Article

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Post-transcriptional regulation of chloroplast gene expression in *Chlamydomonas reinhardtii*

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Key words: Chlamydomonas, chloroplast gene expression, RNA stability, RNA processing, splicing, translation

Contents

Abstract

The biosynthesis of the photosynthetic apparatus depends on the concerted action of the nuclear and chloroplast genetic systems. Numerous nuclear and chloroplast mutants of *Chlamydomonas* deficient in photosynthetic activity have been isolated and characterized. While several of these mutations alter the genes of components of the photosynthetic complexes, a large number of the mutations affect the expression of chloroplast genes involved in photosynthesis. Most of these mutations are nuclear and only affect the expression of a single chloroplast gene. The mutations examined appear to act principally at post-transcriptional steps such as RNA stability, RNA processing, *cis*- and *trans*-splicing and translation. Directed chloroplast DNA surgery through biolistic transformation has provided a powerful tool for identifying important *cis* elements involved in chloroplast gene expression. Insertion of chimeric genes consisting of chloroplast regulatory regions fused to reporter genes into the chloroplast genome has led to the identification of target sites of the nuclear-encoded functions affected in some of the mutants. Biochemical studies have identified a set of RNA-binding proteins that interact with the 5′-untranslated regions of plastid mRNAs. The binding activity of some of these factors appears to be modulated by light and by the growth conditions.

Introduction

The green unicellular alga *Chlamydomonas reinhardtii* has emerged as a powerful model system for studying chloroplast gene expression for several reasons. First, this photosynthetic eukaryotic organism can be grown under controlled laboratory conditions in large amounts and is thus suitable for biochemical analys-

is. Second, *C. reinhardtii* cells, which exist either as mating type + or −, undergo a well-defined sexual cycle and are amenable to extensive genetic analysis. Cells can be propagated as well in the haploid as in the diploid state (for details, see [30]). Third, photosynthetic function in *C. reinhardtii* is dispensable provided a carbon source such as acetate is included in the growth medium. It has therefore been relatively easy to isolate a large number of mutants deficient in photosynthetic activity. Fourth, methods and tools have been developed recently for efficient nuclear and organellar transformation of *C. reinhardtii* [3, 14, 44, 45, 58]. The efficiency of nuclear transformation is sufficiently high to make gene isolation through genomic complementation of mutants with cosmid libraries feasible [68, 94]. Chloroplast transformation can be achieved by bombardment of cells with DNA-coated tungsten or gold particles. The transforming DNA is integrated efficiently into the chloroplast genome by homologous recombination. This technology together with the availability of selectable markers allows one to perform directed gene disruptions and site-directed mutagenesis of any chloroplast gene [26, 59, 76, 86].

It is well established that the biosynthesis of the photosynthetic apparatus depends on the concerted action of the nuclear and chloroplast genetic systems. Both chloroplast and nuclear mutants deficient in photosynthetic activity have been isolated which can be readily distinguished based on the segregation patterns during crosses. While nuclear mutations follow Mendelian inheritance, chloroplast mutations are uniparentally inherited from the mating-type+parent. Nuclear mutations affecting photosynthesis fall into two groups. The first includes mutations located within the genes of structural components of the photosynthetic apparatus whereas the second comprises mutations that affect the expression of chloroplast genes. A striking feature is that these mutations act specifically on the expression of individual genes, i.e. in these mutants the expression of a single chloroplast gene is affected and other chloroplast genes are expressed normally. A large number of mutations of this type corresponding to distinct nuclear loci have been identified. The mutations examined to date appear to act mostly at post-transcriptional steps such as RNA stability, RNA processing, *cis*- and *trans*-splicing and translation (Fig. 1). The analysis of these mutants has provided new insights into the molecular mechanisms underlying chloroplast gene expression, in particular its dependence on nuclear gene products. Since several reviews covering various aspects of chloroplast gene expression in *C. reinhardtii* have appeared recently [23, 57, 60, 70], this article will focus mostly on recent developments in this area.

