-ORF by adding an upstream sequence of *psbA*. The plasmid pGEMI2AAD-ORF was digested with *Eco*RI and *Cla*I, and the 4.8 kb fragment was gel-purified. A 3.3-kb gel-purified fragment of *psbA,* obtained by digesting plasmid pEC23 (R14) (Herrin and Michaels) with *Eco*RI and *Cla*I, was ligated to the 4.8 kb fragment. The new plasmid was called I2-aadA (see the diagram of this insert in Figure 5.1A).

The above plasmids were digested with several restriction enzymes to confirm their insert size and orientation. Also, sequencing was performed to verify the DNA sequence of pGEMI2AAD-ORF and I2-aadA using primer #112 (5′-AGAATTCCG AGTGGTTATACAACGGTGGTCC-3′), a forward primer that annealed to nt 127-148 of exon 2, and #115 (5′-CAGGATCCGAGGTAGCAAAGG GCTCGACTT-3′), a reverse primer that annealed to nt 109-130 of *Cr.psbA3*.

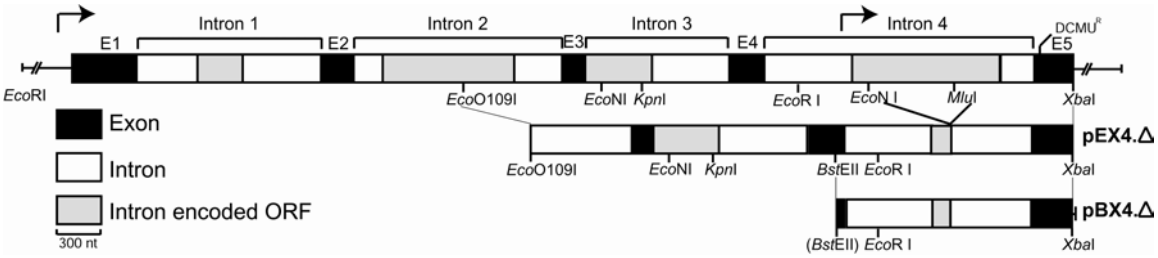


Figure 2.1 Schematic map of the *psbA* gene and cloned fragments used to study *Cr.psbA4* splicing *in vitro* and *in vivo*.The top diagram is the *psbA* gene, and the bottom two are inserts from plasmid clones used for transformation (pEX4.Δ series), mutagenesis and transformation (pBX4.Δ series), and *in vitro* transcription (pBX4.Δ series). The arrows indicate the direction of transcription, and the location of promoters. The internal promoter within *Cr.psbA4*, upstream of the ORF, is believed to be much weaker than the promoter upstream of exon 1 (Odom et al., 2001). A DCMU resistance mutation ($DCMU^R$) in exon 5 (Erickson et al., 1984) is indicated. Δ indicates a deletion of the *Cr.psbA4* ORF between the *Eco*NI and *Mlu*I sites, which also caused a frame-shift in the remaining ORF. The *Bst*EII site in pBX4.Δ is in parenthesis, because it was lost during creation of this plasmid (Holloway et al., 1999).

2.4 *In vitro* synthesis of preRNAs and self-splicing

The pBX4.Δ-based plasmids (Figure 2.1) were linearized with *Not*I, deproteinized, precipitated with ethanol and NaOAc, and then resuspended in H_2O to ~1 mg/mL. The DNA (50 μg/mL final concentration) was transcribed (10 μL vol) with T7 RNA polymerase (1500 units/mL) in 1 mM non-radioactive NTPs (ATP, UTP, CTP, and GTP), 1 μCi/μL of α-^{32}P-GTP (~3000 Ci/mmol, ICN, Inc.), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 40 mM Tris-HCl pH 8 at 37°C for 1 h. The reactions were stopped by adding EDTA (pH 8) to 12 mM and heating to 80°C for 1 min. They were then extracted with chloroform, and precipitated with NH_4OAc and ethanol after adding 10 μg of yeast tRNA as carrier. The RNA was dissolved in 100 μL DEPC-treated water, was precipitated again with ethanol in 300 mM NaOAc (pH 5.2) after adding another 10 μg of yeast tRNA, and then finally dissolved in 50 μL DEPC-treated water.

The standard self-splicing reactions (5 μL) contained ^{32}P-labeled RNA (1.8 nM), 50 mM Tris-HCl (pH 7.5), 1 mM GTP, 15 mM $MgCl_2$, 100 mM KOAc, and were performed at 45°C (unless stated otherwise). A 4-μL mixture of the Tris-HCl pH 7.5, GTP, $MgCl_2$, and KOAc was added first into each 0.65-mL microcentrifuge tube, the tubes were placed on ice, and then 1 μL of RNA (6 nM; ~30,000 cpm) was added (see page 58 of Kuo (1998) for the equation for conversion from radioactivity (cpm) to nanomolar concentration (nM) of a given precursor RNA). After mixing and centrifugation at 6,000 rpm at RT for 5 sec using a table-top mini-centrifuge, the

tubes were incubated at 45°C using a heat block. At selected intervals, the reactions were terminated by adding 6 μL of 90 % formamide, 50 mM EDTA pH 8.0, 0.03 % bromophenol blue, 0.03 % xylene cyanol, heating to 65°C for 3 min, and quick-cooling on ice.

The RNAs were then analyzed by electrophoresis on 4 % polyacrylamide/8 M urea gels at 45°C; equal amounts of radioactive RNA were loaded in each lane. The gels were fixed, transferred to 3MM paper, dried, and exposed to X-ray film (BioMax MS, Kodak) at -70 °C with an intensifying screen (DuPont Cronex). The developed X-ray films were checked to verify the absence of significant degradation in the samples, and they were scanned using a Real Tech 800 scanner with a transparency adapter and Photoshop 4.0 for the Mac (Adobe Inc.). Images were quantified using NIH IMAGE (version 1.5.2). The linearity of detection was verified by electrophoresing a dilution series of unspliced RNA and quantifying the preRNA band.

2.5 Chloroplast transformation

2.5.1 Transformation with the *Cr.psbA4* constructs

The *Cr.psbA4* constructs and the parental plasmid pBX4.Δ were co-introduced into the WT 2137 strain along with plasmid pb4c110, which contains a spectinomycin resistance marker in the 16S *rrn* gene (Thompson and Herrin, 1991; Harris et al.,

1989). Chloroplast transformation was performed by particle bombardment of cells in soft agar (Boynton and Gillham, 1993) with a helium-driven apparatus (Bio-Rad He 1000). The conditions were: 28.5 in of vacuum, 1,100 lb/in^2 rupture disk, the plate on shelf 4, and the macrocarrier in position 2.

The recipient cells were pregrown in liquid TAP to 2×10^6 cells/mL, collected by centrifugation, and resuspended to 1×10^8 cells/ml in TAP. Then, 0.5 ml of cells (~5×10^7 cells) were mixed with 0.5 ml of molten (42°C) 0.25 % agar in TAP-minimal medium, pipetted onto the center of a TAP plate (2 % agar and 100 μg/mL ampicillin), and spread over roughly three-fourths of the surface (110-mm diameter plates). Typically, 7 μg of DNA (total) was precipitated onto 3 mg of tungsten particles (M17; Bio-Rad), and one-eighth of this slurry (~0.4 mg of tungsten and 875 ng of DNA) was shot at each plate. After the shooting, the plates were kept overnight in dim light. The next day, the cell layer was scraped off and respread onto two selective plates (100 μg/ mL spectinomycin in TAP), which were kept in dim light at 23°C. Spectinomycin-resistant transformants usually began appearing after within 2-3 weeks and continued to appear for up to 2 weeks thereafter.

The *Cr.psbA4* P4′-3,4 mutant construct was also transformed into three *Cr.LSU* suppressor mutants (7120, 7151, and 71N1). These recipient strains already carried a spectinomycin-resistance marker, thus transformants were selected on plates containing DCMU (3 μM) in medium light. DCMU-resistant transformants required about 2 weeks to appear and continued to appear for 3 to 4 weeks thereafter.

2.5.2 Transformation with the *Cr.psbA2* constructs

Chloroplast transformation of the IL strain with the *Cr.psbA2* constructs was similar to that described above, except that the following DNAs were used: 3.5 μg of atpX-I2S + 3.5 μg of pb4c110; 3.5 μg of atpX-I2A + 3.5 μg of pb4C110; 3.5 μg of I2-aadA + 3.5 μg of pb4c110; and 5 μg of I2-aadA + 0.5 μg of atpX-I2S + 2.5 μg of pb4c110.

2.6 PCR analysis

Cells were grown on selective plates, and total DNA was prepared as described (Dürrenberger et al., 1996). Standard PCR protocols with *Taq* polymerase were used. The DNA products were analyzed by electrophoresis through 1% agarose gels containing ethidium bromide. When desired, the DNA products were eluted from the gel by capturing them onto 3MM paper (Whatman) supported by a dialysis membrane, eluted with 10 mM Tris-HCl pH 8.0, 1 mM EDTA, and subjected to automated sequencing using the PCR primers.

The following primers were used for confirming the homoplasmicity of the *Cr.psbA4* transformants: #99, a forward primer that annealed to nt 182-205 of exon 4; #176, a forward primer that annealed to nt 114-140 of exon 3; and #100, a reverse primer that annealed to nt 78-53 of exon 5 (Odom et al., 2001). The temperature regimen was as follows: 94°C for 4 min; 24 cycles of 60°C (1 min), 70°C (5 min), and 94°C (30 sec); 60°C for 1 min; and 70°C for 8 min.

To determine if the *Cr.psbA2* transformants were homoplasmic, the following primers were used: #118 (5′-GACGAGTGGTTATACAACGGT GGT-3′), a forward primer that annealed to nt 124-144 of exon 2, and #136 (5′-AGCGATCCATGGA CG CATACC-3′), a reverse primer that annealed to nt 229-249 of exon 3. In some cases, I used reverse primer #177 (5′-GTGCATAGCTGAGAATAATGAACCAC C-3′), which annealed to nt 870-896 of exon 4. After denaturation of the target DNA at 94^{o}C (5 min), amplification was achieved by 25 cycles of 94^{o}C (30 sec), 53^{o}C (2 min), and 70^{o}C (6 min).

The homoplasmicity of the *Cr.LSU* intron in haploid or diploid progeny was checked with by PCR the following primers: #15 (5′-TCGCTCAACGGATAAAA GTT-3′), a forward primer that annealed to nt 215-234 of 23Sδ rRNA, and #14 (5′-ACCTATATAACGGCTTGTCT-3′), a reverse primer that annealed to nt 599-618 of 23Sδ rRNA. After denaturation at 94^{o}C (4 min), amplification was achieved by 36 cycles of 94^{o}C (1 min), 46^{o}C (2 min), and 70^{o}C (2 min).

2.7 RNA gel-blot hybridizations

Total cellular RNAs were isolated, separated by electrophoresis in 1.2-1.4 % agarose/2 % formaldehyde gels, and blotted onto a charged nylon membrane (Zetaprobe or Hybond) as described previously (Herrin et al., 1990; Hwang and Herrin, 1994). The membrane-bound RNA was stained with methylene blue prior to hybridization (Herrin and Schmidt, 1988) to check for equal loading.

DNA probes for the *psbA* gene were as follows. The exons-specific probe was *Hind*III-digested plasmid pBA153, which contains an intronless *psbA* gene (Minagawa and Crofts, 1994). The DNA hybridization probe for *Cr.psbA2* splicing was the 1.7-kb *Xba*I fragment from pGEMR14.2, which contains 81-bp of exon 2, *Cr.psbA2*, exon 3, and 198-bp of *Cr.psbA3* (Herrin and Michaels, 1985). The hybridization probe for intron *Cr.psbA4* was a 1.5-kb PCR product amplified from the plasmid pBX4 (Holloway et al., 1999) using primers #102 (5′-CGACGTTAAACA AATTCAT-3′), a forward primer that annealed to nt 168-187 of the intron, and #103 (5′-AGCTCTAGTCTCTGAACG-3′), a reverse primer that annealed to nt 1666-1683 of the intron (Deshpande et al., 1997). The DNAs were labeled by random priming (Feinberg and Vogelstein, 1983) to 0.25-1 $\times$ 10^9 dpm/μg with α-^{32}P-dCTP (~3000 Ci/mmol, ICN). After hybridization at 65°C with the Church-Gilbert solution (Church and Gilbert, 1984), the blots were washed in 0.1 $\times$ SSPE, 0.1 % SDS (1 $\times$ SSPE = 0.15 M NaCl, 50 mM NaH_2PO_4 pH 7.4, 5 mM EDTA) at 65°C, and then exposed to X-ray film (Kodak BioMax MS) at -70°C with an intensifying screen.

Oligonucleotide hybridization probes for *Cr.LSU* splicing were #8 (5′-ATATTTTGTATTCATAAGATG-3′), a reverse primer that annealed to nt 176-196 of the *Cr.LSU* intron, and #14 (5′-ACCTATATAACGGCTTGTCT-3′), a reverse primer that annealed to nt 599-618 of the 23Sδ exon (Holloway and Herrin, 1998). The oligodeoxynucleotides were 5′ end-labeled with T4 polynucleotide kinase and γ-^{32}P-ATP (NEN; 5000 Ci/mmol) to a specific activity of ~10^7 cpm/μg.

Hybridization with the oligodeoxynuclotides was performed in 6 x SSPE (1 x SSPE is 0.15 M NaCl, 50 mM NaH_2PO_4 pH 7.4, 5 mM EDTA), 1 % SDS, 10 x Denhardt's (0.2 % BSA, 0.2 % Ficoll 400, 0.2 % PVP) at 42°C; the probe concentration was 10 ng/mL. After hybridization, the blots were washed three times (for 3 min each) in 6 x SSPE, 0.2 % SDS at 48 °C, and then three times for 6 min in the same solution and temperature. Exposure of the blots to X-Ray film was at -70°C with an intensifying screen. For sequential hybridization of the two probes to the same blot, the first oligodeoxynucleotide was stripped by incubation of the blot in 0.1 x SSPE, 0.5 % SDS at 100°C for 5 min. The blot was then exposed to X-ray film (Kodak BioMax MS) at -70 °C with an intensifying screen to confirm that no radioactivity remained.

For the quantification of autoradiographs of northern blots, multiple exposures of the blots were made to achieve signals within the linear response range of the x-ray film. The developed films were scanned and quantified as described above for *in vitro* synthesized RNA. In some experiments, the blots were quantified with a phosphorimager and Imagequant software (Molecular Dynamics).

2.8 Pulse-labeling and protein analysis

For *in vivo* pulse-labeling with ^{14}C-acetate, 5×10^6 cells were centrifuged at 8,000 $\times$ g for 5 min, washed with 1 mL of minimal medium, and resuspended in 250 μL of minimal medium. The tubes were shaken at 125 rpm under dim light, and labeling was initiated by adding cycloheximide to 10 μg/mL followed immediately by 12.5

μCi of 1-^{14}C-acetate (60 mCi/mmol, ICN). After either 5 or 30 min at 23°C, the tubes were placed on ice, the cells centrifuged at 16,000 × g for 2 min (4°C), and then resuspended in ice-cold 100 mM Tris-HCl pH 8.6, 100 mM DTT, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride. The cells were then either frozen at -70°C, or solubilized immediately for gel electrophoresis. Radioactivity in total protein was determined by hot trichloroacetic acid precipitation (Herrin et al., 1981).

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the buffers described by Laemmli (1970), and an acrylamide/bisacrylamide ratio of 30:0.8. The stacking gel contained 6 % acrylamide, and the resolving gels were linear gradients of 7.5-15 % acrylamide. Samples were prepared for electrophoresis by incubation at 60°C (10 min) in 2.5 % (w/v) lithium dodecylsulfate, 12 % sucrose, 0.01 % bromophenol blue, 60 mM Tris-HCl pH 8.6, 60 mM DTT, 5 mM benzamidine, 1 mM phenylmethylsulfonylfluoride. After electrophoresis at 4°C, the gels were fixed and stained with coomassie blue to check the protein loads. They were then impregnated with Fluorohance (Research Products International), dried, and exposed to X-ray film (BioMax MR, Kodak) at room temperature or -70°C. The fluorographs were quantified as described above for *in vitro* synthesized RNAs.

2.9 Genetic analysis

2.9.1 Tetrad analysis

Techniques for mating and tetrad analysis were generally performed as described by Harris (1989). Cells for matings were grown for seven days on selective solid TAP medium (2 % agar) in medium or dim light. To induce gametogenesis, the cells were gently scraped off with a tungsten wire and resuspended in 3 mL (in a 15 mL tube) of nitrogen-free Sager's medium except for the following substitutions (final concentrations): 0.1 % (w/v) KCl instead of 0.1 % (w/v) NH_4NO_3, and 0.075 % (w/v) $(Na)_2MoO_4$, instead of 0.080 % (w/v) $(NH_4)_2$ MoO_4 (Harris, 1989). After overnight incubation in medium light, gametes were mixed and left in medium light for 2 h. Then, the cells were harvested by centrifugation at 3000 rpm (23 °C) for 5 min using a Beckman GPR centrifuge, resuspended in 0.5 ml TAP, and pipetted onto a maturation plate that contained 4 % agar (in TAP, plus 100 μg/mL L-arginine when needed). After 24 h in medium light, the plates were doubly wrapped in aluminum foil, and incubated for 4 days at 23 °C. To remove gametes from the maturation plate, a clean razor blade was dipped in 100 % ethanol, flamed briefly, and then the razor blade was drawn across the surface of the agar with a firm but gentle motion. The zygotes were collected along with a small amount of agar from the maturation plates and transferred to gridded germination plates using a ~0.5 mm diameter glass needle and a dissecting microscope (~50 × magnification). To make the

germination plates, five plates (1.5 % agar in TAP containing 100 μg/mL ampicillin or 100 μg/mL spectinomycin and 200 μg/mL erythromycin) were gridded by cutting parallel vertical lines 5-8 mm apart, and parallel horizontal lines 1.5-2 cm apart, with a sterile razor blade. The glass needles were made by drawing out a Pasteur pipette with a bunsen burner and forceps. Each zygote was deposited at the top of a grid. The plates were placed in dim or bright light overnight. The next day, the germinated zygotes were touched with the glass needle, and the meiospore daughter cells were separated using a ~ 0.2 mm diameter glass needle. The plates were then kept in dim light until colonies were visible.