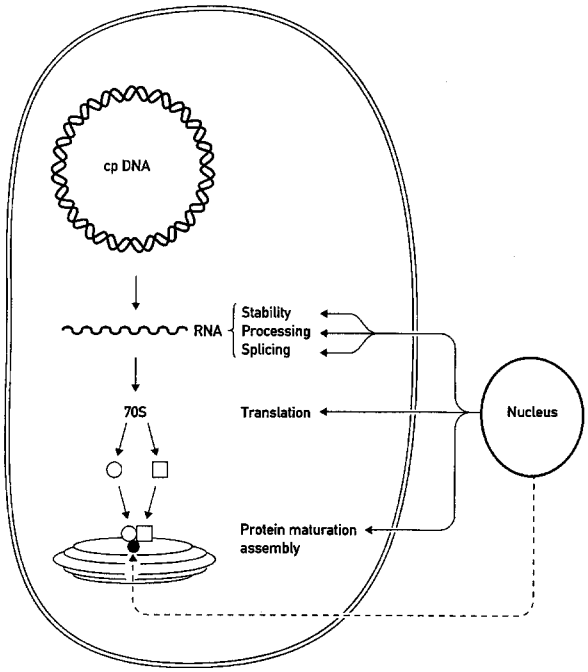

Figure 1. Scheme of biosynthesis of photosynthetic complexes. Photosynthetic complexes consist of chloroplast (open symbols) and nuclear-encoded subunits (black symbols). Numerous nuclear-encoded functions are required for the expression of chloroplast genes at the level of RNA stability, processing, splicing, translation, protein maturation and assembly of complexes. cp, chloroplast DNA; 70S, chloroplast ribosome.

Chloroplast RNA stability and processing

It is well documented that the levels of chloroplast transcripts vary considerably during plastid development and differentiation in higher plants (see [29]). Although it has been recognized that this differential accumulation of chloroplast mRNAs is partly controlled at the transcriptional level, a major part of this regulation occurs through stabilization or destabilization of transcripts. Further, chloroplast RNA stability is closely linked to RNA processing and translation. *C. reinhardtii* offers interesting possibilities to study this problem using genetic, molecular and biochemical approaches.

Role of 3′-untranslated region

A characteristic feature of most chloroplast mRNAs is the presence of inverted repeats within the 3′-untranslated region that can fold into stem-loop structures. Similar structures are also found in prokaryotes where they appear to play an important role in rho-independent transcription termination. The role of the 3′ inverted repeat of the *atpB* mRNA of *C. reinhardtii* was studied using both chloroplast transformation and *in vitro* assays [83]. Deletion of this structure reduces the amount of the *atpB* mRNA to 20–35% of wild-type levels without affecting the transcription rate and leads to the appearance of *atpB* RNA of heterogeneous size [83]. These data indicate that the 3′ inverted repeat of *atpB* plays a role both in RNA stabilization and in 3′-end formation.

Although run-on transcription analysis has shown that the *atpB* 3′ inverted repeat is involved in transcription termination, this only occurs with a frequency of 50%. Hence, the 3′ inverted repeat is also required for 3′-end processing (see below). *In vitro* studies revealed that the 3′-end maturation of the *atpB* mRNA is a two-step process [84]. Endonucleolytic cleavage at a site downstream of the 3′ inverted repeat is followed by exonucleolytic trimming to the stem of the 3′ inverted repeat.

Blowers *et al.* [2] examined the role of the 3′-untranslated region of *rbcL* and *psaB* and their associated inverted repeat elements. They introduced into the chloroplast of *C. reinhardtii* chimeric GUS genes containing the *rbcL* or *psaB* 3′-untranslated regions with one or more 3′ inverted repeats or with the inverted repeat in opposite orientation. The analysis of these transformants revealed that these 3′ stem-loop structures act principally as signals for RNA 3′-end formation by transcription termination or endonucleolytic cleavage. Since their removal does not affect RNA decay, it was concluded that they play no major role in RNA stabilization [2] in contrast to the results obtained with the *atpB* 3′ end [83]. Further, efficient 3′-end formation occurred only with constructs containing the 3′ inverted repeat in the normal forward orientation. It is interesting to contrast these results with those obtained with the spinach *petD* 3′ inverted repeat which was shown to promote correct 3′-end formation both in the forward and reverse orientation when inserted downstream of *atpB* in *C. reinhardtii* [83].

The picture which emerges from these studies is that each chloroplast transcript 3′ end is distinct and that a number of mechanisms underlie the formation of discrete ends and the stabilization of the transcripts. From the work performed in higher plants it is clear that the 3′ inverted repeat alone is not sufficient to account for the stabilization and processing of mRNA [82]. Indeed several proteins that bind specifically to the 3′-untranslated region of mRNAs have been identified in higher plants and include polypeptides of 100, 54, 33, 28 and 24 kDa. The 28 kDa protein appears to be involved in 3′-end processing [79] and a 54 kDa protein from mustard was shown to copurify with an endoribonuclease activity [65]. Little is known on RNA-binding proteins of this sort in *C. reinhardtii*.