2.9.2 Isolation and characterization of diploids using complementing *nit* mutations

For the isolation of diploids, the mating procedure was similar to that used for tetrad analysis above. After mating of the suppressor strains, which are *nit* /*nit2*, with a *nit4* strain (*nit* mutants lack nitrate reductase), the mating mixtures were plated on Sager's medium (plus 2 % agar) with nitrate as the only nitrogen source. The medium was modified by using 0.1 % (w/v) KNO_3 instead of 0.1 % (w/v) NH_4NO_3, and 0.075 % $(Na)_2MoO_4$, instead of 0.080 % $(NH_4)_2$ MoO_4 (Harris, 1989). The plates were placed in dim light for up to three weeks.

The putative diploids were grown in liquid TAP medium for five days in medium light, and then were observed microscopically. The images were captured

with a CCD camera (cool) connected to a Nikon Optiphot phase contrast microscope at 400 × magnification (0.65 NA Phase 3) (Garden City, NY). The lengths of 9 cells from each diploid clone, and 27 haploid WT cells were measured using NIH Image.

In addition, the mating type of each diploid clone was determined by mixing gametes (induced the same way as haploid cells) of each diploid with gametes of the WT 2137 mt+ strain, and observing the presence of the zygote pellicle at the top of the tube.

2.10 Other

Plasmid DNAs were isolated from *E. coli* DH5α cultures using standard techniques (Sambrook and Russell, 2001). Transformation of *E. coli* DH5α was performed according to the protocol of Life Technologies. Plasmids and PCR products were sequenced at the University of Texas at Austin DNA Analysis Center using PCR- based reactions, fluorescent dideoxynucleotides, and automated gel or capillary electrophoresis-laser systems (ABI).

Chapter 3 Mutagenesis of the Light-Regulated *Cr.psbA4* Intron Reveals the Importance of Efficient Splicing of *psbA* PremRNA

3.1 Summary

The chloroplast-encoded *psbA* gene encodes the D1 polypeptide of the photosystem II reaction center, which is synthesized at high rates in the light. In *Chlamydomonas reinhardtii*, the *psbA* gene contains four self-splicing group I introns whose rates of splicing *in vivo* are increased at least 6-fold to 10-fold by light. However, because *psbA* is an abundant mRNA, and some chloroplast mRNAs appear to be in great excess of what is needed to sustain translation rates, the physiological and developmental significance of light-promoted splicing has not been clear. To address this and other questions, potentially destabilizing nucleotide substitutions were made in the predicted P3, P4, P6, P6a, P7 and P11 helices of the fourth *psbA* intron, *Cr.psbA4,* and their effects on *in vitro* and *in vivo* splicing assessed. Two-nucleotide substitutions in P4 and P7 were necessary to substantially reduce splicing of this intron *in vivo*, although most mutations reduced self-splicing *in vitro*. Further analysis of the partially splicing-deficient strains revealed approximate correlations between *in vivo* splicing efficiency, synthesis of full-length D1, and autotrophic growth rates. Most informative was the P4'-3,4 mutant, which exhibited a 45 % reduction in spliced *psbA* mRNA, a 28 % reduction in synthesis of full-length D1, and an 18 % reduction in growth rate, compared to the control strain. We conclude

from these results that the highly efficient splicing of the *psbA* introns, which is afforded by light conditions, is essential to maintain robust autotrophic growth.

3.2 Introduction

The *psbA* gene encodes a critical and highly conserved component of the photosystem II reaction center, polypeptide D1. This ~36 kDa polypeptide is believed to span the thylakoid membrane five times, and to bind quinone *b*, photosynthetic pigments, and possibly metals (reviewed in Ruffle and Sayre, 1998). D1 is synthesized on thylakoid-bound ribosomes at high rates in the light (Herrin and Michaels, 1985; Herrin et al., 1986; Klein et al., 1988), and its synthesis increases at higher light fluxes (Mattoo et al., 1984). The accelerated production of D1 during the daytime is part of the daily production of thylakoid components (Lee and Herrin, 2002, and references therein), and also serves to replace damaged D1. At very high, photoinhibitory light fluxes, the rate of D1 damage exceeds the rate of removal of damaged D1, with concomitant loss of photosynthetic capacity (Schuster et al., 1988).

Numerous studies have revealed the dynamic regulation of *psbA* gene expression that occurs in response to light (early work was reviewed in Erickson and Rochaix (1992)). Light stimulation of *psbA* gene expression occurs at the transcriptional (e.g., Deshpande et al., 1997; Klein and Mullet, 1990; Tiller and Link, 1993), and several post-transcriptional levels, including RNA splicing (Deshpande et al., 1997), RNA stability (Baumgartner et al., 1993; Salvador et al., 1993), and translation (e.g.,

Danon and Mayfield, 1994b; Klein et al., 1988; Staub and Maliga, 1994; Zhang et al., 2000). Hence, in addition to its key role in photosynthesis, *psbA* has been an important model for understanding gene regulation in chloroplasts.

The unicellular chlorophyte, *Chlamydomonas reinhardtii* (*Chlamydomonas*), is an excellent organism for studying organelle biogenesis and regulation, since genetic, molecular and cellular approaches can be employed (Rochaix et al., 1998). Moreover, since photosynthesis is dispensable, and chloroplast transformation occurs by homologous recombination, chloroplast-encoded genes for photosynthesis can be manipulated, and the phenotypic effects observed without interference from wild type gene copies (Goldschmidt-Clermont, 1998).

The *psbA* gene in *Chlamydomonas* contains 4 large group I introns (Figure 3.1), *Cr.psbA1*-*Cr.psbA4*, that can self-splice *in vitro* but probably require *trans*-acting factors for efficient splicing *in vivo* (Deshpande et al., 1997; Herrin et al., 1991; Li et al., 2002). Interestingly, in cells growing autotrophically under light-dark cycles the *in vivo* splicing rate of all four of the introns is very slow in the dark period, but increases 6-fold to 10-fold within 30 min of light administration via a process that requires photosynthetic electron transport (Deshpande et al., 1997). It was suggested that the role of light stimulation of splicing, which does not happen for the chloroplast 23S rRNA intron (*Cr.LSU*, also a self-splicing group I intron), is to ensure an adequate supply of spliced *psbA* mRNA for the high rates of translation in the light (Deshpande et al., 1997). However, there has been no direct evidence to support

this hypothesis. The question of the developmental significance of light-promoted *psbA* splicing is underscored by the fact that *psbA* mRNA is highly abundant (Herrin et al., 1986; Jensen et al., 1986), and by evidence suggesting that some chloroplast mRNAs, including *psbA*, may be in considerable (up to 10-fold) excess of what is needed for translation (Eberhard et al., 2002).

In order to address the question of whether highly efficient splicing of *psbA* introns is critical for autotrophic growth, we systematically substituted nucleotides in core helices of the *Cr.psbA4* intron, and analyzed the effects of these potentially destabilizing mutations on splicing *in vivo* and *in vitro*. This intron was selected because it can be replaced *in vivo* with a version that has most of the open reading frame in the loop of P5b deleted (ORF-minus), and that splices similarly to the wild type intron (Odom et al., 2001; O.W. Odom and D.L. Herrin, unpublished results). The ORF-minus version is ~500 bp shorter than the wild type intron, and this size difference facilitates the identification of homoplasmic transformants.

Most mutagenesis studies of group I introns have examined the effects of mutations only on self-splicing *in vitro*; only a few reports have looked at corresponding *in vivo* effects in the normal host organism (Brion et al., 1999; Mohr et al., 1992; Myers et al., 1996). Those reports concerned the phage T4 thymidylate synthase intron (*T4.td*), and the conclusion was that the effects of the mutations *in vitro* and in *Escherichia coli* were quite similar. In a recent study of the *Cr.LSU* intron, most of the nucleotide substitutions could not be effectively

analyzed *in vivo* because of the heteroplasmicity of the transformants, due apparently to the lethal nature of the mutations in a homoplasmic cell (Li et al., 2002). Because *psbA* is not essential in *C. reinhardtii*, it was possible to analyze the most interesting mutants *in vivo* as well as *in vitro*.

3.3 Materials and Methods

The materials and methods for this chapter are decribed in Chapter 2.

3.4 Results

3.4.1 Requirements for efficient self-splicing of *Cr.psbA4*

Cr.psbA4 was previously shown to self-splice *in vitro* using a set of conditions that had worked for other group I introns (25 mM Tris-HCl pH 7.5, 100 mM $(NH_4)_2SO_4$, 200 μM GTP, and 25 mM $MgCl_2$ at 45°C) (Thompson and Herrin, 1991; Herrin et al., 1991). However, to potentially compare the effects of the mutations *in vitro* to *in vivo*, we wanted to use conditions that were more similar to organellar conditions (at least as far as possible, considering that group I introns usually don't self-splice with much efficiency at temperatures that are optimal for *Chlamydomonas* growth). Thus, it was necessary to evaluate the effects of certain key parameters on *Cr.psbA4* splicing *in vitro*. These effects, as well as those of the mutations (see below), were assessed using RNA synthesized from linearized pBX4.Δ DNA

(Figure 3.1). The intron in pBX4.Δ is wild type, except for a deletion of most of the open reading frame (ORF) from the non-conserved loop of P5b (Figure 3.2). Deleting the ORF did not have an appreciable effect on *Cr.psbA4* splicing *in vivo* (see below), although it seemed to slightly improve *in vitro* splicing (J. Lee, O.W. Odom and D.L. Herrin, unpublished results), and was done to facilitate identification of homoplasmic transformants. This form of the intron and preRNA will be referred to as wild type (WT), when comparing with the substituted introns.

Figure 3.3 shows the effects of varying the concentrations of GTP and Mg^{2+}, as well as the reaction temperature, on *Cr.psbA4* self-splicing. These reactions also contained 100 mM KOAc as monovalent salt, a concentration that is close to optimal for chloroplast protein synthesis (Klein et al., 1988; Bhaya and Jagendorf, 1984; Herrin et al., 1981), and were pH 7.5; typically, there is little effect of pH on self-splicing in the range of 6-9 (Herrin et al., 1998). Since the reactions were for a relatively long period of time, i.e., 30 min, the data reflect the extent of splicing more so than the initial rate, and are based on accumulation of the ligated-exons product (see Figure 3.4A,B). Figure 3.3A shows that the GTP concentration was saturating at 0.1 mM, and above that concentration, the splicing efficiency declined slightly (up to 5 mM, which was the highest concentration tested). This result indicates that the ribozyme has a high affinity for GTP, and that nucleotide levels are unlikely to be regulatory *in vivo*.

The Mg^{2+} concentration was varied from 0-25 mM and the effect on self-splicing

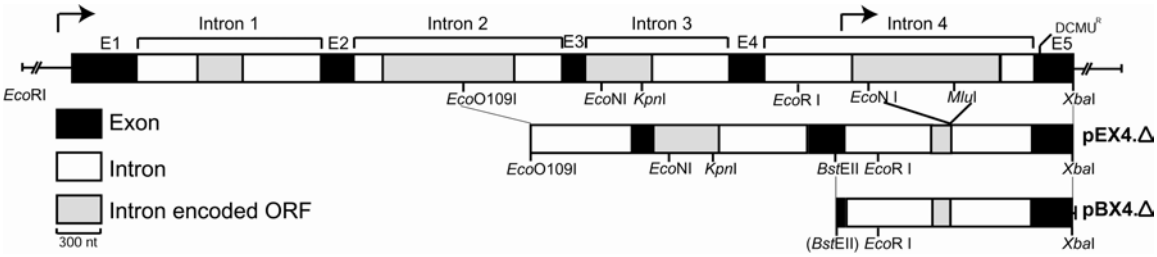


Figure 3.1 Schematic map of the *psbA* gene and cloned fragments used to study *Cr.psbA4* splicing *in vitro* and *in vivo*.The top diagram is the *psbA* gene, and the bottom two are inserts from plasmid clones used for transformation (pEX4.Δ), mutagenesis and transformation (pBX4.Δ), and *in vitro* transcription (pBX4.Δ). The arrows indicate the direction of transcription, and the location of promoters. The internal promoter within *Cr.psbA4*, upstream of the ORF, is believed to be much weaker than the promoter upstream of exon 1 (Odom et al., 2001). A DCMU resistance mutation ($DCMU^R$) in exon 5 (Erickson et al., 1984) is indicated. The plasmid clones are identified to the right; Δ indicates a deletion of the *Cr.psbA4* ORF between the *Eco*NI and *Mlu*I sites, which also caused a frame-shift in the remaining ORF. The *Bst*EII site in pBX4.Δ is in parenthesis, because it was lost during creation of this plasmid (Holloway et al., 1999).

is shown in Figure 3.3B. The greatest splicing efficiency was obtained at 10 mM Mg^{2+}, and above that concentration, splicing declined gradually to ~38 % of the maximum (at 25 mM Mg^{2+}). There was no splicing observed at 5 mM Mg^{2+}, which was also the case for the *Cr.psbA2* (Bao, 1993) and *Cr.LSU* introns (Thompson and Herrin, 1991).

Figure 3.3C shows the effect of temperature on *Cr.psbA4* self-splicing. There was a sharp optimum around 55°C, and splicing was poor at the physiological temperatures of 23°C and 30°C. The dramatic increase in splicing efficiency at 45°C compared to 37°C is not readily explained by an effect on the chemical steps of splicing, but more likely reflects poor folding of the intron at 37°C. At the other end of the spectrum, the dramatic drop in splicing at 62°C probably reflects unfolding of tertiary and structural elements (Jaeger et al., 1993).

Finally, it should be noted that monovalent ions (K^+ and NH_4^+) had relatively minor effects on *Cr.psbA4* self-splicing, although they increased the minimum Mg^{2+} requirement as expected (data not shown). Based on these data, and published estimates of the nucleotide concentrations in higher plant chloroplasts (~1 mM; Hampp et al., 1982), and the optimal Mg^{2+} concentration for translation by chloroplast polysomes (~12 mM; Bhaya and Jagendorf, 1984), the conditions used to evaluate the effects of the mutations on *Cr.psbA4* self-splicing were 25 mM Tris-HCl pH 7.5, 1 mM GTP, 100 mM KOAc, 15 mM $MgCl_2$, at 45°C. It was also reasoned that these conditions would allow most of the mutants, except for perhaps the most

severely effected ones, to self-splice.

3.4.2 Effects of nucleotide substitutions on *Cr.psbA4* self-splicing *in vitro*

Figure 3.2 shows the locations of the nucleotide substitutions on the predicted secondary structure of *Cr.psbA4* (Holloway et al., 1999). The conserved ribozyme core is composed of two major, stacked-helices domains, P5-P4-P6, and P9.0-P7-P3-P8, respectively (Michel and Westhof, 1990); the guanosine binding site resides mainly on ^{1674}G (WT numbering) in P7 (Michel et al., 1989a). *Cr.psbA4* also contains several less conserved, peripheral domains, including P5a, P5b, P6a, P7.1, P9.1, and the signature (for subgroup IA1 introns) P11 pairing (Michel and Westhof, 1990). Initially, single-nucleotide substitutions were made in the P3, P4, P6, P6a, P7, and P11 helices, and then two more mutants were created that had two-nucleotide substitutions in P4 and P7 (the P4'-3,4 and P7-4,5 mutants, respectively). These latter mutants were constructed because of the relatively weak effects of most of the single-nucleotide substitutions.

Figure 3.4A shows representative time-course splicing reactions for WT (i.e., pBX4.Δ) and 2 of the mutant introns, P7-4,5 and P6-2, respectively. The principal products of the reactions are the linear intron (I4) and ligated-exon (E4-E5) molecules. Quantification of the ligated-exons product is shown in Figure 3.4B for these reactions as well as for 4 other mutants (P4'-2; P4'-3,4; P6a-2; and P7-4). These results show that splicing of the WT preRNA is quite fast under these conditions,

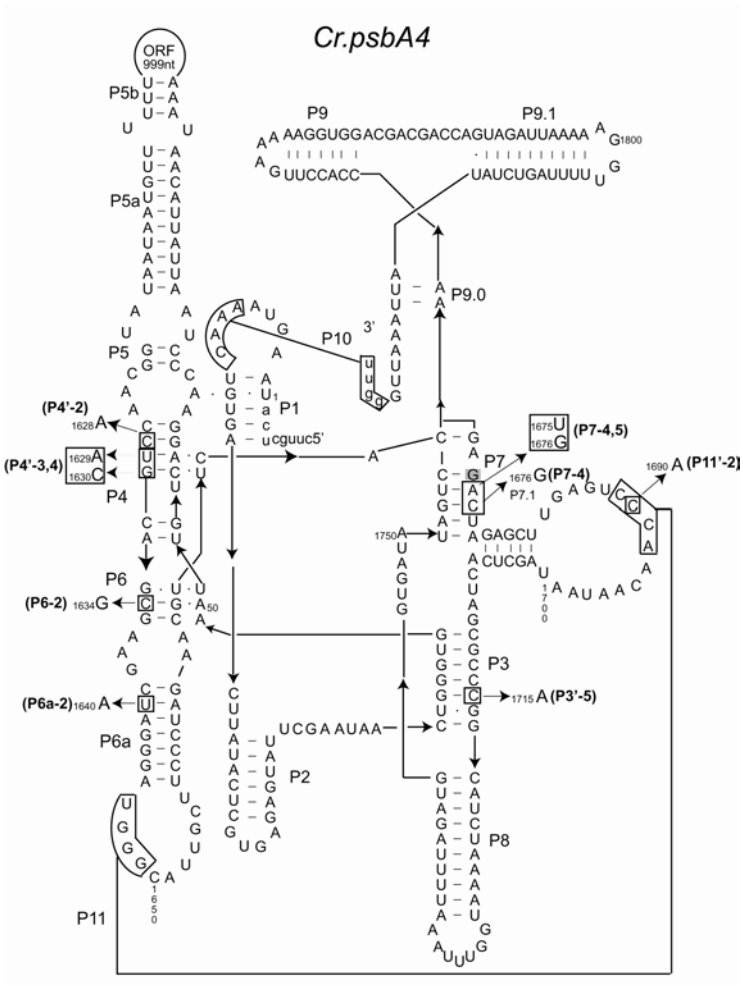


Figure 3.2 Predicted structure of the *Cr.psbA4* intron, and the locations of nucleotide substitutions in the mutants.The paired regions are labeled (P1-P11), and the two that are within terminal loops, P10 and P11, are also boxed. The 3' half of a paired duplex is indicated by a prime symbol (‘), and the nucleotides within a paired region are numbered from 5' to 3'. All the nucleotides in the intron sequence are also consecutively numbered from the 5'-end of the intron using the wild type numbering scheme. The sites of 1 or 2-nucleotide substitutions are indicated by shaded boxes, and the new nucleotides are indicated by arrows. The nomenclature is based on group I intron conventions (Burke et al., 1987). The GTP binding site is the shaded ^{1674}G in P7.

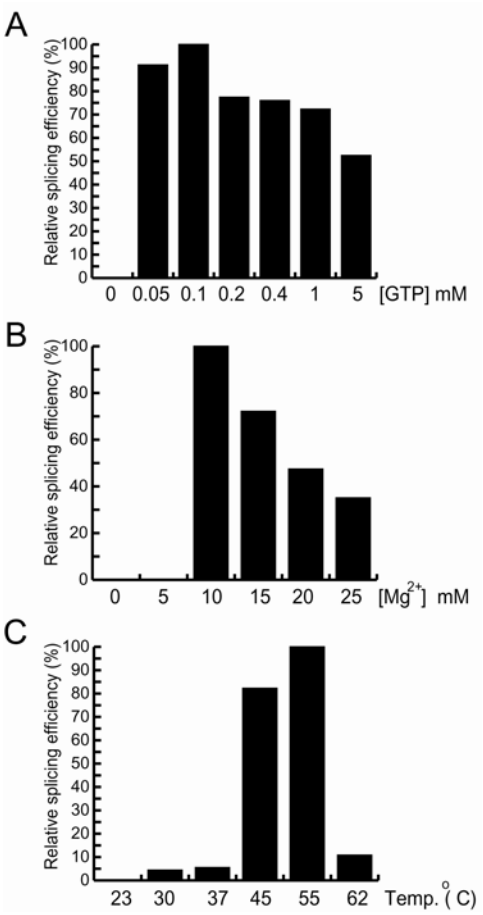


Figure 3.3 Effect of varying GTP, Mg^{2+}, and temperature on self-splicing of *Cr.psbA4*. The preRNA transcribed from pBX4.Δ (Figure 3.1) was incubated for 30 min in the standard splicing reaction mixture, except for varying the indicated parameter, and then analyzed by denaturing gel electrophoresis. Splicing efficiency was estimated based on the accumulation of the 319-nucleotide ligated-exons product (see Figure 3.4A). The data were normalized relative to the highest value, which was set to 100 %.

(A) The effect of varying the GTP concentration on *Cr.psbA4* self-splicing.

(B) The effect of varying the Mg^{2+} concentration on *Cr.psbA4* self-splicing.

(C) The effect of varying the reaction temperature on *Cr.psbA4* self-splicing.

being essentially finished in 10 min (*k* of ~0.15/min); ~65 % of the preRNA spliced. All 6 of the substituted introns showed reduced splicing compared to WT; the reductions ranged from moderate, in the case of the P6a-2 and P4'-2 mutants, to a complete loss of self-splicing with the P7-4,5 mutant. The significant effect of the P6a-2 substitution was somewhat surprising, since this paired region is not part of the conserved core. Figure 3.4B also shows that the kinetics of the P7-4 mutant were unique; there was a lag period of ~3-4 min before any product was formed, and the subsequent course was biphasic. This pattern was highly reproducible under these conditions, but not quite as biphasic under other conditions (described below). The kinetics of the P7-4 mutant are consistent with it being a slow-folding mutant. It should also be noted that heterogenous kinetics are not uncommon for self-splicing group I introns (Kuo and Herrin, 2000; Thompson and Herrin, 1991), and presumably reflect the presence of conformational isomers. A fraction of relatively inactive preRNA is also not uncommon, even for wild type preRNAs, and is likely molecules trapped in misfolded states (Thirumalai and Woodson, 2000).

The splicing efficiencies of the other 2 mutants, P3'-5 and P11'-2, together with the 6 discussed above were also assessed in time-course reactions in a commonly used splicing buffer (25 mM Tris-HCl pH 7.5, 100 mM $(NH_4)_2SO_4$, 200 μM GTP, and 25 mM $MgCl_2$) at 45°C. The P3'-5 mutant was indistinguishable from WT, and the P11'-2 mutant self-spliced with an efficiency similar to the P7-4 mutant, but without the biphasic kinetics (data not shown). Of the 6 mutants analyzed above

(Figure 3.4), only the P7-4 and P6-2 mutants showed significant differences in their splicing efficiencies relative to WT in this buffer, compared to the more physiological solution; the P7-4 mutant was ~2-fold less active, whereas the P6-2 mutant was a dramatic 8-fold lower in splicing efficiency (data not shown). Thus, these results demonstrate the importance of the reaction composition used for self-splicing. Finally, increasing the Mg^{2+} concentration (up to 100 mM) increased the self-splicing efficiency of most of the mutants, except for the P7-4,5 mutant (data not shown), which was still inactive, consistent with their structure being destabilized.

3.4.3 Effects of the nucleotide Substitutions on *in vivo* Splicing of *Cr.psbA4*

To assess the *in vivo* effects of the nucleotide substitutions on *Cr.psbA4* splicing, the various constructs were transformed into chloroplasts of the wild type 2317 strain using co-transformation with a 16S *rrn* gene that confers spectinomycin-resistance, but otherwise has little effect on the phenotype (Harris et al., 1989).

The initial transformations were with pBX4.Δ -based DNAs (Figure 3.1), which contain a very short fragment of exon 4. However, most of these transformants did not have the entire intron replaced, although many had the DCMU-resistance marker in exon 5 (Figure 3.1). Thus, the upstream *Eco*O1091-*Bst*EII fragment of *psbA* was added to create the pEX4.Δ-based series of plasmids (Figure 3.1), which have a much greater amount of 5' flanking homology with the target DNA. These transformations

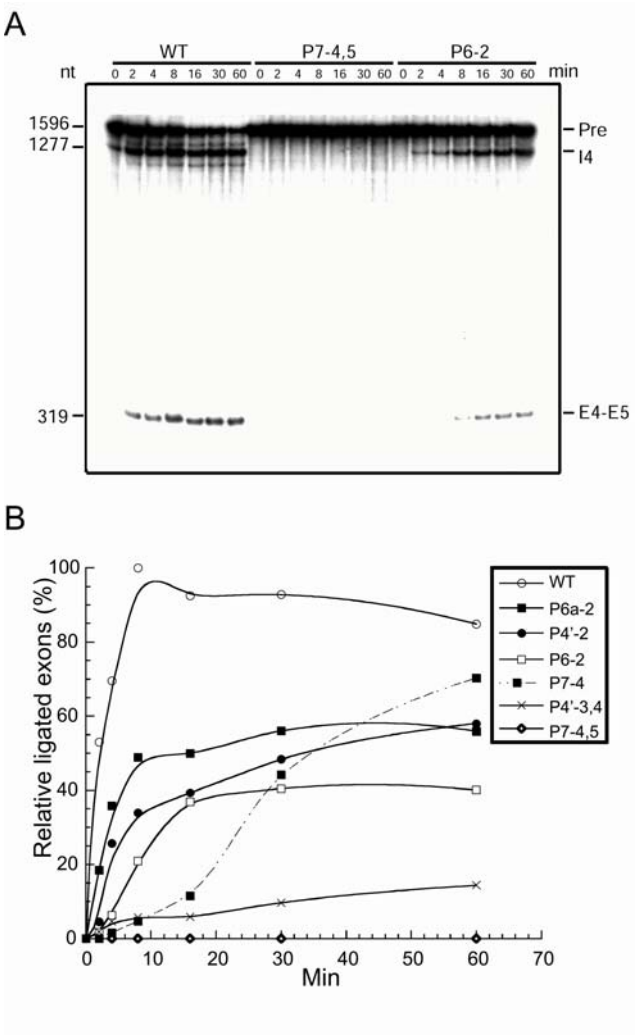


Figure 3.4 Effects of the nucleotide substitutions on *Cr.psbA4* splicing *in vitro*. The splicing reactions were performed and analyzed as described in Chapter 2.

(A) Autoradiograph of the splicing reactions for the wild type (with respect to the substitutions), and the P7-4,5 and P6-2 mutant preRNAs. The sizes of the major RNAs are indicated to the left in nucleotides (nt). Pre, preRNA; I4, linear free intron; E4-E5, ligated exons.

(B) Time courses of self-splicing by the mutants and wild type *Cr.psbA4* preRNAs. The ligated-exons product was quantified as described in Methods, and the data were normalized relative to the control preRNA (WT), which was considered to be 100 %.

were successful, and the homoplasmicity of the transformants was verified by PCR with primers that flank the *Cr.psbA4* intron (Odom et al., 2001); only transformants that contained exclusively the ORF-deleted form of the intron were analyzed further. The PCR products were also sequenced to verify the presence of the nucleotide substitutions (data not shown).

To examine the effects of the substitutions on *Cr.psbA4* splicing *in vivo*, RNA gel-blot hybridizations were performed on cultures growing in acetate-containing media under moderately bright light (~40 μmoles/m^2/sec). Figure 3.5 shows the results obtained with an intronless *psbA* gene (Exons 1-5 probe), and an intron-specific probe. The P7-4,5, P4'-3,4, P7-4, and P6-2 mutants showed evidence of reduced *Cr.psbA4* splicing, based on the accumulation of a 2.4-kb premRNA and corresponding decreases in the 1.1-kb mature mRNA. The hybridization with the intron-specific DNA (Intron 4 probe) confirmed that the 2.4-kb RNA is unspliced precursor. Even with a longer exposure, the P6a-2, P4'-2, and P3'-5 mutants did not show detectable levels of unspliced precursor. It should also be noted that, since the cells were grown in bright light, the control strain does not accumulate unspliced premRNA.

The data in Figure 3.5 also show that splicing of the P7-4,5 mutant is essentially blocked, whereas splicing of the P4'-3,4 mutant is inefficient (~40 % of the WT levels of mature *psbA* mRNA). The numbers below the lanes of the exons-only hybridization are the levels of the mature *psbA* mRNA relative to the control

strain (WT), and were determined from two to three separate RNA preparations. It is also noteworthy that the sum of the mature and premRNA in the different strains are within 10 % of each other, suggesting that the unspliced RNA is as stable as the mature mRNA. The identities of the minor bands that migrate between the 2.4 and 1.1-kb RNAs in the splicing-deficient mutants are not known with certainty. They may be degradation products of the premRNA; although the somewhat diffuse band of ~2 kb might be the intron-exon 5 splicing intermediate (Deshpande et al., 1997).

3.4.4 D1 synthesis in the splicing-deficient *Cr.psbA4* mutants

To investigate the effects of the intron mutations on D1 synthesis, the strains with deficiencies in *Cr.psbA4* splicing (and the control strain) were grown as described for Figure 3.5, and pulse-labeled for 30 min with ^{14}C-acetate in the presence of cycloheximide. Equal numbers of cells were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and a representative fluorograph is shown in Figure 3.6. The most obvious effect is the disappearance of D1 in the P7'-4,5 mutant, and the concomitant appearance of a polypeptide that migrates with an apparent M_r of ~24 kDa. The *Cr.psbA4* intron contains an in-frame termination codon in the second subjective codon position of the intron. The predicted size of a polypeptide terminating at this site is 27 kDa. However, it is well established that D1 migrates faster than its predicted size by SDS-PAGE (Erickson and Rochaix, 1992). Hence, the

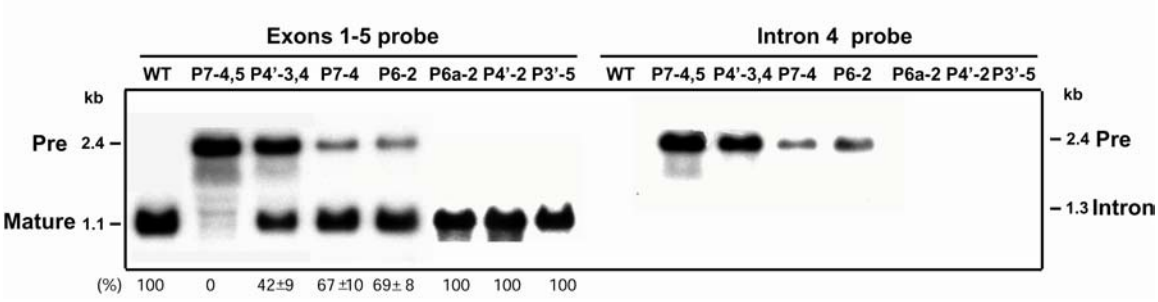


Figure 3.5 Effects of the nucleotide substitutions on *Cr.psbA4* splicing in cells growing mixotrophically.

Total RNA was isolated from early log phase cultures of the indicated chloroplast transformants and subjected to RNA gel-blot analysis. The WT strain is wild type with respect to the substitutions, but is otherwise like the other strains in being ORF-minus and carrying the spectinomycin and DCMU resistance markers (see Figure 3.1). The cells were grown in liquid acetate-containing medium (TAP+ 100 μg/mL spectinomycin) at a light intensity of ~40 μmoles/m^2/sec. An equal amount of RNA (5 μg) was loaded in each lane, and the blots were hybridized with an intronless *psbA* gene (Exons 1-5 probe), and a PCR product from the *Cr.psbA4* intron (Intron 4 probe). The positions of the major *psbA* precursor (2.4-kb RNA), the mature *psbA* mRNA (1.1-kb RNA), and the free Intron (1.3-kb) are indicated. *Chlamydomonas* rRNAs, which were visible on the methylene blue-stained blots, were used as size markers. The relative amounts of mature mRNA are given below the lanes of the exons-only hybridization; they were quantified and normalized relative to the control strain (WT), which was considered to be 100 %. For those mutants that showed an effect on *Cr.psbA4* splicing, the mean ± the standard error was obtained from two to three independent analyses.

new ~24-kDa protein is most likely a truncated D1 (tD1) containing the first four exons. This protein can also be seen in the P4'-3,4 mutant, but at a lower level than the P7'-4,5 mutant. The relative rates of synthesis of full-length D1, normalized using the large subunit of ribulose-1,5-bisphosphate carboxylase (LS) to correct for slight differences in acetate uptake and incorporation, are shown below the lanes. The data indicate that there are reduced amounts of full-length D1 synthesis in the P7-4 and P6-2 mutants, as well as in the P4'-3,4 mutant. Finally, it should be noted that these results indicate that unspliced *psbA* pre-mRNA can be translated *in vivo*.

3.4.5 Comparing autotrophic growth rates, *psbA* mRNA levels, and D1 synthesis in the partially- deficient splicing mutants

The analyses in Figures 3.5 and 3.6 were performed with cells growing mixotrophically (light + acetate), because the P7-4,5 mutant did not grow at all on minimal medium. However, it was important to assess the effects of the partially-deficient *Cr.psbA4* mutations in autotrophically growing cells, which would be dependent on photosynthesis. Growth curves in liquid minimal medium (gently bubbled with 5 % CO_2) are shown in Figure 7A. The growth rates of the P7-4 and P6-2 mutants were similar to the control strain (WT), whereas the P4'-3,4 mutant showed a clear reduction in growth, having only 57 % of the cell number of the control strain after 4 days (Figure 3.7A). Using the exponential part of the growth curves, this corresponds to a doubling time that is 18 % longer than the control strain (WT).

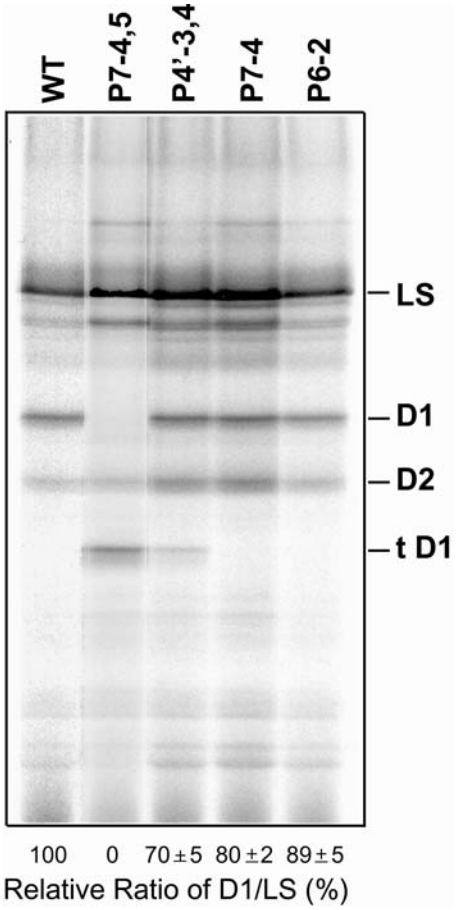


Figure 3.6 D1 protein synthesis in selected *Cr.psbA4* mutants.

Aliquots of log phase cultures of the indicated strains, grown as described in Figure 3.5, were removed and pulse-labeled with ^{14}C-acetate for 30 min in the presence of cycloheximide. An equal number of cells (1×10^6) were separated by SDS-PAGE, and a fluorograph of a gel is shown. The ratios of D1/LS polypeptide labeling were determined from 3 gels and normalized relative to the ratio in the control strain (WT), which was set to 100 %. The polypeptides identified to the right are: LS - large subunit of ribulose-1,5-bisphosphate carboxylase; D1, D2 - photosystem II reaction center polypeptides; tD1 - truncated D1.

The growth rates of the partial splicing mutants were also analyzed in cultures growing in air (with mild shaking). Although the absolute growth rates were slower as expected, growth of the P4'-3,4 mutant was still significantly (15 %) slower than the control strain. We also noticed that in older cultures of the P4'-3,4 mutant, senescence occurred earlier and was more pronounced than in the control strain or the P7-4 and P6-2 mutants.

The *psbA* transcripts were also analyzed in the autotrophic growth experiment of Figure 3.7A during the exponential phase (indicated by the arrow). RNA gel-blot hybridizations with the intronless *psbA* gene (exons 1-5 probe), and the intron-specific probe are shown in Figure 3.7B; the exposures were selected to show the accumulated precursor in the P7-4 mutant. The mature mRNA band was quantified with a phosphorimager, and its levels relative to the control strain (WT) are given below the lanes. The results were generally similar to those obtained with cells growing mixotrophically (Figure 3.5), except that the splicing efficiency was somewhat improved. For example, the P4'-3,4 mutant has ~55 % of the control levels of mature *psbA* mRNA under autotrophic conditions, compared to ~42 % in the mixotrophic cultures (Figure 3.5); the P4'-3,4 mutant also has correspondingly reduced levels of unspliced precursor in the autotrophic condition.

To determine the effects of the mutations on D1 synthesis under autotrphic growth conditions, additional aliquots were taken from the cultures at the indicated time (arrow in Figure 3.7A), and the cells were pulse-labeled for 5 min with ^{14}C-

acetate in the presence of cycloheximide. The labeled proteins were separated by SDS-PAGE, and a fluorograph of a gel is shown in Figure 3.7C. The synthesis of full-length D1 is reduced ~28 % in the P4'-3,4 mutant, and <15 % in the P6-2 and P7-4 mutants. These results are similar to those obtained with mixotrophically growing cells (Figure. 3.6), except for a modest increase in relative D1 synthesis in the P7-4 mutant.