Role of 5′-untranslated region

Evidence that determinants for chloroplast mRNA stability reside within the 5′-untranslated region arose from the analysis of a nuclear mutant of *C. reinhardtii* deficient in PSII activity [46]. This mutant, nac2–26, was shown to lack *psbD* mRNA and appears to be specifically affected in the stabilization of this RNA since *psbD* RNA is transcribed and other chloroplast RNAs accumulate to wild-type levels [46]. To identify the determinants for degradation within the *psbD* message, chimeric genes consisting of the *psbD* 5′-untranslated region fused to the reporter gene *aadA* (conferring resistance to spectinomycin [26]) were introduced into the chloroplast genome through biolistic transformation [66]. The transformants of mating type + were subsequently crossed to the nac2–26 mutant strain. Tetrad analysis revealed cosegregation between photosystem II deficiency and spectinomycin sensitivity. Furthermore, it was shown that the chimeric message is destabilized in the nac2–26 nuclear background, but not in the wild-type background thus indicating that the 74 nucleotide *psbD* leader contains one of the major target sites for *psbD* RNA degradation in the absence of wild-type NAC 2 function. Similar experiments in which the *psbD* 3′-untranslated region was fused to *aadA* did not reveal cosegregation between the two phenotypes indicating that the 3′-untranslated region is not sufficient to promote RNA degradation in the nac2–26 nuclear background.

The selective instability of *psbD* 5′ leader RNA could also be demonstrated *in vitro* [66]. Incubation of this RNA with lysates from purified chloroplasts of wild type and nac2–26 revealed that the RNA is degraded considerably faster with the mutant extract. This rapid degradation appears to be mediated by specific endonucleolytic cleavages within the *psbD* 5′ leader. UV crosslinking analysis of proteins binding

to the *psbD* leader revealed a 47 kDa protein in wild-type extracts whose binding activity was altered in the mutant. Binding activity could only be recovered from lysates treated with non-ionic detergents, suggesting that the 47 kDa protein may be associated with membranes.

The *psbD* RNA is produced as a precursor with a 74 nucleotide leader which is processed at residue −47 (relative to the initiation codon) to give rise to the mature RNA. The 47 kDa protein binds only to the −74 leader, but not to the mature −47 leader suggesting that the binding site is in the 5′ region of the leader and/or overlapping the processing site [66]. Processing at −47 could not be achieved *in vitro*, possibly because the activity and/or essential cofactors are lost during the preparation of the lysate. Surprisingly, deletion of the 5′-terminal region of the *psbD* leader from −74 to −47 leads to destabilization of *psbD* message *in vivo* although *psbD* is still transcribed at wild-type levels under these conditions (J. Nickelsen and J.-D. Rochaix, unpublished results). We are thus left with a paradox since in wild-type the *psbD* precursor RNA is processed to produce the mature RNA which is stable. One way to reconcile these results is to assume that there is an obligate maturation pathway for *psbD* mRNA in which *psbD* RNA processing is closely coupled to some early event in the initiation of translation, possibly binding of a factor or protein complex to the 5′ end of the mature *psbD* mRNA which may protect the RNA against degradation and make it competent for translation.

The nuclear gene deficient in nac2–26 has recently been isolated through genomic complementation using a wild-type cosmid library (J. Nickelsen and J.-D. Rochaix, unpublished results). Preliminary data indicate that the encoded polypeptide does not encode known RNA-binding motifs.

Other nuclear mutants have been characterized which fail to accumulate the chloroplast transcripts of either *psbB* [42, 63], *psbC* [81] or *atpA* [17]. Preliminary results indicate that in the case of mutant 222E the target site of the nuclear-encoded function defined by the mutation lies within the 5′-untranslated region of the *psbB* mRNA (F. Vaistij, M. Goldschmidt-Clermont and J. D. Rochaix, unpublished results). These genetic data suggest the existence of nuclear encoded factors which interact specifically with the 5′-untranslated regions of chloroplast mRNAs and play a major role in transcript stabilization.

Additional evidence that 5′ sequences of chloroplast transcripts contain important positive and negative determinants for RNA stability has emerged from studies of chimeric plastid genes in transgenic *C. reinhardtii* [77]. It was found that fusion of the 5′ *rbcL* leader to various reporter genes strongly destabilizes the chimeric transcript upon illumination of cells previously grown in the dark. However, addition of 5′ sequences from the coding part of *rbcL* prevented this transcript destabilization.