Figure 3.7D summarizes the data on growth rates, synthesis of full-length D1, and mature *psbA* mRNA levels in the three mutants, compared to the control strain. It should be noted that if the *in vivo* splicing efficiency was expressed as the fraction of *psbA* RNA spliced (i.e, mature/mature + precursor) rather than levels of mature RNA, the results were essentially identical. Analysis of the P4'-3,4 mutant data shows evidence of parallel, although not perfect, correlations between autotrophic growth rate, full-length D1 synthesis, and mature *psbA* mRNA levels. Most importantly, however, the P4'-3,4 mutant indicates that a reduction in *Cr.psbA4* splicing of ~45 % negatively affected the autotrophic growth rate. The data also suggest that the rate of synthesis of full-length D1 is slightly (~10 % of WT) greater than is needed to sustain growth, and that mature *psbA* mRNA levels are also somewhat (~17 % of WT) greater than is needed to sustain full-length D1 synthesis.

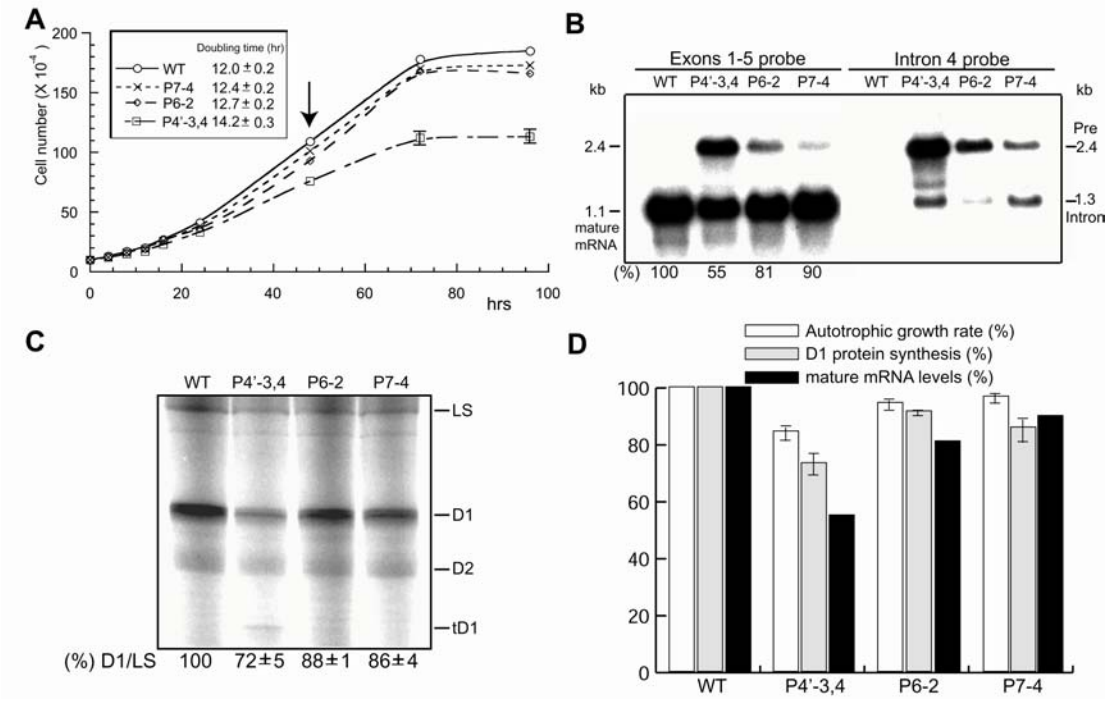


Figure 3.7 The effects of certain *Cr.psbA4* substitutions on growth rate, splicing efficiency, and D1 (full-length) synthesis in autotrophically growing cells.

WT is wild type with respect to the nucleotide substitutions; all of the strains are ORF-minus and carry the spectinomycin and DCMU resistance markers.

(A) Growth curves for selected *Cr.psbA4* mutants. 2×10^7 cells of each strain were inoculated into 200 mL of minimal medium (+ 100 µg/mL spectinomycin), and grown as described in Methods. The cell number at each time point represents the mean (± standard error) of replicate measurements; where the error bars are not visible, they were smaller than the symbols. Doubling time (in h) was calculated from the exponential phase of the curves. The arrow indicates the time at which aliquots of the cultures were removed for analyses of RNA (B) and protein synthesis (C).

(B) RNA gel-blots of total RNA (5 µg/lane) from the indicated strains, hybridized with the intronless *psbA* gene (Exons 1-5 probe), and the *Cr.psbA4* intron-specific PCR product (Intron 4 probe). The cells were taken for RNA analysis at the time indicated by the arrow in (A). The blots were quantified with a phosphorimager, and amounts of mature *psbA* mRNA, relative to the control strain (WT), which was set to 100 %, are given below the lanes. Pre, unspliced precursor; Intron, free intron; Mature, spliced *psbA* mRNA.

(C) D1 synthesis in the *Cr.psbA4* mutants. Culture aliquots were removed at the time indicated in (A) by the arrow, and pulse-labeled with ^{14}C-acetate for 5 min in the presence of cycloheximide. Equal cell numbers ((1×10^6) were separated by SDS-PAGE, and a fluorograph of a gel is shown. The ratios of D1/LS polypeptide labeling were determined from two different pulse-labelings and normalized relative to the ratio in the control strain (WT), which was set to 100 %. The proteins were identified as in Figure 6.

(D) Comparison of growth rates, mature *psbA* mRNA levels (*Cr.psbA4* splicing efficiency), and full-length D1 synthesis in the *Cr.psbA4* substitution mutants growing autotrophically. The values were obtained from panels (A), (B) and (C), and normalized relative to those for the control strain (WT), which were set to 100 %.

3.5 Discussion

The *psbA* gene of *Chlamydomonas reinhardtii* contains four group I introns whose splicing rates are very slow in the dark (in autotrophically growing cells), but increase coordinately at least 6-fold to 10-fold with the administration of light and resulting electron transport (Deshpande et al., 1997). Although this is a relatively strong effect on gene expression, the biological significance of light-promoted splicing has not been clear, in part because *psbA* mRNA is very abundant (in light or dark) (Herrin et al., 1986; Malnöe et al., 1988), and because of evidence suggesting that several chloroplast mRNAs are in excess, some as much as 10-fold, over what is needed to sustain translation rates (Eberhard et al., 2002). The present study, which describes the effects of mutations in the fourth *psbA* intron, demonstrates that highly efficient splicing of *Cr.psbA4*, and, by inference, the other *psbA* introns is also necessary for optimal photosynthetic growth.

The most informative splicing-deficient mutant is the two-nucleotide substituted P4'-3,4 mutant, which exhibited a 45 % reduction in mature *psbA* mRNA, a 28 % reduction in synthesis of full-length D1, and an 18 % reduction in growth rate under autotrophic conditions. Clearly, a 6-fold to 10-fold reduction in splicing of this intron, which could be considered the dark or basal rate, would be expected to severely limit autotrophic growth even further. Moreover, it should be emphasized that we have reduced the splicing efficiency of only one of the four introns in *psbA*; a similar but coordinate reduction in splicing should further increase this effect, since each of

these introns are spliced independently and at similar rates (Deshpande, 1997). These results provide strong support for the suggestion that light-promoted splicing of *psbA* introns functions to ensure an ample supply of spliced *psbA* mRNA for the greatly increased translation in the light (Deshpande et al., 1997).

Previous work with *Chlamydomonas* has shown that light and the circadian clock increase transcription of the *psbA* gene 2-fold to 3-fold in the early light period (Deshpande et al., 1997; Hwang et al., 1996), and that light greatly (> 10-fold) stimulates translation of *psbA* mRNA, which is also very low in the dark (Herrin et al., 1986; Malnöe et al., 1988). Based on these data in this report, we propose that the documented increases in transcription, splicing, and translation are all necessary to provide sufficient D1 to maintain optimal phototrophic growth in the light.

These results also indicate that most of the *psbA* mRNA is translated in the light. A previous report, which looked at the distribution of *psbA* mRNA between polysomes and non-polysomal fractions in *Chlamydomonas*, found only ~35 % of *psbA* mRNA in the polysomal fraction in cells growing in the light with acetate (Yohn et al., 1996). A low proportion of *psbA* mRNA on polysomes was also reported by Klein and Mullet (1990) for dark-grown barley shoots transferred to light. However, it is not clear whether the recovery of *psbA* polysomes in these studies was complete. Indeed, this is something that has rarely been shown in polysome studies. In a more recent report (Eberhard et al., 2002), a 40-50 % drop in *psbA* mRNA levels,

obtained by treating *Chlamydomonas* with rifampicin for several h, was accompanied by a decrease in D1 synthesis under mixotrophic (light+acetate) conditions, but, like several other chloroplast-encoded proteins, an increase in D1 synthesis was obtained in autotrophic conditions. Those results would suggest that the *psbA* mRNA level is close to limiting for translation in mixotrophic conditions, but not in autotrophic conditions. It is difficult to compare the present data with those results because of the quite different experimental approaches used (Eberhard et al., 2002). However, the genetic approach used here is clearly more specific than rifampicin, which inhibits essentially all chloroplast transcription.

A previous attempt to perform a mutagenic analysis of the *Cr.LSU* rRNA intron was limited by the apparent lethality of most of the mutations *in vivo*, which manifested as heteroplasmic transformants (Li et al., 2002). However, we can compare the *in vitro* effects of the substitutions on the two introns. The mutations in *Cr.psbA4* that are similar to the *Cr.LSU* study are the single-nucleotide substitutions in the P4, P6, and P7 regions, respectively (Li et al., 2002): the P6 and P7 mutations resulted in 100 % and >95 % inhibition of *Cr.LSU* splicing, compared to ~60 % and 80 % for *Cr.psbA4*, and the P4 mutations inhibited *Cr.LSU* self-splicing > 95 %, compared to 50 % for *Cr.psbA4*. This comparison indicates that *Cr.psbA4* has a more stable structure than *Cr.LSU*. In fact, the stability of *Cr.psbA4* toward point mutations is much more similar to the *Tetrahymena* intron (Flor et al., 1989; Oe et al., 2001) than to *Cr.LSU*. A greater stability for *Cr.psbA4* is also suggested by the ~10°C

higher temperature optimum for self-splicing by *Cr.psbA4* (Thompson and Herrin, 1991).

What is the biological significance of a greater structural stability for *Cr.psbA4* than *Cr.LSU*? The answer is not clear at present, but one possibility is that it helps to maintain the splicing efficiency of *Cr.psbA4* at high temperature. The effect of heat stress on splicing of these introns *in vivo* has yet to be examined, but evidence has been presented that splicing of the chloroplast *psaA* introns, which are *trans*-spliced group II introns, is partially inhibited at elevated temperature (Choquet et al., 1988). Alternatively, the data could indicate that the role of *trans*-acting factors in promoting splicing of *Cr.psbA4* is somewhat less than with *Cr.LSU*, which may require more protein assistance to stabilize its structure.

The effects of the substitutions on *Cr.psbA4* splicing *in vivo* are less than the *in vitro* effects, suggestive of considerable stabilization of the intron's structure *in vivo*. It should be noted, however, that the *in vitro* splicing reactions required a non-physiologically high temperature (45°C) to be efficient, and this could have had a differential effect on the mutant preRNAs compared to wild type. However, the fact that this difference was consistently seen with all of the mutations tends to support the validity of this comparison. The simplest explanation for the greater stability of *Cr.psbA4* in vivo is that one or more proteins bind to the partially (or completed) folded intron and steady its tertiary structure, as has been shown for several fungal mitochondrial introns (reviewed in Lambowitz et al., 1999). Candidates for such

a protein in *Chlamydomonas* are the *css* genes that were recently identified by suppressor genetics (Li et al., 2002). These nuclear mutants suppressed the splicing inefficiency of the *Cr.psbA4* P4'-3,4 mutant intron when it was transformed into them.

The effects of the core mutations on splicing of *Cr.psbA4* seem to differ from the phage intron where nucleotide substitutions in the *T4.td* intron's core produced similar effects *in vivo* and *in vitro* (Mohr et al., 1992; Myers et al., 1996). A likely explanation for this difference between chloroplasts and *E. coli* is that *E. coli* does not seem to contain a protein that specifically binds to the *T4.td* intron's core region to stabilize its structure. Presumably, it is this feature of *E. coli* that makes it possible to study the ability of the group I splicing factor, CYT18, from *Neurospora* to suppress nucleotide substitutions in the core domains of the *T4.td* intron (Mohr et al., 1992; Myers et al., 1996).

An interesting question is how light-dependent RNA splicing evolved in the *Chlamydomonas* chloroplast. Based on studies with fungi, it has been suggested that group I intron splicing factors evolved from pre-existing proteins that had other functions (Lambowitz et al., 1999). It is possible then that light-activated RNA-binding proteins (Danon and Mayfield, 1994b; Zerges et al., 2002) were recruited as splicing factors for *psbA* introns, and that these proteins have retained their light-dependent properties. Of course, this hypothesis also assumes that evolution of the *psbA* gene occurred insertion of the introns into a pre-existing *psbA* gene that was

intronless. The isolation of splicing factors for these introns, coupled with studies of *psbA* genes in other *Chlamydomonas* species, may provide support for this hypothesis.

Chapter 4 Suppression of the *Cr.psbA4* P4′-3,4 Mutation by the Nuclear Suppressors of the *Cr.LSU* P4A Intron and Genetic Analysis of the 7120 Suppressor

4.1 Summary

Previously, three suppressor strains of *C. reinhardtii* (7151, 7120 and 71N1), that restored the splicing of a deficient chloroplast rRNA intron (*Cr.LSU*), were isolated by F. Li and shown to be nuclear mutants. Here I show that all three suppressor strains can also suppress the splicing-deficient P4′-3,4 mutation in the *Cr.psbA4* intron, indicating that these genes play a role in splicing of multiple group I introns in the chloroplast. In addition, further genetic analysis was performed with the 7120 suppressor, which had been only partially characterized previously. Tetrad analysis of a cross of 7120 (mt+) with wild-type (mt-) indicated that it is a single nuclear gene mutation, based on the 2:2 ratio of progeny phenotypes. Also, complementation analysis in diploids, which were obtained from a cross of 7120, (which is also *nit1 nit2*) with a *nit4* strain, indicated that the 7120 suppressor is dominant. We propose to call this gene *css2* (for chloroplast splicing suppressor 2).

4.2 Introduction

Splicing factors for the *psbA* introns, or for that matter any chloroplast group I introns, have not been identified. F. Li (2002) isolated three suppressor strains of the splicing-deficient *Cr.LSU* intron (7120, 7151, and 71N1), and since these mutations are nuclear, they likely represent proteins that promote splicing of the *Cr.LSU* intron. I wanted to determine if these strains would also suppress the P4′-3,4 mutation in the *Cr.psbA4* intron. The reason why I chose the P4′-3,4 mutant for this test is that it is significantly, but only partially deficient in splicing, and therefore more likely to be suppressed than the completely blocked P7-4,5 mutant. Also, I was unable to isolate suppressors of the P7-4,5 mutant after UV mutagenesis (Chapter 3). And finally, since the three *Cr.LSU* suppressors were isolated based on their suppression of a mutation in the P4 helix of the *Cr.LSU* intron, it seemed reasonable that they might suppress the the P4′-3,4 mutant of *Cr.psbA4*.

The 7151 suppressor was well characterized genetically, and shown to be a single dominant mutation; it was proposed to call the gene *css1* (Li et al., 2002). The 71N1 suppressor mutation also appeared to be a single nuclear gene, since it showed a 2:2 ratio of progeny phenotypes in tetrads (Li, 2002). However, it was not determined if the 7120 suppressor was a single-gene mutation, or if it is dominant or recessive. Therefore, I decided to obtain this information as it would be necessary for developing a strategy to clone this gene.

4.3 Materials and Methods

The materials and methods for this chapter are described in Chapter 2.

4.4 Results

4.4.1 The *Cr.LSU* P4 suppressor strains also suppress the P4′-3,4 mutation in the *Cr.psbA4* intron

To determine if the *Cr.LSU* suppressors could suppress a similar mutation in one of the light-regulated *psbA* introns, the P4′-3,4 mutant construct was transformed into the 7151, 7120 and 71N1 strains. The creation and reduced splicing abilities of this mutant form of *Cr.psbA4* were described in detail in Chapter 3. The pEX4.Δ plasmid, containing the P4′-3,4 mutation (pEX4.Δ P4′-3,4) and the DCMU-resistant marker in exon 5, was bombarbed into the three strains, and the transformants were selected on minimal media plus 3 μM DCMU. The homoplasmicity of representative transformants was shown by PCR using primers that flank the intron (Figure 4.1AB). Figure 4.1B shows that the transformants contained only the short, mutant form of *Cr.psbA4*. On the other hand, the recipient strains (7120, 7151, and 71N1 in Figure 4.1B) have only the WT size of the *Cr.psbA4* intron.

The transformants containing the P4′-3,4 did not show any difference in growth phenotype on acetate-containing media (TAP) compared to three *Cr.LSU* suppressors containing the WT *Cr.psbA4* intron (data not shown).

Figure 4.2 shows the results of a RNA blot analysis of *psbA* transcripts in the transformed suppressors (lanes 2-4 and 6-8), and for comparison in a strain carrying the *Cr.psbA4* P4'-3,4 mutation in the WT background (lanes 1 and 5). The cultures used for RNA isolation were grown in medium light. Hybridization was with an intronless *psbA* gene (exons 1-5 probe; lanes 1-4 of Figure 4.2A), and with an intron-specific probe (lanes 5-8 of Figure 4.2A). The autoradiographs show that the P4'-3,4 mutant intron is inefficiently spliced in the WT background, accumulating slightly more transcript as precursor than as mature mRNA, whereas the opposite is true in the suppressor strains. The relative splicing efficiency, which is quantified in Figure 4.2B, and is based on the ratio: mature mRNA/mature mRNA + precursor, shows that in all three suppressor backgrounds, there was a significant increase (2-2.5-fold) in *Cr.psbA4* splicing efficiency. WT was considered to be 100 % efficient, since we have never observed much precursor for any of the four *psbA* introns in WT grown in the light. Also, the 7120 and 71N1 suppressors increased the splicing efficiency of the P4'-3,4 mutant slightly more than the 7151 suppressor, which is similar to their effect on the *Cr.LSU* P4A mutation (Li et al., 2002). These results indicate that these nuclear mutations can suppress similarly located mutations in at least one *psbA* intron, as well as *Cr.LSU*.