Recently, a nuclear photosynthetic deficient mutant was identified that accumulates minute amounts of *rbcL* mRNA [38]. Pulse-labelling experiments indicated that the rate of *rbcL* mRNA synthesis is apparently reduced in this mutant. Whether this phenotype is indeed due to a specific alteration of the rate of transcription of *rbcL* (which has never been observed before for any chloroplast gene) or whether the *rbcL* mRNA is highly unstable in this mutant strain remains to be explored.

Considerable changes in the levels of chloroplast mRNAs of *C. reinhardtii* cells grown in a 12 h light/12 h dark regime have been found to be due to changes in both transcription and decay rates [78]. A striking drop in stability in the light was observed for the transcripts of *rbcL*, *atpB*, *tufA*, *psaB*, *psbA* and 16S rDNA. In contrast to the genetically defined factors involved in the stability of specific transcripts, this fast light-induced acceleration of RNA degradation does not appear to be transcript specific as all transcripts tested were affected.

Maturation of polycistronic transcripts

In contrast to higher plants only few chloroplast genes of *C. reinhardtii* have been shown to be organized as operons that are transcribed into polycistronic primary transcripts. Besides the ribosomal operon, polycistronic transcription units include *psbB-ycf8* [43, 68], *psbD*-exon 2 of *psaA* [7], *psaC*-ORF58-*ycf7* [86], *rps7*-atpE [69], *tscA-chlN* (M. Goldschmidt-Clermont, unpublished results) and *petA-petD* [33, 75, 85] (Fig. 2). In the latter case, *petD* can be transcribed from its own promoter, or cotranscribed with *petA*. Use of specific deletions of the 5′ region of *petD* allowed the identification of an RNA processing site which is used to generate monocistronic *petD* mRNA from the *petA-petD* dicistronic RNA and most likely also from the transcript initiated from the *petD* promoter [75]. The nature of the processing site was further investigated by inserting various chimeric genes, consisting of the 5′-untranslated region of *petD* fused to *uidA*, downstream of *petA* into the chloroplast genome. Trans-

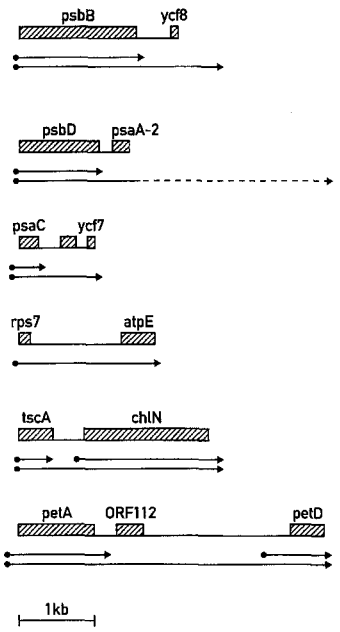

Figure 2. Di-and tri-cistronic chloroplast transcription units in *C. reinhardtii*. The 5′ ends are indicated by dots and the 3′ ends by arrows. In the case of *psbD-psaA–2*, several large transcripts with sizes ranging between 2.6 and 7 kb have been detected [7]. However, their ends have not been mapped precisely.

formants with the deletion of the *petD* promoter still accumulated monocistronic *uidA* transcript whereas in transformants with a larger deletion removing in addition the first 25 bases downstream of the mature *petD* 5′ end only dicistronic *petA-uidA* transcript could be detected. These results suggest that the recognition site for processing is localized at least partially within the first 25 bases of the 5′-untranslated region of *petD*. It is likely that additional polycistronic transcription units will be found in the chloroplast of *C. reinhardtii*.

Chloroplast RNA splicing

Chloroplast introns of higher plants and algae fall into two distinct classes, group I and group II introns, each of which has characteristic secondary structure features [61]. In the chloroplast of *C. reinhardtii* only two genes, the 23S rRNA gene [71] and *psbA* [21] contain group I introns and one gene, *psaA*, contains two unusual group II introns that are split [48] (see below). It is not yet known whether other group II introns are present in the chloroplast genome of *C. reinhardtii*.