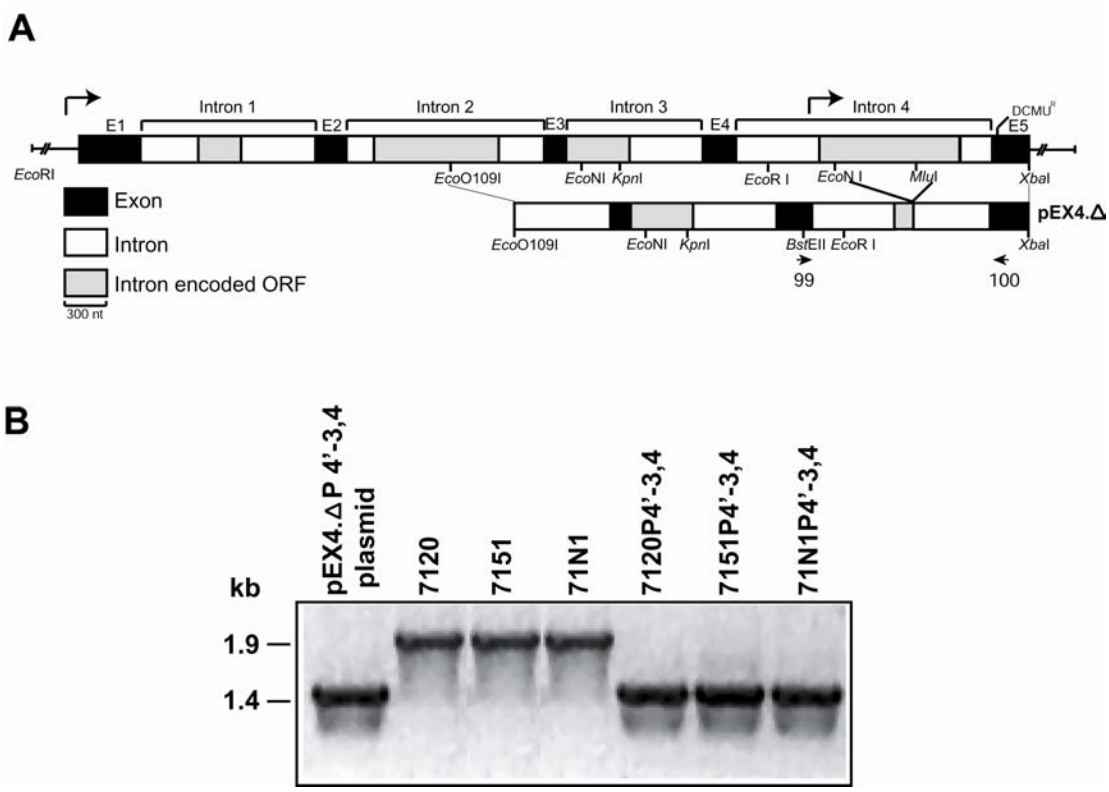


Figure 4.1 PCR analysis of the homoplasmicity of the suppressor strains (7120, 7151, 71N1) transformed with the P4′-3,4 mutant of the *Cr.psbA4* intron (7120P4′-3,4, 7151 P4′-3, and 71N1 P4′-3,4). The pEX4.Δ plasmid, containing the P4′-3,4 mutation (pEX4.ΔP4′-3,4) and the DCMU-resistant marker, was transformed into the three suppressors (7120, 7151, and 71N1), and the transformants were selected on minimal medium plus 3 μM DCMU.

(A) Schematic map of the *psbA* gene (top), and the pEX4Δ fragment used to replace WT *Cr.psbA4* intron with the P4'-3,4 mutant. The arrows indicate the direction of transcription, and the location of promoters. The internal promoter within *Cr.psbA4*, upstream of the ORF, is believed to be much weaker than the promoter upstream of exon 1 (Odom et al., 2001). A DCMU resistance mutation ($DCMU^R$) in exon 5 (Erickson et al., 1984) is indicated. The locations of primers used for PCR (#99 and #100) are indicated below pEX4.Δ. The Δ indicates a deletion of the *Cr.psbA4* ORF between the *Eco*NI and *Mlu*I sites, which also caused a frame-shift in the remaining ORF.

(B) PCR analysis of *Cr.psbA4* homoplasmicity. PCR was performed with primers #99 and #100 (see A) on DNA from the three suppressor strains (7120, 7151, 71N1) and transformants bombarded with the P4′-3,4 mutant of the *Cr.psbA4* intron (7120P4′-3,4, 7151 P4′-3,4 and 71N1 P4′-3,4). The plasmid pEXΔP4′-3,4 was used as a control. The PCR products were separated through a 1 % agarose gel containing ethidium bromide. The figure shows the only PCR product that was obtained with each DNA. Molecular size markers (not shown) were used to verify that the products were the sizes expected for the transformants.

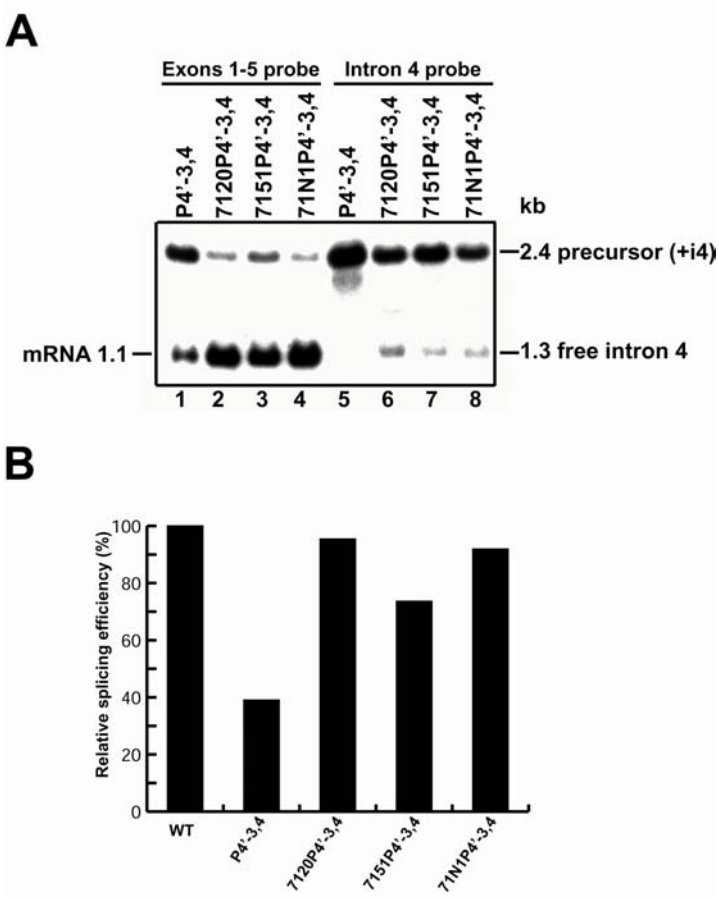


Figure 4.2 RNA analysis of the three suppressor strains transformed with the P4′-3,4 mutant of *Cr.psbA4* (7120P4′-3,4; 7151 P4′-3; and 71N1 P4′-3,4), and in a transformant carrying the *Cr.psbA4* P4′-3,4 mutation in a WT background (P4′-3,4).

(A) RNA blot analysis. The cultures were grown in acetate-containing medium (TAP) in dim light, and an equal amount of RNA (3 μg) was applied to each lane. Lanes 1-4 were hybridized with an intronless-*psbA* gene (exons 1-5 probe), and lanes 5-8 were hybridized with a *Cr.psbA4* intron-internal DNA (intron 4 probe). The 2.4-kb RNA is a precursor containing only intron *Cr.psbA4*; the excised intron is 1.3 kb. The mature *psbA* mRNA is 1.1 kb.

(B) Quantification of the splicing efficiency of the *Cr.psbA4* P4′-3,4 intron in the suppressor mutants (71.20P4′-3,4; 71.51 P4′-3; and 71.N1 P4′-3,4) and in the WT background (P4′-3,4); WT refers to the WT *Cr.psbA4* intron in a WT nuclear background. The relative splicing efficiency is based on the ratio: mature mRNA/mature mRNA+precursor, with the data normalized by considering the WT value to be 100 %.

4.4.2 The 7120 suppressor phenotype is from a single-gene mutation

The chloroplast genome of *C. reinhardtii* is uniparentally inherited from the mt + parent. Therefore, the progeny of a cross of the 7120 suppressor (mt+) with WT (mt-) should contain the mutant intron. If the suppressor mutation was nuclear and a single gene, a 2 : 2 ratio of suppressed:mutant progeny would be expected. 7120 (mt+) was crossed to WT (mt-), and 94 zygotes were transferred from maturation plates (4 % agar in TAP) to germination plates (1.5 % agar in TAP plus 100 μg/mL spectinomycin and 200 μg/mL erythromycin). The spectinomycin and erythromycin resistant markers were used in replacing the wild-type *Cr.LSU* intron with the P4 A^{125} mutant (Li et al., 2002), and thus they should also be in all the progeny. After ~12 h in dim light, I dissected 37 germinated zygotes (by touching them with a 0.2-mm diameter glass needle), and separated 36 tetrads and 1 octet.

After 11 days in dim light, 14 complete tetrads grew up to colonies and replica were plated to bright light. The progeny from 14 tetrads showed a 2 : 2 ratio of suppressed:mutant growth phenotypes under bright light (data not shown); the mutant *Cr.LSU* P4A is sensitive to bright light (Li et al., 2002).The growth phenotype of one representative tetrad for this cross using the spot test is shown in Figure 4.3A. Two of the four progeny (T1 and T2) grew in bright light, whereas two (T3 and T4) did not.

To show that the growth phenotypes co-segregated with *Cr.LSU* splicing efficiency, RNA blot hybridization of the same tetrad was performed (Figure 4.3B and 4.3C). The bottom panel in Figure 4.3B shows the methylene blue-stained RNA

pattern, whereas the upper panels show the autoradiographs obtained by hybridization with the 23S δ exon oligodeoxynucleotide (LSU exon probe), and with a *Cr.LSU* intron oligodeoxynucleotide (LSU intron probe), respectively. The 23S rRNA gene in *C. reinhardtii* contains four mature RNAs: 7S, 3S, γ, and δ, separated by three internal transcribed spacers (ITS-1, ITS-2, and ITS3) as well as the *Cr.LSU* intron (Rochaix and Malnoe, 1978; Rochaix and Darlix, 1982; Turmel et al., 1993). The observed RNAs are diagrammed in Figure 4.3C. The results show that only the two light-sensitive progeny (T3 and T4) accumulated unspliced *Cr.LSU* transcripts, and had reduced levels of the mature δ fragment; the other two progeny resembled WT. The faint ~1.3-kb RNA in the right panel of Figure 4.3B is unknown; it could be a degradation product. The stained RNA profile also shows that there is a partial reduction in chloroplast 16S rRNA levels for the splicing-deficient clones (T3 and T4), as seen before (Li et al., 2002). These results indicate that the 7120 suppressor mutation is in a single nuclear gene, which we propose to call *css2* (for chloroplast splicing suppressor).

4.4.3 The *css2* mutation is dominant in stable diploids

The dominance or recessiveness of the *css2* mutation was assessed in vegetative diploids carrying the suppressor mutation and its corresponding WT allele (and the P4A mutation in the *Cr.LSU* intron). Since stable diploids appear at a low

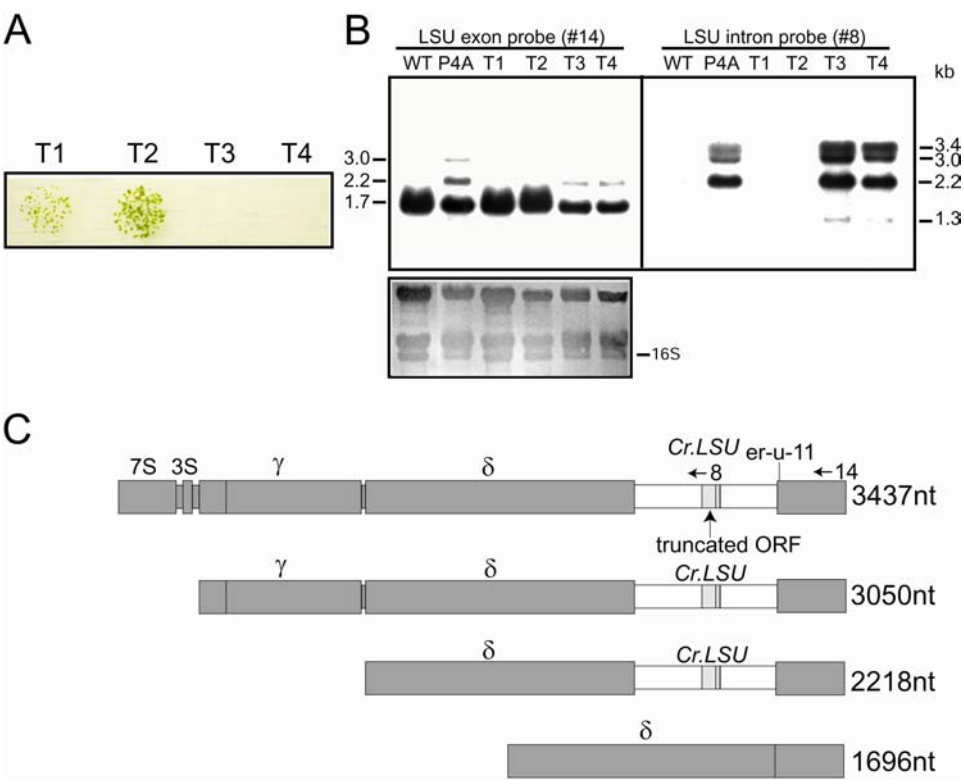


Figure 4.3 Tetrad analysis of the 7120 suppressor. The 7120 strain (mt+) was crossed to WT (mt-), and 14 complete tetrads were dissected.

(A) Growth of a representative tetrad progeny in bright light using the spot test. The progeny from a complete tetrad grown in liquid TAP medium (~1 × 10^4 cells) were pipetted onto a TAP plate, incubated in bright light for 4 days, and then photographed.

(B) RNA blot analysis of the tetrad progeny. Total RNA was isolated from the same tetrad progeny in (A) grown in liquid TAP medium in dim light. The RNAs (3 μg) were separated on a 1.4 % denaturing agarose gel, blotted to a nylon membrane, and stained with methylene blue (bottom panel). The blot was hybridized to a ^{32}P-oligodeoxynucleotide-specific for the δ fragment of the exon (LSU exon probe). After stripping the blot, it was hybridized to a ^{32}P-oligodeoxynucleotide specific for the *Cr.LSU* intron (LSU intron probe). The size of each band is indicated on both sides of the autoradiograph. The labels in the figure are as follows: WT, wild-type strain; P4A, mutant with the P4A mutation in *Cr.LSU*; T1-T4, individual progeny from the tetrad; 16S, 16S rRNA.

(C) Diagrams of the major observed RNAs. The length of each RNA (nt) is given to the right. The locations of the oligonucleotides used in (B) are indicated (8 and 14) in the top diagram. The labels are as follows: 7S, 7S rRNA (α fragment of the 23S rRNA); 3S, 3S rRNA (β fragment of the 23S rRNA); γ, γ fragment of the 23S rRNA; δ, δ fragment of the 23S rRNA. The intron is indicated as the open rectangle in δ. *er-u-11* indicates the erythromycin-resistant marker.

frequency (~5 %) in crosses with *C. reinhardtii* (Harris, 1989), they were selected for using complementing mutations (Fernandez and Matagné, 1986). Also, the diploids show some unique characteristics that are typically used in identifying them; the length of the cell is ~50-100 % longer than haploid cells, and they are mating type minus (Harris, 1989).

The WT strain used to isolate the *css2* mutation also carries the *nit1* and *nit2* mutations, which result in a loss of nitrate reductase activity and therefore an inability to grow on nitrate as sole nitrogen source (Harris, 1989). Thus, the 7120 suppressor was crossed with a complementing *nit4* (mt-) mutant, and the mating mixtures plated on nitrate-only (i.e., no ammonia) medium. Initially, this cross did not produce any vegetative diploids probably due to an unknown nuclear mutation(s) in the suppressor. Therefore, I determined to remove the interfering mutation(s) from 7120 by backcrossing 7120 (mt+) with WT (mt-), dissecting tetrads, and identifying progeny that grew well under bright light (and 100 μg/mL spectinomycin and 200 μg/mL erythromycin). I then identified those progeny that were also mating type plus (mt+) [by mating the gametes to mating type-minus gametes and observing the zygote pellicle (Harris, 1989)]. I also checked for the inability of the progeny to grow on nitrate as sole nitrogen source, which was as expected since both parents are *nit1 nit2*. I proposed to call the desired product 7120_2G.

The 7120_2G strain (mt+) was then crossed with the complementing *nit4* (mt-) strain, and diploids were selected on nitrate-only (i.e., no ammonia) medium. A

number of putative diploid clones were obtained, and three of them were analyzed with respect to cell size and mating type. The average size of the diploids was ~50 % larger than haploid cells, one of which is shown in Figure 4.4A. The mating type was verified as minus by observing the zygote pellicle after mating with the WT strain (mt+) (data not shown). Also, since the inheritance of chloroplast DNA in diploid progeny is not as strongly biased for the mt+ parent as in meiotic zygotes (Van Winkle-Swift, 1978), PCR was used to confirm that the short (i.e., mutant) form of *Cr.LSU* was the only one present in the diploid clones (Figure 4.4B).

The growth phenotypes of the diploids on solid TAP medium in bright light are shown in Figure 4.4C; their growth was similar to WT. RNA blot analyses, presented in Figure 4.3D, show that the 23S rRNA profiles in the diploids are also similar to WT; they did not accumulate unspliced precursors (Figure 4.3D). These results indicate that the 7120 suppressor mutation is dominant.