Efficient *in vitro* self-splicing was demonstrated for the ribosomal intron [19, 35] and for three *psbA* introns [34]. These findings do not necessarily imply that self-splicing also occurs *in vivo*. The existence of nuclear mutations that prevent splicing of mitochondrial introns known to self-splice *in vitro* is well-documented in fungi [9]. Also, a nuclear mutant of *C. reinhardtii* partially deficient in chloroplast ribosomes was shown to accumulate unspliced 23SrRNA precursor [35].

The *psaA* gene of *C. reinhardtii*, which encodes one of the major reaction center polypeptides of photosystem I, consists of three exons that are widely separated on the chloroplast genome [48]. The observation that exons 1 and 2 are directed in opposite orientation on the chloroplast genome implies that transcription of these exons is discontinuous [7]. Each of the *psaA* exons is surrounded by typical 5′- and/or 3′-terminal sequences of group II introns [48]. Hence, maturation of the *psaA* mRNA depends on two *trans*-splicing reactions.

Surprisingly, amongst nuclear mutants deficient in photosystem I activity more than one fourth are affected in the maturation of *psaA* mRNA [27]. These mutants fall into three classes based on their *psaA* RNA phenotype. Class A mutants are defective in exon 2-exon 3 splicing, class B mutants are unable to perform either of the two *trans*-splicing reactions and class C mutants are deficient in exon 1-exon 2 splicing. These mutations fall into a large number of complementation groups: at least 5 for class A, 2 for class B and 7 for class C [27, 28]. Since several of these complementation groups include a single allele, it is likely that many more nuclear loci exist that are involved, directly or indirectly, in *psaA trans*-splicing. Besides these nuclear loci, a chloroplast locus, *tscA*, was identified and shown to be involved in *trans*-splicing [27, 28]. This locus is distinct from either *psaA* exon. Mutants with deletions in *tscA* are unable to splice exons 1 and 2. Analysis of the *tscA* mutants revealed that this locus encodes a 430 nucleotide RNA required for the first *trans*-splicing reaction [28].

A model was proposed in which the *tscA* RNA interacts with the 5′ and 3′ intron regions flanking the first and second *psaA* exons so that a composite structure with features of group II introns is reconstituted [28]. This secondary structure consists of a characteristic wheel and six protruding stem-loop domains. In this model the *tscA* RNA includes domains II and III of the intron and its terminal regions reconstitute the disrupted helices corresponding to domains I and IV by base-pairing with the 5′ and 3′ parts of the intron (Fig. 3). The model is based on the presence of short conserved sequence motifs of group II introns with-