4.5 Discussion

4.5.1 Suppression of a splicing-deficient *Cr.psbA4* intron: implications for *css1* function

These data demonstrate that the 7151, 71N1 and 7120 suppressors are not specific for the *Cr.LSU* intron, and that they also suppress a destabilizing mutation

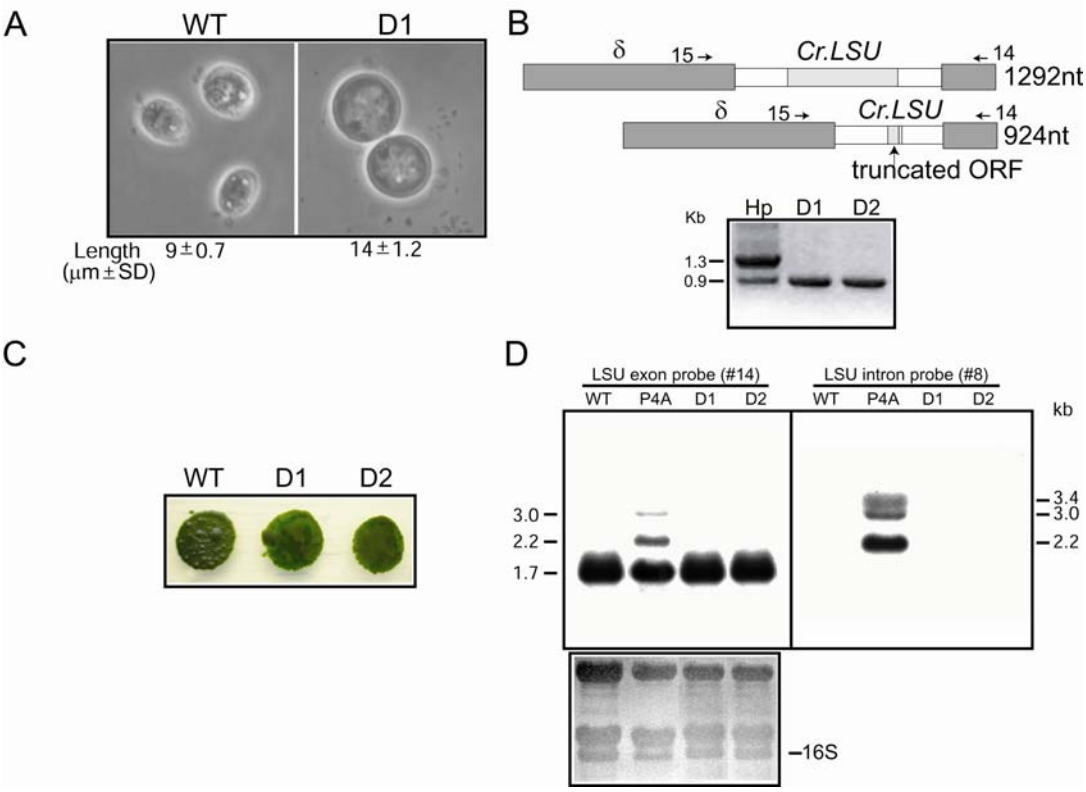


Figure 4.4 Characterization of vegetative diploids containing the mutant *Cr.LSU* intron and the 7120 suppressor. The diploids were obtained from a cross of the 7120_2G suppressor strain (mt+), which is *nit1*/*nit2*, with a complementing *nit4* mutant, and the diploids were selected on plates containing nitrate as the sole nitrogen source.

(A) The cell size of the vegetative dipoids. Three putative diploids (D1, D2, and D3) and haploid WT cells were grown in liquid TAP media for five days in medium light. The images were captured with a cool CCD camera connected to a phase contrast microscope (400 × magnification). A representative image of diploid D1 is shown. The lengths of 9 cells from each diploid clone and 27 haploid WT were measured using NIH Image. Units are in μm± standard deviation (μm±SD).

(B) PCR analysis to verify the homoplasmicity of the *Cr.LSU* P4A intron in two diploids. PCR was performed with primers (#14 and #15) that flank the *Cr.LSU* intron (see diagram) using DNA from diploid clones D1 and D2. A heteroplasmic haploid transformant (Hp) was used for size comparison. The diagrams are of the PCR products expected from the WT intron (1292 nt), and from the ORF-truncated intron (924 nt). The figure shows the only PCR products that were obtained with each DNA. Molecular size markers (not shown) were used to verify that the products were the sizes expected for the WT and mutant introns, respectively.

(C) Growth phenotype of D1 and D2 (the same dipoids used in A and B) in bright light. Each strain (1×10^4 cells in liquid TAP) was pipetted ontosolid TAP medium and grown for six days in bright light.

(D) RNA blot analysis of the diploids. Total RNA from D1, D2, haploid WT, and the *Cr.LSU* P4A mutant were separated on a 1.4 % denaturing agarose gel, and blotted onto a nylon membrane that was stained with methylene blue (bottom panel in D). The blot was hybridized with the δ exon-specific oligodeoxynucleotide (LSU exon probe (#14)). After stripping, the blot was hybridized with the *Cr.LSU* intron-specific oligodeoxynucleotide (LSU intron probe (#8)). The size of each band is indicated, and diagrams of the RNAs are given in Figure 4.3C.

in the P4 region of the *Cr.psbA4* intron (i.e., they increased the splicing efficiency of the *Cr.psbA4* P4′-3,4 mutant). This result is significant for two reasons. First, it suggests that there is a role for these genes in splicing of the light-regulated *Cr.psbA4* intron. Moreover, since the core structure of *Cr.psbA4* is very similar to that of *Cr.psbA1* (Holloway *et al*., 1999), this role likely extends to *Cr.psbA1*. Further, *Cr.LSU* is in a different structural subgroup (IA2) from *Cr.psbA4* and *Cr.psbA1* (which are in subgroup IA1), although it is in the same subgroup as *Cr.psbA3* (Holloway *et al*., 1999). Thus, these genes might promote splicing of all four *psbA* introns as well as *Cr.LSU*. Further research will be required to determine if this is indeed the case.

4.5.2 *Css2*: *a* single dominant nuclear suppressor of the splicing-deficient *Cr.psbA4* and *Cr.LSU* introns

In previous work (Li et al., 2002), it was not determined if the 7120 suppressor was a single-gene mutation or if multiple loci were involved. Here I showed that the 7120 suppressor mt(+) is a single nuclear gene by crossing it to the WT mt(-) and dissecting tetrads. All of the tetrads showed a 2:2 ratio of suppressor:mutant phenotypes.

The 7120 mutation was also shown to be dominant in vegetative diploids. These were obtained from a cross of a suppressor strain carrying the 7120 mutation (that was also *nit1 nit2*) (mt+) with the complementing *nit4* (mt-) strain. The

diploids, which were selected based on their ability to utilize nitrate, grew well in bright light and showed no evidence of reduced *Cr.LSU* splicing. This latter information is important, in part, because it suggests that one could clone the *css2* gene by complementation of a strain containing the mutant intron using a cosmid library from the 7120 suppressor (Zhang *et al*., 1994).

I also tried to perform diploid analysis of the 71N1 suppressor (mt+), to determine whether it is a dominant mutation. However, no diploids were obtained, probably due to unknown mutation(s) in 71N1 that blocked formation of stable diploids. Although I tried to remove these mutations by a back-cross with WT, no dipoids were obtained. Further back-crosses of 71N1 with WT may be needed before diploid analysis will be possible with this mutant.

4.5.3 The possible mechanism of action of *css2*

A likely explanation for the function of *css2* is that it encodes a protein that binds to the core of these introns (including P4), and either stabilizes the correctly folded structure or promotes correct folding (Guo and Lambowitz, 1992; Mohr et al., 1992; Weeks and Cech, 1995a, b, 1996). Another possibility is that *css2* functions by interacting with the exons and preventing them from interfering with folding of the intron, i.e., as a chaperone (Coetzee *et al*., 1994**;** Zhang *et al*., 1995**;** Semrad and Schroeder, 1998); exon sequences have been shown to affect the efficiency of *Cr.LSU* self-splicing *in vitro* (Kuo and Herrin, 2000). However, since the exonic context of

Cr.psbA4 is completely different from *Cr.LSU* (i.e. mRNA versus rRNA), it seems unlikely that *css2* acts mainly on the exons. Another possible mechanism is that *css2* is a Mg^{2+} transporter of the chloroplast envelope, and that the suppressing mutation produces elevated Mg^{2+} levels in the organelle, which, in turn, improve splicing of the mutant introns. This idea derives from studies with yeast where multicopy suppressors of a mutant group II intron turned out to be ion (probably Mg^{2+}) transporters (Bui et al., 1999; Gregan et al., 2001; Kolisek et al., 2003). However, to our knowledge, screens for group I intron suppressors in yeast did not turn up similar proteins (reviewed in Dujardin and Herbert, 1996). Moreover, self-splicing group II introns generally have much higher Mg^{2+} requirements than group I introns (Michel and Ferat, 1995). Nonetheless, we cannot rule out the possibility that *css1* or *css2* act indirectly to promote splicing. The cloning of these genes, which is feasible in *C. reinhardtii*, should enable their mechanism of action to be determined precisely.

4.5.4 How many genes are involved in *Cr.LSU* and *Cr.psbA4* intron splicing?

F. Li (2002) proposed that there was at least one other loci besides *css1* that promoted splicing of the deficient *Cr.LSU*. Here I show that *css2* is the other locus to promote splicing of *Cr.LSU*, that both loci promote splicing of *Cr.psbA4*. It is not clear, however, whether the 7151 and 71N1 suppressor mutations are in the same gene, so it will be necessary to analyze more tetrads from crosses of 7151 with 71N1 to determine if this is the case. Also, this screen may not have been saturating, and

there could be additional suppressor genes to be identified. It may be relevant that 14 nuclear genes are required for splicing the two *psaA1* introns in the *C. reinhardtii* chloroplast (reviewed in Herrin et al., 1998); although it should be noted that these introns are group II and are spliced *in trans* (Kück et al., 1987). However, multiple genes have also been implicated in the splicing of mitochondrial group I introns in yeast and *Neurospora* (Burke, 1988**;** Lambowitz et al., 1999).

Chapter 5 Replacement of the ORF of the *Cr.psbA2* Intron with a Selectable Marker: Implications for Intron ORF Expression and Function

5.1 Summary

The ORF of the *Cr.psbA2* intron, whose function is unknown, was replaced with the bacterial *aadA* gene, which confers streptomycin and spectinomycin resistance. I was unable to replace the *Cr.psbA2* intron in wild type cells with this *Cr.psbA2-aadA* construct for reasons that are not clear. However, it was possible to integrate the *aadA*-containing intron into the intronless *psbA* gene when the *Cr.psbA2* ORF was provided on a separate plasmid. These transformants grew normally on minimal medium, and analysis of *psbA* RNA demonstrated that splicing of the *Cr.psbA2-aadA* intron was efficient *in vivo*. The *Cr.psbA2-aadA* transformants did not show any evidence of streptomycin resistance, however; indicating that expression of *aadA* from within the intron was poor. This finding may have implications for why the chloroplast genome tolerates the presence of group I introns with their potentially toxic homing endonucleases. These data also confirm that *Cr.psbA2* is a mobile intron, and, moreover, indicate that the *Cr.psbA2* ORF does not encode an essential maturase.

5.2 Introduction

Some group I introns encode site-specific homing endonucleases that promote intron mobility by specifically cutting the intronless allele near the point of intron insertion (Dujon, 1989). Odom et al. (2001) used constructs containing *Cr.psbA4* and introduced these into the intronless-*psbA* strain, IL. It was shown that *Cr.psbA4* integrated correctly into the intronless *psbA* gene, and that integration was dependent on its ORF (Odom et al., 2001).

Also, N. Deshpande (1996) showed that the *Cr.psbA2* intron correctly integrated into the intronless *psbA* gene when it was introduced by biolistic transformation. The construct contained flanking exon sequences and the *atpA* promoter to drive expression. Intron integration was dependent on the intron construct being in the sense orientation. This suggested that *Cr.psbA2* was possibly a mobile intron; however, it was not shown that integration of the intron was specifically dependent on the ORF

Four different types of endonucleases have been found in group I introns, and these are distinguished by the presence of a conserved amino acid motif: LAGLIDADG, GIY-YIG, H-N-H, or His-Cys. Also, several of the homing endonucleases, all of which are LAGLIDADG proteins and found in fungal mitochondrial introns, have been shown to promote splicing of the pre-mRNAs that encode them. These proteins are called maturases. A maturase has yet to be identified in a chloroplast group I intron, and the Herrin lab has ruled out the *Cr.LSU*,

Cr.psbA3 and *Cr.psbA4* introns as possibly having important maturases by deleting most of those ORFs from each intron *in vivo* (Thompson and Herrin, 1991; O.W. Odom, unpublished results). Therefore, I also wanted to determine if the ORF of the *Cr.psbA2* intron, which contains a GIY-YIG motif, encodes an essential maturase.

To test for a possible maturase function for the *Cr.psbA2* ORF, I attempted to replace it with a selectable marker, the bacterial *aadA* gene, which confers spectinomycin and streptomycin resistance. This was done, rather than just deleting the ORF, because I wanted to see if a selectable marker could be expressed from within a chloroplast group I intron. If so, it might be possible to use it to isolate mutants deficient in splicing (i.e., they would over-accumulate the precursor) and/or turnover of the excised intron, since both the precursor and exised intron RNAs could be translated to produce the *aadA* enzyme.

5.3 Materials and Methods

The materials and methods for this chapter are described in Chapter 2.

5.4 Results

5.4.1 Generating a strain with the *aadA* gene replacing the ORF in *Cr.psbA2*

The DNA that was constructed to replace the *Cr.psbA2* ORF with the *aadA* gene, I2-aadA (the details of its construction are given in section 2.3.2), is

shown in Figure 5.1A. It has 3.3-kb more upstream sequence than pGEMI2AAD-ORF (the details of its construction are in Appendix I) from which it was derived. The I2-aadA plasmid was shot into WT 2137 by itself, as well as by co-transformation with the spectinomycin-resistance marker (pb4c110). The transformants were selected on TAP medium plus 100 μg/mL spectinomycin. DNAs from 50 spectinomycin-resistant transformants were isolated and subjected to PCR using primers #118 and #136, which anneal on either side of the intron (Figure 5.1A). All 50 transformants gave only a 1.54-kb PCR product typical of WT, indicating no replacement of the *aadA*-containing intron (data not shown). The reason for this result was not obvious, but it might have been due to a recombination cold spot between sequences upstream of *psbA* exon 1 and the *Cr.psbA3* intron (Newman et al., 1992). The shorter pGEMI2AAD-ORF plasmid also did not replace the WT *Cr.psbA2* intron when it was bombarded into cells.

An alternative approach that proved successful was to rely on intron homing to integrate the *aadA*-containing intron into the intronless *psbA* gene. This was accomplished by co-transforming in three plasmids: I2-aadA, atpX-i2S, which provided the *Cr.psbA2* ORF *in trans* (Figure 5.1A), and pb4c110 to provide strong spectinomycin resistance. I increased the probability of integration of the ORF-substituted intron over the WT version from atpX-i2S by bombarding cells with 10-fold more I2-aadA than atpX-i2S. This was necessary because the intron from atpX-i2S integrates efficiently by itself (Odom et al., 2001).

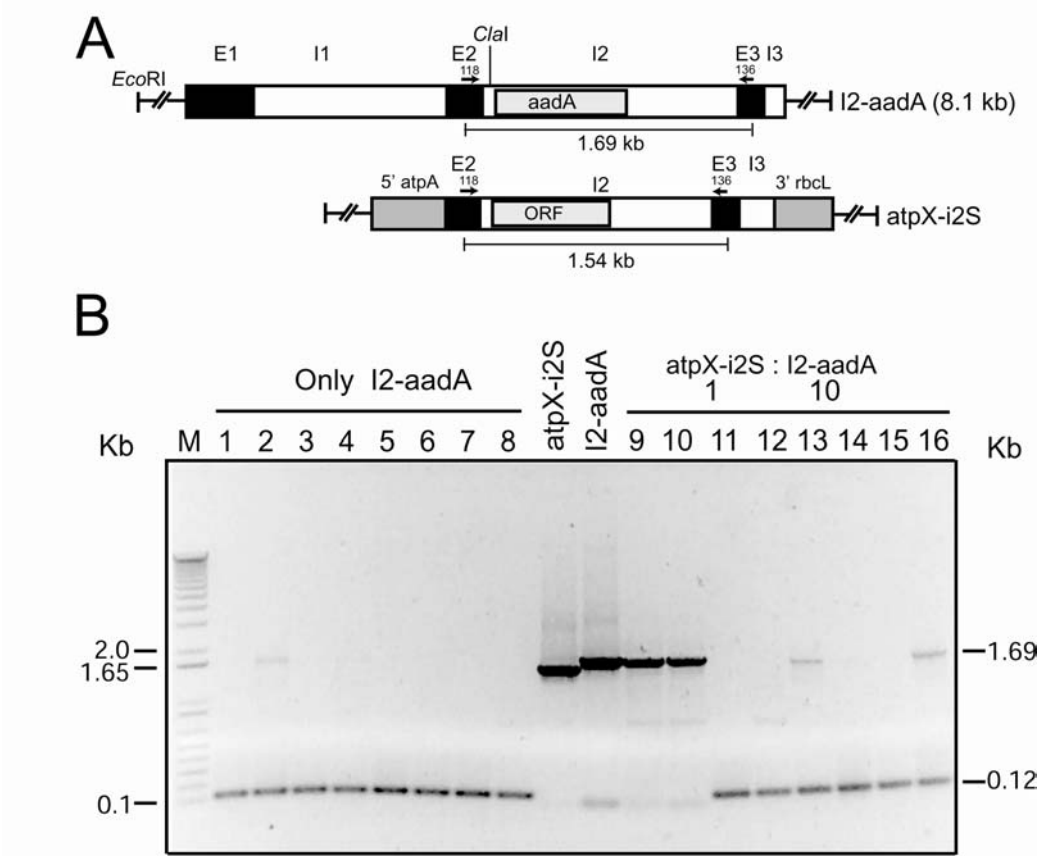


Figure 5.1 PCR analysis of the integration of the *Cr.psbA2-aadA* intron into the IL *psbA* gene. The IL strain was co-transformed with plasmids pb4c110, atpX-i2S, and I2-aadA (a ratio of 1:10), or just pb4c110 and I2-aadA. Plasmid pb4c110 provided spectinomycin resistance. atpX-i2S provided expressed wild type intron, and I2-aadA provided the *aadA*-substituted intron, both with flanking exon sequences. Transformants were selected on spectinomycin, and then analyzed by PCR for intron integration.

(A) Diagram of the inserts of plasmids atpX-i2S and I2-aadA. Locations of the primers used for PCR and the expected sizes of the PCR products are indicated. Restriction sites (*Eco*RI and *Cla*I) used to add to the shorter insert from pGEMI2AAD-ORF (Appendix I) are indicated.

(B) Agarose gel analysis of the PCR products. Lanes 1-8 are of spectinomycin-resistant transformants bombarded with I2-aadA and pb4c110, whereas lanes 9-16 were from bombardments of all three plasmids. The PCR products from plasmids atpX-i2S and I2-aadA were also analyzed as size markers. The positions and sizes (in kilobases) of selected DNA markers (lane M) are indicated to the left. The 0.12 kb product is from the intronless *psbA* gene. The faint band migrating just faster than the 0.12 kb band may be from primer-dimer as it was variable and not specific to transformant DNA.

Transformants were selected on TAP plates containing 100 μg/mL spectinomycin and PCR analysis was performed using primers that flank the *Cr.psbA2* intron (Figure 5.2). Six of eleven transformants from the 3-plasmid bombardment contained the *Cr.psbA2-aadA* intron, four were homoplasmic and two were heteroplasmic. PCR analysis of 8 of the transformants is shown in Figure 5.2B (transformants #9-16). In contrast, only one intron integration event was observed among 48 spectinomycin-resistant transformants that were bombarded with only I2-aadA and pb4c110, and not atpX-i2S. This result confirms that the *Cr.psbA2* intron is mobile and that the ORF probably encodes an endonuclease, at least.

5.4.2 The ORF of the WT *Cr.psbA2* intron does not encode an essential maturase

RNA blot analysis of a homoplasmic transformant with the *aadA*-containing intron was performed to assess the effect, if any, of the ORF substitution on *Cr.psbA2* splicing. The DNA hybridization probe was a 1.7-kb internal *Xba*I fragment of the *psbA* gene that contained 81 bp of exon 2, *Cr.psbA2*, exon 3, and 198 bp of *Cr.psbA3* (Herrin and Michaels, 1985). Figure 5.2 shows the results of RNA blot analysis of the *aadA*-containing transformant (I2aadA) and WT for comparison. The I2aadA strain showed a small amount of a 2.7-kb RNA, which is the size expected for the *aadA*-containing precursor (see diagram in Figure 5.2), as well as mature *psbA* mRNA (1.1 kb). Although the I2aadA strain appeared to have less mature *psbA* mRNA than WT, there was no substantial difference in the ratio of precursor to mature RNA in these

two strains. The I2aadA lane may have had somewhat less RNA loaded, based on the staining of the 18S and 16S rRNAs (shown below the autoradiograph in Figure 5.2). Also, although the DNA probe had a small amount of intron *Cr.psbA3*, we did not detect a RNA in WT that would have contained unspliced *Cr.psbA3*, which would have been 2.2 kb. This result indicates that the ORF of *Cr.psbA2* does not encode an essential maturase. However, we cannot rule out the possibility that it has maturase activity that is not needed.

To determine if there was significant expression of the *aadA* gene beyond splicing of the *Cr.psbA2-aadA* intron, two I2-aadA transformants (I2aadA-1 and I2aadA-2) and the recipient IL strain were grown on a TAP plate containing 30 μg/mL streptomycin, and on minimal media for 4 days in medium light. Prior to this test, I had determined that this was the minimum concentration of streptomycin needed to effectively kill the IL strain. Also, the I2aadA-1 and I2aadA-2 transformants were already spectinomycin resistant from the marker in the 16S rRNA gene. Figure 5.3A shows that neither I2aadA-1 nor I2aadA-2 grew on 30 μg/mL streptomycin, indicating that expression of the *aadA* gene in *Cr.psbA2* must be poor.

Figure 5.3B also shows that the transformants grew as well on minimal medium as the recipient IL strain, indicating that the ORF substitution did not negatively effect photoautotrophic growth.

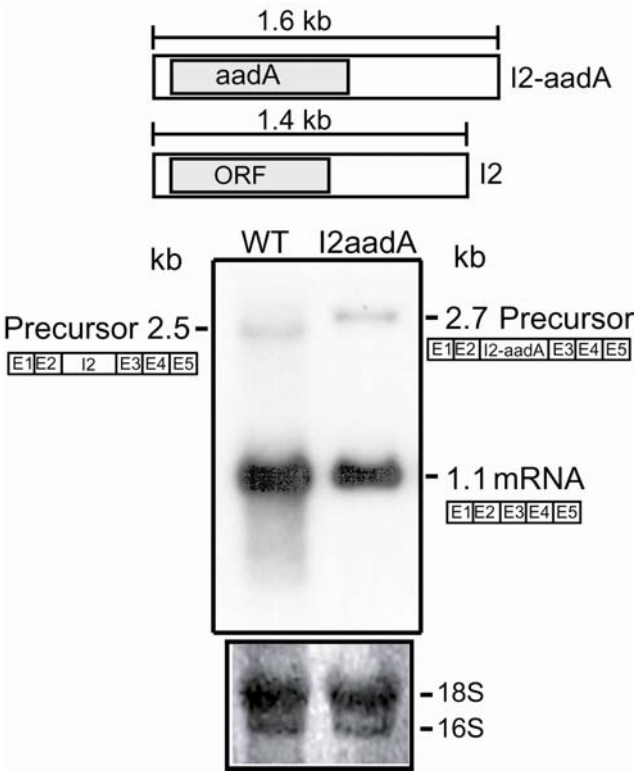


Figure 5.2 Analysis of *psbA* transcripts in an I2-aadA transformant and WT. Total RNA was isolated from cultures of WT and a *Cr.psbA2-aadA* transformant grown in medium light. The RNAs (4 μg) were separated on a denaturing agarose gel, blotted to a nylon membrane, and stained with methylene blue (the 18S and 16S rRNAs are shown in the bottom panel). The blot was hybridized to ^{32}P-labeled DNA containing exon 2, *Cr.psbA2*, exon 3, and small portion of *Cr.psbA3*. The sizes of the hybridizing RNAs were determined from the known sizes of the *C. reinhardtii* rRNAs. The structures of the RNAs are explained at the top and on both sides of the autoradiograph. A precursor containing intron *Cr.psbA3* was not observed in WT RNA, which would have been 2.2 kb.

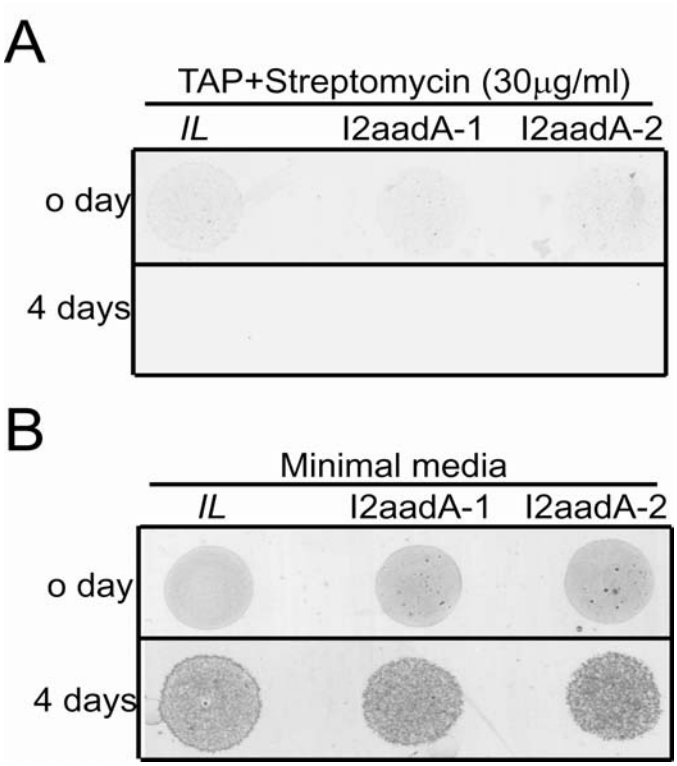


Figure 5.3 The I2aadA-containing transformants are not resistant to streptomycin, but grow well photoautotrophically in the absence of drug. Two *Cr.psbA2-aadA* transformants (I2aadA-1 and I2aadA-2), and the recipient IL strain, were spotted on solid medium, which was scanned (0 day), then incubated in the light for 4 days and scanned again (4 days).

(A) Each strain was spotted onto a TAP plate containing 30 μg/mL streptomycin.

(B) Each strain was spotted onto a plate of minimal medium.

To exclude the possibility that the *aadA* ORF itself was somehow inactive, I determined whether the construct used to make the I2-aadA plasmid, pGEMI2AAD-ORF, could confer resistance to streptomycin and spectinomycin on *E. coli*. The plasmid was transformed into *E. coli* DH5α, and the presence of the plasmid was verified with DNA mini-preps. Figure 5.4 shows the growth of a pGEMI2AAD-ORF transformant in spectinomycin (100 μg/mL) and streptomycin (25 μg/mL), respectively, compared to a clone transformed with the parental pGEM3zf(+) vector (vector only lines in Figure 5.4B). The absorbance at 550 nm (A_{550}) was measured at intervals of 1h to check growth rates. The data shows that the pGEMI2AAD-ORF transformant grew well in liquid LB medium containing 100 μg/mL spectinomycin and somewhat slower in 25 μg/mL streptomycin, whereas the vector-only transformant did not grow at all with either drug. The growth rate of the pGEMI2AAD-ORF transformant in the presence of spectinomycin in Figure 5.4B was similar to the growth rate in the absence of either drug (data not shown), indicating a complete resistance to spectinomycin at the concentrations used (up to 100 μg/mL). However, the figure also shows that 25 μg/mL streptomycin partially inhibited growth of the pGEMI2AAD-ORF transformant, and even greater inhibition occurred at higher concentrations of streptomycin (50 and 100 μg/mL) (data not shown). These relative differences in spectinomycin versus streptomycin resistance have been seen before with chloroplast transformants expressing *aadA* (Goldschmidt-Clermont, 1991).

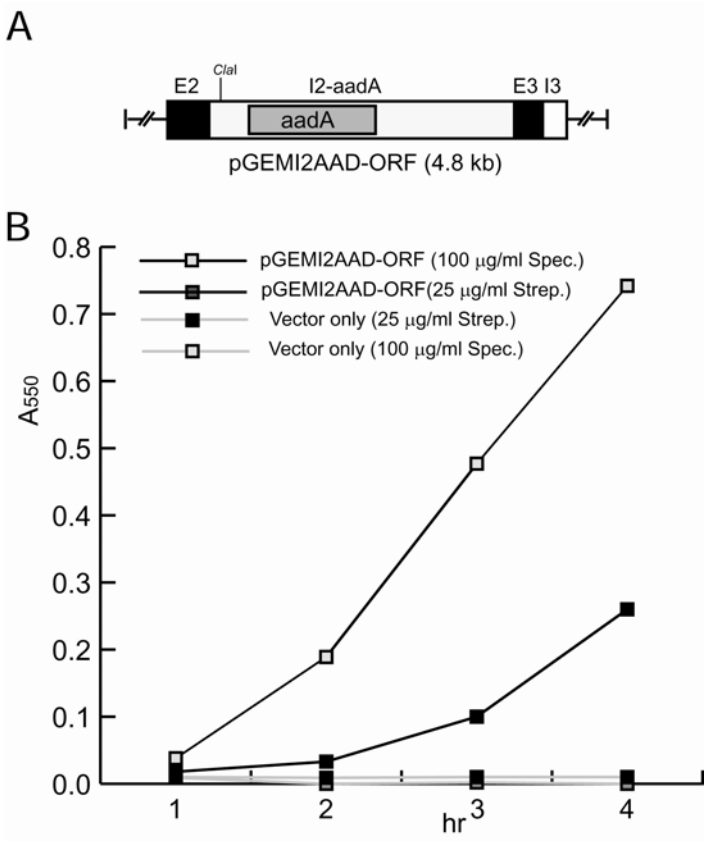


Figure 5.4 Spectinomycin and streptomycin resistance of an *E. coli* transformant with the *aadA*-containing *Cr.psbA2* intron.

(A) Diagram of the insert of pGEMI2AAD-ORF, which contains the *aadA*-containing *Cr.psbA2* intron in the pGEM3zf(+) vector.

(B) Growth of an *E.coli* transformant containing the pGEMI2AAD-ORF plasmid, and one containing the vector pGEM3zf (+) only (Vector only). 50 μl of stationary phase liquid cultures were inoculated into 5 mL of LB (plus 100 μg/mL ampicillin) with the addition of the indicated concentrations of either spectinomycin or streptomycin. The cultures were incubated at 37°C with shaking, and absorbance at 550 nm (A_{550}) was measured at intervals of 1 h.

5.5 Discussion

5.5.1 *Cr.psbA2* is a mobile intron

N. Deshpande (1996) showed previously that *Cr.psbA2* correctly integrates into an intronless *psbA* gene from a chloroplast expression vector when it was introduced by biolistic transformation. In this study, I showed that expression of the intact *Cr.psbA2* intron can mediate, *in trans*, the integration of a disabled (i.e, ORF-substituted) *Cr.psbA2* intron into the intronless *psbA* gene. These results indicate further that *Cr.psbA2* is a mobile intron.

Cr.psbA2 aadA integration (~36%) *in trans* into the IL gene was less efficient than a similar experiment with the *Cr.psbA4* intron (Odom et al., 2001). In fact, for the *Cr.psbA4 trans*-homing experiment a weak internal promoter was sufficient, whereas in this case, a strong external *atpA* promoter was used to express the intron ORF. Possibly, the endonuclease activity of the *Cr.psbA2* ORF is much poorer than the endonuclease encoded by *Cr.psbA4*. Indeed, H-H. Kim (personal communication) was unable to get endonuclease activity with the *Cr.psbA2* ORF gene product expressed in *E. coli*, although there could be technical rather than biological reasons for this.

5.5.2 The ORF of the *Cr.psbA2* intron does not encode an essential maturase

So far, a few group I intron ORFs containing the LAGLIDADG motif have been found to have essential maturase activity, based on the fact that mutating the ORF led to splicing defects *in vivo* (Perlman and Lambowitz, 1990). Here I showed that the *Cr.psbA2* ORF, which contains a GIY-YIG motif, could be replaced with the *aadA* gene, and there was no significant effect on *Cr.psbA2* splicing *in vivo*. Thus, the *Cr.psbA2* ORF is not an essential maturase. All of the large ORFs in the chloroplast group I introns of *C. reinhardtii* have now been tested for *in vivo* maturase activity, and no evidence has been obtained for such a function (Thompson and Herrin, 1991; O.W. Odom, unpublished rsults; this study). Proteins that promote splicing of these introns are likely to be nuclear-encoded proteins, such as the *css1* and *css2* genes.

One motivation for this work was to see if a selectable marker could be expressed from within a chloroplast group I intron, and then to use isolate mutants with enhanced expression of the intron marker. These mutants might be deficient in splicing or turnover of the excised intron. The *Cr.psbA2-aadA* transformants did not show any evidence of streptomycin resistance, indicating that expression of the *aadA* gene from within the intron was quite low, although the cells did accumulate a detectable amount of unspliced precursor. The I2aad-1 and I2aad-2 transformants were also mutagenized with UV light and plated on TAP + 30 μg/mL streptomycin in dim light. I obtained 5 streptomycin-resistant mutants, but RNA blot analysis revealed no over-accumulation of unspliced or free *Cr.psbA2* intron

(unpublished results).

I verified that the *Cr.psbA2-aadA* construct was functional by showing that it provided *E. coli* with drug resistance. Since there was no external promoter in the right orientation to transcribe the pGEMI2AAD-ORF insert, it is possible that *Cr.psbA2* has an internal promoter similar to *Cr.psbA4* (Odom et al., 2001). In fact, there is a putative -35 (TTGATT) and -10 (TTATAT) sequence upstream of the ORF in *Cr.psbA2*. It might be interesting to see if this is indeed an internal promoter by using primer extension analysis and other methods.

The potential evolutionary significance of these results is that poor expression of certain endonuclease genes within introns might be adaptive, since these proteins could be detrimental to the integrity of the chloroplast genome. I-*Cre*I, the homing endonuclease encoded within the *Cr.LSU* intron, was not detected in *C. reinhardtii* extracts using an antibody (Dürrenberger and Rochaix, 1991). Apparently, however, the *Cr.psbA4* endonuclease is not so toxic, possibly because it is a "very, very rare cutter".

Chapter 6 Summary and Conclusions

The *psbA* gene of *C. reinhardtii* encodes the D1 protein of photosystem II, and has four large group I introns whose splicing is stimulated 6-10-fold by light (Deshpande et al., 1997). Although this is a relatively strong effect on gene expression, there have been reports suggesting that some chloroplast mRNAs are in great excess over what is needed for translation. Thus, one major goal of my research was to try to determine the physiological significance of light-promoted splicing of *psbA* pre-mRNA, given that the *psbA* mRNA is abundant and translationally regulated. Also, there have been very few comparisons of the effects of mutations on splicing of a group I intron *in vitro* and *in vivo*.

I generated several point mutations (P3, P4, P6, P6a, P7, P11 helices) in the wild-type *Cr.psbA4* intron, and tested their effects on self-splicing. I also replaced the wild-type intron *in vivo* with each mutant by co-transforming them into chloroplasts with a spectinomycin-resistance marker. There were parallel effects of the mutations on *in vitro* and *in vivo* splicing; however, there was generally less effect *in vivo*, suggestive of stabilization of the intron's active structure. Interestingly, there were approximate correlations between *in vivo Cr.psbA4* splicing efficiency, synthesis of full-length D1, and photoautotrophic growth rates. The most informative mutant was the P4′-3,4 mutant, which showed a 45 % reduction in *psbA* mRNA, a 28 % decline in synthesis of full–length D1, and a 18 % decline in photoautotrophic growth rate. We infer from these results that *psbA* mRNA is not in great excess (although it

may be in slight excess under our standard autotrophic growth conditions), and that the majority of *psbA* mRNA molecules are translated. In addition, the data suggest that the efficient splicing of *psbA* introns, which is afforded by light, is essential for optimal photoautotrophic growth. It would be interesting to clone a gene responsible for the light-promoted splicing of *psbA* pre-mRNA, knock out this gene, and observe whether it shows a similar or stronger physiological effect than the *Cr.psbA4* P4′-3,4 mutant.

The P7-4,5 mutant of *Cr.psbA4* was completely blocked in splicing *in vivo*, consistent with the essential role of this paired region. I tried to isolate suppressors of the *Cr.psbA4* P7-4,5 mutant by UV mutagenesis, but this approach was not successful, indicating that there might be no suppressor capable of compensating for such a severe mutation. The P4′-3,4 mutant's phenotype was not strong enough to attempt suppressor isolation.

Previously, F. Li (2002) used the light sensitivity of the P4 mutants of the *Cr.LSU* intron (*Cr.LSU* P4A) to isolate three suppressors (71.20, 71.51 and 71.N1) with substantially restored splicing. I determined if these suppressors also suppress the P4′-3,4 mutation in the *Cr.psbA4* intron. This was accomplished by replacing the wild-type *Cr.psbA4* intron in the suppressors with the P4′-3,4 mutant; higher steady-state levels of spliced *psbA* mRNA was observed in all three suppressors. This result indicates that these genes also promote the splicing of a light-regulated intron. Moreover, since *Cr.psbA4* is very similar to *Cr.psbA1* (Holloway et al., 1999),

these genes probably act on *Cr.psbA1*. Additionally, *Cr.LSU* is in a different structural subgroup (IA2) than *Cr.psbA4* and *Cr.psbA1* (which are in subgroup IA1), but it is in the same subgroup as *Cr.psbA3* (Holloway et al., 1999). Thus, these genes may play a general role in splicing of group I introns in *Chlamydomonas reinhardtii*. If one was to clone these genes, it should be possible to tell if the proteins encoded by them stimulate splicing of all of the chloroplast group I introns *in vitro*.