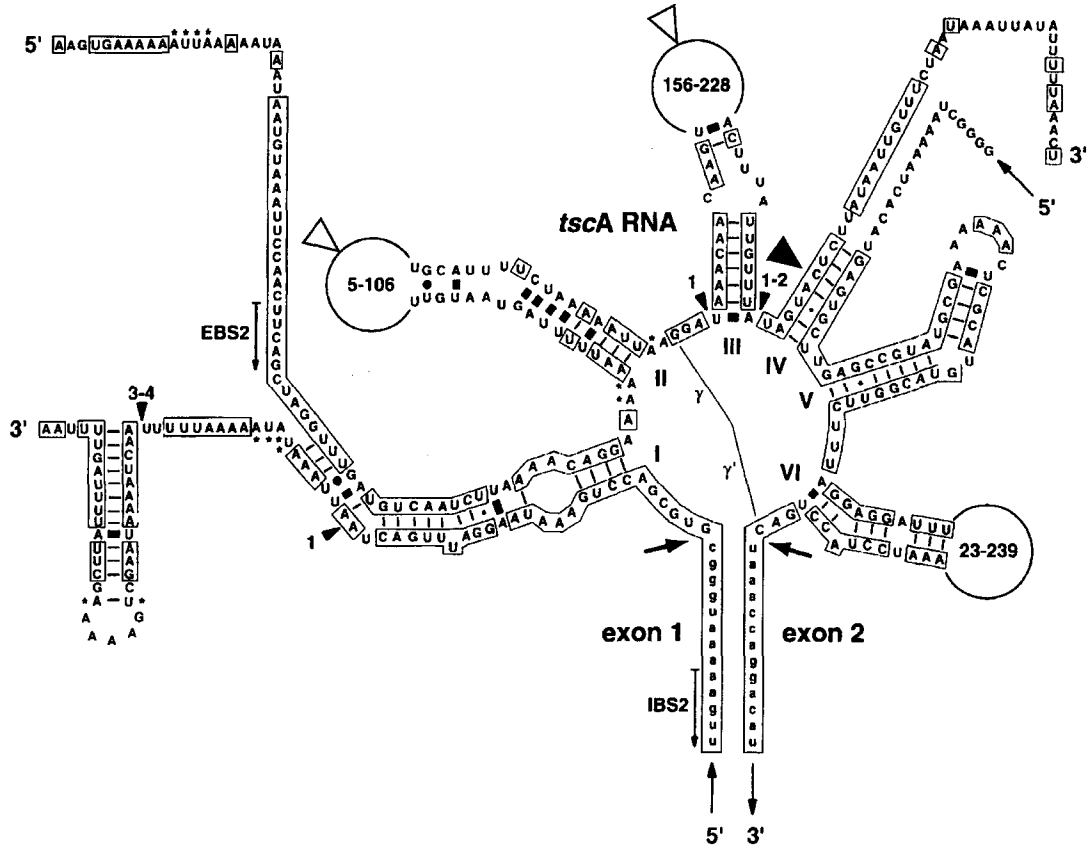

Figure 3. Secondary structure model of the tripartite intron 1 of *psaA* in *C. reinhardtii*, *C. gelatinosa* and *C. zebra*. Roman numerals indicate the six major secondary structure domains of group II introns. Conserved bases in the three *Chlamydomonas* are boxed. Residues that are missing in *C. gelatinosa* and/or *C. zebra* are marked by asterisks, whereas those representing additions in the latter two algae relative to *C. reinhardtii* are indicated by small solid triangles with their accompanying numbers indicating the size of the insertions in nucleotides. Numbers in the loops indicate the minimum and maximum size of these regions. Blocked arrows denote a potential pairing between EBS2 (exon-binding site) and IBS3 (intron-binding site). The long-range tertiary interaction γ-γ' is indicated. The thick arrows point to the intron-exon junctions. Thin arrows indicate the 5′- and 3′-strand polarity of the separate transcripts containing exon 1 and exon 2. Closed and open wedges indicate the sites of insertion of the *aadA* cassette which block and which do not inhibit *trans*-splicing, respectively (adapted from Turmel *et al.* [89], with permission).

in *tscA* and on the observation that disruption of *tscA* by the *aadA* expression cassette within the external loops of domains II and III does not interfere with *trans*-splicing, whereas disruption of *tscA* within the stem of domain IV blocks *trans*-splicing. It is known from studies of mitochondrial group II introns that the external loops are variable in size and that they do not appear to play an active role in splicing, in contrast to the more conserved intron core [62]. Further support for this model has arisen from the sequence comparison of the equivalent introns in the *psaA* genes from *C. gelatinosa* and *C. zebra* [89] which are also tripartite and which can be folded in a similar way (Fig. 3). It is noticeable that high sequence conservation within *tscA* is found in the intron core region and also in its 5′ and 3′ parts suggesting that these regions may have some important functional role perhaps by interacting with specific (*trans*)splicing factors.

A striking feature of the split intron 1 of *psaA* is that it lacks any recognizable domain I structure which is usually well conserved within group II introns. This raises the possibility that this domain has diverged considerably in *C. reinhardtii* or that another *trans*-acting RNA specifies domain I. The latter possibility is of special interest in the context of the idea that group II intron domains may have evolved into snRNAs during the evolution of nuclear pre-mRNA introns (80).

The *tscA* gene of *C. reinhardtii* appears to be cotranscribed with the downstream *chlN* gene. This is suggested by the existence of a nuclear mutant deficient in

splicing exon 1 and exon 2 of *psaA* which accumulates *tscA-chlN* cotranscripts (M. Goldschmidt-Clermont, unpublished results). Whether the splicing defect is directly linked to the failure to process *tscA* RNA or whether the accumulation of the *tscA* precursor RNA results from a secondary effect of the mutation is not yet known.

Besides the two discontinuous group II introns of *psaA*, no other group II intron has been detected in the chloroplast genome of *C. reinhardtii*. To determine whether the group II *trans*-splicing machinery of *C. reinhardtii* is capable of splicing a heterologous group II *cis* intron, the *atpF* gene of spinach was introduced into the chloroplast genome of *C. reinhardtii* [16]. This gene contains a 764 bp group II intron which does not self-splice *in vitro*. Although the precursor RNA was expressed, no spliced product could be detected suggesting that group II *cis*-intron splicing is either species specific or distinct from *trans*-splicing.

In contrast, the mitochondrial group II intron rI1 from *Scenedesmus obliquus* was shown to splice efficiently when introduced into the *C. reinhardtii* chloroplast [32]. This intron is capable of self-splicing *in vitro* [49] and the question remains whether splicing of this intron is dependent on specific factors in *C. reinhardtii*.

Translation

Several nuclear mutants of C. reinhardtii *are affected at the level of translation initiation*

Translation is a key step in the control of expression of chloroplast genes (cf. [22, 60]). Several mutants of *C. reinhardtii* affected in translation have been characterized and have provided valuable insights into the underlying molecular mechanisms.

At least two nuclear mutations, F34 and F64, and one chloroplast mutation, Fud34, were shown to block synthesis of the photosystem II core subunit P6 (CP43 in higher plants) which is encoded by the chloroplast *psbC* gene [1, 8, 15]. The chloroplast mutation has been mapped in a stem-loop structure within the 550 base *psbC* 5′ leader region [73]. Moreover, a point mutation within the same stem-loop structure partially suppresses the F34 mutation [73]. To confirm that these mutations interfere with translation rather than turnover of the protein, chimeric genes consisting of the *psbC* 5′ leader region fused to the reporter *aadA* were introduced into the chloroplast via biolistic transformation. Expression of the reporter gene was shown to occur only with the wild type, but not with the mutant F34 and F64 alleles thus indicating that the *psbC* 5′ leader contains *cis*-acting target(s) for these F34- and F64-dependent *trans*-acting factors [93].

To gain further insight into these target regions various deletions were introduced in the *psbC* 5′-untranslated region of the chimeric *aadA* construct (W. Zerges and J.-D. Rochaix, unpublished results). Removal of the stem-loop considerably reduced expression of *aadA* suggesting that this structure is required for efficient translation. This deletion partially relieved the dependence on F34 function. Deletion of the 3′ part of the leader severely diminished this dependence indicating that the F34 factor interacts with this region including the stem loop. In contrast none of the deletions tested relieved the dependence of translation on the F64 function suggesting that the F64 factors acts on several sites of the *psbC* leader. UV crosslinking experiments with chloroplast extracts and the *psbC* leader revealed a binding activity at 46 kDa in the F64 extract that was absent in wild type [93]. One possibility is that F64 encodes an anti-repressor which prevents tight binding of the 46 kDa protein to the *psbC* leader in wild type, but not in the mutant. Alternatively the F64 factor, together with the 46 kDa protein, may be part of a protein complex required for translation which is non-functional in the F64 mutant and releases the 46 kDa RNA-binding protein.

Another nuclear mutant, F15, is unable to synthesize the PsaB protein, one of the reaction center subunits of photosystem I. Since the *psaB* mRNA accumulates to wild-type levels in F15, the mutation must act at a post-transcriptional step. Similar experiments as described above for *psbC* with chimeric genes consisting of the *psaB* leader fused to *aadA* revealed that in this case, too, the *psaB* 5′-untranslated region mediates the translational requirement for the nuclear-encoded F15 function (O. Stampacchia, unpublished results). A chloroplast suppressor mutation of F15 was found within the 5′-untranslated region of *psaB* near a potential Shine-Dalgarno sequence (J.L. Zanasco, P. Bennoun, J.-D. Rochaix, unpublished results). The latter appears to be occluded in the wild type because of base-pairing with the upstream region. The suppressor mutation could destabilize this pairing and may thereby allow interactions between the Shine-Dalgarno sequence and the small ribosomal subunit. The function affected in F15 may be required for promoting this interaction.

At least two nuclear loci NAC1 and AC115 were shown to be required for the synthesis of the *psbD*

product D2 [47]. Pulse-labelling experiments revealed that synthesis of theD2 protein is severely reduced in both mutants [47, 91]. Mutations at the NAC1 locus appear to act at a step following the initiation of translation since chimeric genes consisting of the *psbD* 5′-untranslated region fused to *aadA* are still expressed in the presence of the mutant *nac1* allele (J.-D. Rochaix and J. van Dillewijn, unpublished results). A nuclear suppressor was isolated which is able to overcome the effects of two different allelic mutations at the NAC1 locus as well as mutations in AC-115. The behavior of this suppressor, which is neither allele- nor gene-specific, is consistent with a bypass suppressor [91]. The molecular basis of this interesting suppression remains to be elucidated.