I also tried to select a cosmid from a library made with genomic DNA of the 71.51 suppressor (prepared by F. Li (2002) and this study) that complements or suppresses the *Cr.LSU*P4A mutation. However, I was not successful, since the *cw15 Cr.LSU* P4A mutant, which was obtained by F. Li (2002) from a cross of the 7150 suppressor with a *cw15 arg2* strain (CC424), did not yield zeocin-resistant transformants after electroporation with pools of cosmid DNA (the CC424 strain yielded ~100 transformants with the same amount of DNA (see the details of the conditions in Appendix II). The *cw15 Cr.LSU*P4A mutant possibly contains extra mutation(s) that make it a poor target for transformation. Thus, in order to generate a better strain for nuclear transformation, I transformed the *Cr.LSU*P4A mutant intron into the WT 2137 (mt+) strain, and then crossed the new homoplasmic mutant with CC424 (*cw15 arg2*) (mt-) to obtain a new *cw15 Cr.LSU*P4A (mt+) strain (characterization of this strain is in Appendix IV). It will be necessary to identify the optimal conditions for transformation of this strain before again attempting transformation with the indexed library.

F. Li (2002) reported that crosses between the 7151, 7120 and 71N1 suppressors indicated that the mutations were in at least 2 nuclear genes. However, it was not shown whether 7120 was a single-gene mutant, or if 71N1 and 7120 were dominant. The 71.51 suppressor, which defines the *css1* (chloroplast splicing suppressor) gene, was shown to have no discernible phenotype with the wild-type intron (this character forced him to make an indexed library with genomic DNA from this strain), and to be dominant in vegetative diploids containing the mutant intron. Although the yeast two-hybrid technique is a feasible tool to clone other genes that interact with *css1*, it would be useful to determine if the 7120 suppressor is a single-gene mutation, and if it is dominant. I showed here that the 71.20 suppressor carries a single dominant gene mutation, and proposed to call this gene *css2*. The dominance of the 7120 mutation provides the opportunity to clone the *css2* gene by complementing the *Cr.LSU*P4A mutation with a cosmid library of the 7120 suppressor. To verify that suppressor 71N1, which is linked to the 7151 suppressor mutation (Li et al., 2002), is a distinct gene will require more crosses between these two strains.

I also showed that integration of an ORF-substituted (with the *aadA* gene) *Cr.psbA2* intron into an intronless *psbA* gene could be promoted *in trans* by an intact *Cr.psbA2* intron expressed from the *atpA* promoter. Thus, *Cr.psbA2* is a mobile intron. Analysis of RNA from these *Cr.psbA2-aadA* transformants revealed that the *Cr.psbA2* ORF does not encode an essential maturase, as there was no

significant effect on *Cr.psbA2* splicing *in vivo*. Expression of *aadA* from within the intron was poor, however; based on the lack of streptomycin resistance in the transformants. Attempts to obtain mutants (by UV mutagenesis) from these transformants that had detectable streptomycin resistance were unsuccessful. Muscarella and Vogt (1993) also had difficulty expressing proteins from within the *Physarum* rRNA group I intron in its homologous host. These findings, now in two different systems, suggest that there may be some adaptive value to keeping expression of intron-encoded homing endonucleases, which are potentially toxic, very low. Indeed, this may be necessary for the host organism to tolerate mobile introns.

Appendix I Construction of the recombinant plasmid, pGEMI2AAD -ORF (by H. Han)

A 300-bp PCR product was obtained using pGEMR14.2 plasmid as template and primers #112 (5′-AGAATTCCGAGTGGTTATACAACGGTGGTCC-3′), a forward primer that annealed to nt 127-148 of the *psbA* exon 2, and #113 (5′-GACTCCATGGCGAAGGGGTT CAGTCAAACACC-3′), a reverse primer that annealed to nt 198-219 of the *Cr.psbA2* intron (Figure A.1). The PCR product was ligated into pUC-AAD-atpX (containing vector pUC18) which had been digested with *Eco*RI and *Nco*I to delete the promoter and 5' UTR of the *atpA* gene in *C. reinhardtii*. This construct was called pUCXENAAD-atpX.

The plasmid pUCXENAAD-atpX was then digested with *Bam*HI and partially digested with *Hind*III to remove the 3′ UTR of the gene for large subunit of ribulose 1, 5-bisphosphate carboxylase (*rbcL*) of *C. reinhardtii*. The larger fragment, called pUCXENAAD-atpX-rbcL, was gel-purified, and ligated to a 800-bp PCR product, that was obtained using primers #114 (5′-GACAAGCTTTTGGGAAAG CGTGAGTGCTG-3′), a forward primer that annealed to nt 873-891 of the *Cr.psbA2* intron, and #115 (5′-CAGGATCCGAGGTAGCAAAGGGCT CGACTT-3′), a reverse primer that annealed to nt 109-130 of the *Cr.psbA3* intron (Figure A.1). This construct was named pUCI2AAD-ORF. The insert in the plasmid pUCI2AAD-ORF was then subcloned into the vector pGEM3zf(+) using *Eco*RI and *Hind*III restriction sites. The final recombinant plasmid was called pGEMI2AAD-ORF (Figure A.1).

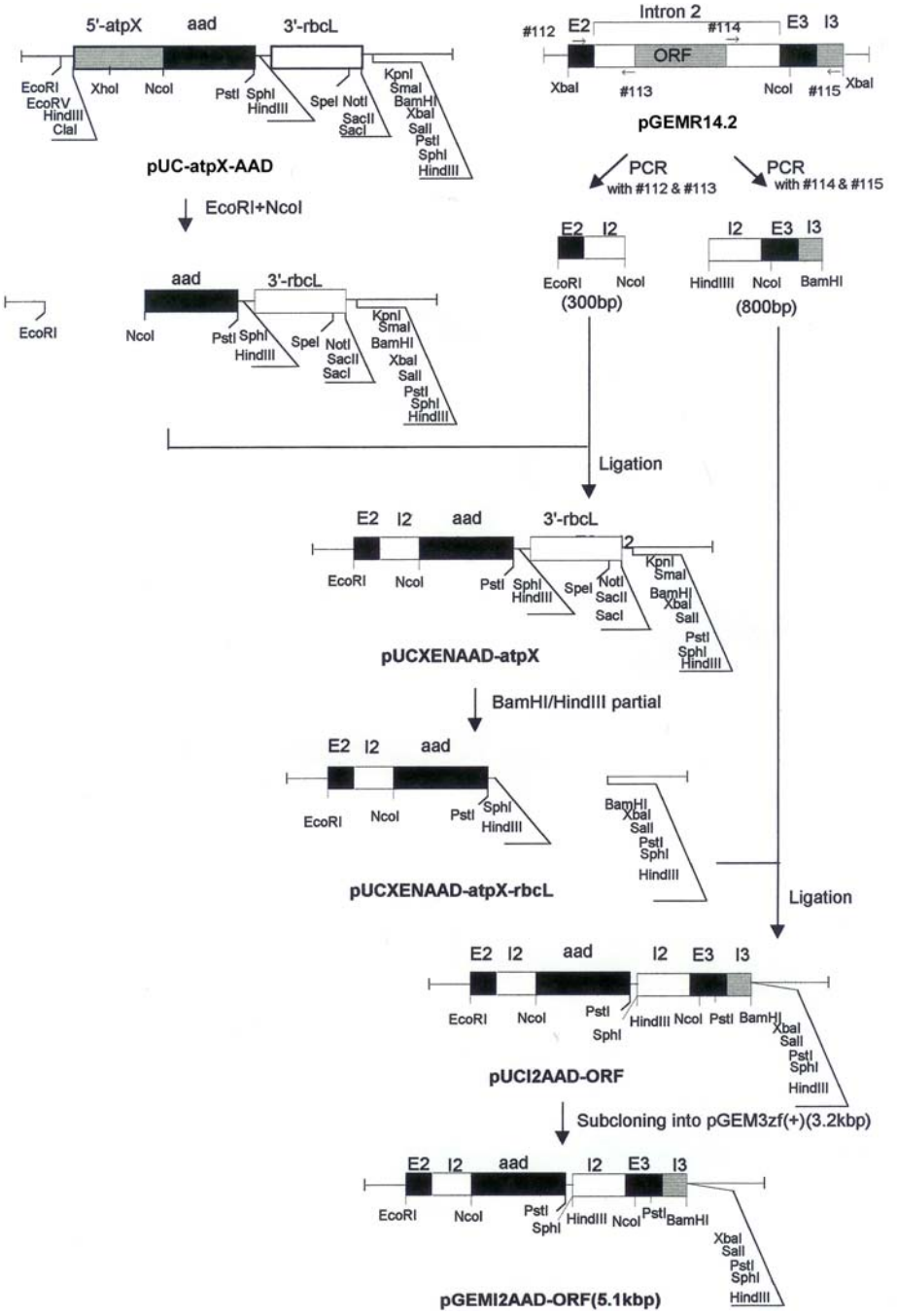


Figure A.1 A schematic chart of the construction of the plasmid pIGEMI2AAD-ORF.

The labels in the figure are as follows: 5′-atpX, the promoter sequence and 5′-UTR of the *atpA* gene in *Chlamydomonas reinhardtii*; aad, bacterial *aadA* gene; 3′-rbcL, 3′-UTR of the *rbcL* gene of *C. reinhardtii*; E2, exon 2 of *psbA*; E3, exon 3 of *psbA*; I3, intron 3 of *psbA*; +, double digestion with the restriction enzyme; /, sequential digestion with the restriction enzymes.

Appendix II A standard protocol for nuclear transformation of the cell wall-deficient mutant (CC424) by electroporation

1. Grow CC424 (cell wall-less strain) in liquid TAP containing 100 μg/mL arginine until the cell density reaches ~2×10^6 cells/mL (~ 50 mL culture per electroporation).

2. Before starting electroporation, prepare sterile electroporation cuvettes with a 0.4 mm electrode gap. Set up water bath at 10°C.

3. Transfer cells to 50 mL sterile centrifuge tube. Spin down for 5 min at 3,000 rpm using Beckman GPR centrifuge at 23°C to harvest the cells.

4. Pour off supernatant, drain well and gently resuspend cells in TAP medium containing 60mM sucrose. Resuspend cells of 200 mL culture in 1 mL TAP-sucrose.

5. Add 20 μL of 5 mg/mL heat-denatured salmon sperm DNA, 12 μg of cosmid into an electroporation cuvette. Then add 250 μL of the resuspended cells to the cuvette and mix gently but thoroughly well.

6. Put the cuvette in the 10°C water bath for 5 min and remix cells.

7. Place cuvette in the Bio-Rad Gene Pulser II. Electroporate using settings of 0.72 kV (1800 V/cm), 1000 Ω, and time constant should be 2.5-2.7 ms.

8. For recovery period, transfer electroporated cells into 20 mL TAP+ 60 mM sucrose medium containing 100 μg/mL of L-arginine in 50 mL tubes. Incubate cells for 22-24 h in dim light at 23°C with shaking (100 rpm).

9. Spin cells down for 5 min at 3000 rpm at 23°C. Pour off supernatant, leave ~0.4 ml supernatant with cells, resuspend, and mix in 0.8 ml of a [a]25 % cornstarch solution,

which also contains 0.4 % (w/v) PEG 8000, and gently pipette cells on a [b]plate containing selection medium (e.g. TAP + 35 μg/mL zeocin + 100 μg/mL arginine). Use pipette tip to gently spread cells on plate.

10. Dry plates under a sterile hood at least for 40 min.

11. Put the plates in medium light (~40 $\mu E/m^2/sec$).

[a] weigh 10 g of cornstarch and put into a 50 mL Falcon tube. Add 100 % ethanol to the tube, suspend the starch completely and store for at least 24 hours. The ethanol is replaced with an appropriate medium (TAP+60mM sucrose) by four repeated rounds of centrifugation (3000 rpm for 1 min). The starch is finally resuspended to 25 % (w/v) in the same medium used for washing the starch (40 mL).

[b] Plates should be prepared within two weeks before electroporation.

* Steps 1-7 should be performed within 30 min.

Appendix III Summary of nuclear transformation by electroporation

Table A.1 Summary of nuclear transformation by electroporation.*

	No. of transformants	
Kind of DNA (2.5 μg)	pSP124S (linearized by *Kpn* I)	Library DNA
	672±18	10±1
Amount of cosmid library DNA	2.5 μg	10 μg
	13±1	70±2
Strains	CC424 (cw15 arg2 sr-u-2-60 mt -)	CC849 (cw10 mt-)
1875V/cm, 1000Ω, 25μF, 8.3-8.6 ms	86±6	16±1
1800V/cm, 1000Ω, 10μF, 2.6-2.7 ms	76±6	2±1
Plating method	Starch embedding	Agarose embedding
pSP124S (linearized DNA)	672±18 (100 %)	295±18 (44 %)
Library DNA (2.5 μg)	10±1 (100 %)	4±1 (40 %)
Electroporation condition	1800V/cm, 1000Ω, 10μF, 2.6-2.7 ms	1875V/cm, 1000Ω, 25μF, 8.3-8.6 ms
	76±6	86±6

* A part of this result was published in the paper by Chang et al. (2003).

Table A.2 Standard conditions for nuclear transformation by electroporation.

Sample conditions	
Sample size	250 μl in 4 mm-gap cuvette
Cell number	1×10^8 cells/mL
Exogenous DNA	2.5 μg for plasmid and 12 μg for cosmid library DNA
Carrier DNA (salmon sperm DNA)	400 μg/ml
Transformation medium	TAP + 60 mM sucrose + arginine if needed (100 μg/mL)
Temperature just before electroporation	10-15 °C
Electric field conditions	
Electric field strength	1800 V/cm
Resistance	1000 Ω
Capacitance	10 μF
Time constant	2.5-2.6 msec
After electroporation	
Incubation	23 °C for 22-24 hrs in 20 ml TAP + 60 mM sucrose + arginine (100 μg/mL)
Plating	20 % starch + 60 mM sucrose + arginine (100 μg/mL) + 0.4 % PEG 8000 in TAP
Time to begin to see colonies (if not a slow growing mutant)	5 days

Appendix IV Generation of the *Cr.LSU* splicing-deficient and cell wall-deficient strain

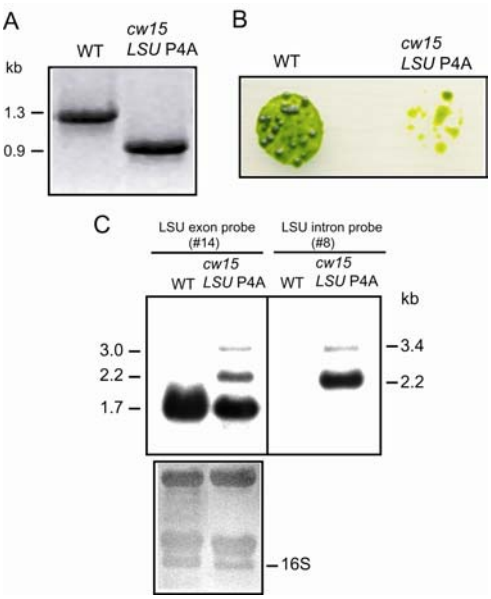


Figure A.2 Molecular analysis of the mutant strain containing a mutation in helix P4 (^{125}C→A) of the *Cr.LSU* intron (*Cr.LSU* P4A), and the *cw15* cell wall-deficient mutation(s) (*cw15 Cr.LSU* P4A). The mutant strain containing a splicing-deficient *Cr.LSU* intron was generated by transformation of the *Cr.LSU* mutant plasmid (P4mut 23srRNA) into the WT strain. The plasmid contains spectinomycin and erythromycin-resistant markers. The homoplasmic mutant DNA was confirmed by PCR analysis using primers #14 and #15, and slow growth compared to WT in bright light. Also, more accumulated precursor RNAs were observed by RNA blot analysis. (data not shown). The mutant strain was then crossed with the cell wall-deficient mutant (CC424) mt(-) and the progeny were selected on TAP plates containing 100 μg/mL spectinomycin in dim light at 23°C.

(A) PCR analysis to verify the homoplasmicity of the *Cr.LSU* intron. The PCR analysis was performed with primers #14 and #15, and the WT strain was used for a control. The figure shows the only PCR product that was obtained with each DNA. Molecular size markers (not shown) were used to verify that the products were the sizes expected for the WT and mutant introns, respectively. The mutant intron is ORF-minus and ~350 bp smaller than WT.

(B) The growth phenotype of the mutant. The mutant strain and WT (~1× 10^4 cells in TAP) were pipetted onto a TAP plate (1.5 % agar), which was incubated in bright light for 6 days, and photographed. The mutant showed a slow growth compared to WT. (In addition, this mutant was not arginine auxotroph because it grew well in arginine-deficient medium (1.5 % agar in TAP). It was also lysed in 1 % NP-40; no green color was observed in pellet after centrifugation, indicating that it is cell wall-deficient) (data not shown).

(C) RNA blot analysis of the mutant. Total RNAs from the mutant and WT were separated on a denaturing agarose gel and blotted onto a nylon membrane that was stained with methylene blue (Stain, bottom). The blot was hybridized with the ^{32}P-labeled *Cr.LSU* 23Sδ. exon-specific oligodeoxynucleotide (LSU exon probe). After stripping the blot, it was also hybridized with the *Cr.LSU* intron-specific oligodeoxynucleotide (LSU intron probe). The mutant *cw15 Cr.LSU* P4A accumulated more precursors compared to WT (top panel), and less 16S rRNA by staining pattern than WT (bottom panel) indicating that it is the splicing-deficient mutant.

Appendix V Mutant cryopreservation (by B. Piasecki, a graduate student in Dr. Brand's lab)

***cw15 Cr.LSU* P4A (labeled as " CW15LSUP4M")**

Cryopreservation: Six 1.8 mL (total volume) sample at a final culture density of 5×10^5 cells/mL were simultaneously cryopreserved on Feb. 11, 2003 in TAP and 5 % v/v MeOH. Some tubes were revived on Feb. 12, 2003, the MeOH was removed, and viability of the cells was shown to be at ~34 %. Five samples remain in cryopreservation in the dewar named: Big Mamma, Port 2, Level 2, #10-14.

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