Cis *elements in the 5′-untranslated regions of chloroplast mRNAs*

The finding of potential Shine-Dalgarno (SD) sequences in close vicinity upstream of the start codons of many, but not all, chloroplast genes of *C. reinhardtii* suggests that the initiation of translation in the chloroplast may occur in a similar way as in prokaryotes where pairing between the 3′ end of 16S rRNA with the SD sequence facilitates the positioning of the 30S ribosomal subunit on mRNA [25]. The 3′ end of 16S rRNA of *C. reinhardtii* and *E. coli* are highly homologous and pairings with putative chloroplast SD sequences are possible [18]. The functional role of SD sequences has been tested only recently in *C. reinhardtii*. Mutations in the potential SD sequences of *petD* (GGA to TTA) did not affect translation of *petD* [74]. Similarly, a change in the *psbD* SD sequence from GGAG to AAAG did not affect phototrophic growth and photosystem II activity (J.-D. Rochaix, unpublished results). In contrast, deletion of the *psbA* SD sequence abolished *psbA* translation [59]. However, since *psbA* mRNA was strongly reduced in this mutant, the significance of the result is not clear.

The 362 nucleotide *petD* 5′ leader was shown to be both necessary and sufficient to drive expression of GUS in transgenic chloroplasts [76]. However, removal of the first two thirds of the *petD* leader in the chimeric gene considerably reduced the expression of GUS [76]. Mutational studies of other regions of the *petD* 5′ leader revealed that two regions are required for translation, one located between 150 and 200 nucleotides, the other ca. 40 nucleotides upstream of the initiation codon. Site-directed mutations of the *psbA* [59] and *psbD* 5′-untranslated regions (J. Nickelsen and J.-D. Rochaix, unpublished results) also revealed short sequence motifs that are important for translation.

Initiation codons

Although AUG is used in most cases as initiation codon, GUG can also be utilized efficiently, for example for *psbC* translation in *C. reinhardtii* [73]. Initiation codon recognition in *C. reinhardtii* was examined by changing the AUG initiation codon of *petD* to either AUU or AUC [5]. These mutations reduce the rate of translation initiation to 10 to 20% of wild-type level. Phototrophic growth of these mutants is reduced at room temperature and completely blocked at 35 °C. In the case of *psbD*, replacement of the AUG initiation codon by AUA severely reduced expression of *psbD* whereas the AUC replacement had only a minor effect (J.-D. Rochaix, unpublished results). In a more thorough study, five mutations were created in the initiation codon of *petA*, which encodes cytochrome *f* of the cytochrome *b6/f* complex [6]. Mutant strains with single-basepair substitutions, AUU and ACG, accumulated cytochrome *f* to 20 and 2–5% of wild-type levels. In mutant strains with double-basepair changes, ACC and ACU, cytochrome *f* levels were 1–2% and 0.8% relative to wild-type. The mutant strain with the UUC codon did not accumulate detectable levels of cytochrome *f*. Of these mutants only the AUU codon mutant was able to grow phototrophically. The use of alternative initiation codons in these mutants could be ruled out by introducing stop codons either immediately upstream or downstream of the initiation codon: cytochrome *f* could be detected with the upstream, but not with the downstream stop codon [6]. These results suggest that the initiation codon in *C. reinhardtii* is important for determining the efficiency of translation initiation. However, its role in establishing the location of the initiation site seems to be minimal. The latter appears to be determined by the sequence context around the initiation site, such as the Shine Dalgarno sequence. For those mRNAs lacking an SD sequence other interactions between rRNA and mRNA may occur [6].

Relationship between mRNA stability and translational capacity

In prokaryotes mRNA stability is usually closely correlated to the translatability of the mRNA with strains deficient in translation accumulating less of the corresponding RNA (cf. [67]). However, the mechan-

ism by which a given mRNA is degraded can affect this correlation. Amongst the mutants of *C. reinhardtii* deficient in translation of specific chloroplast mRNAs, three categories have been described. The first contains mutants in which the corresponding mRNA level is decreased. These include mutants with altered 5′-untranslated region of *psbA* [59] and *petD* [74] and strain Fud47 harboring a frame-shift mutation within *psbD* [20]. In the second category, the mRNA levels in the mutants are unaltered relative to wild type or only slightly reduced. Here one finds a mutant deficient in *psbA* translation [24] and the nuclear mutants F34 and F64 affected in the initiation of translation of *psbC* [73]. However, chimeric transcripts consisting of the 5′-untranslated region of *psbC* fused to *aadA* accumulate to higher levels in the F34 and F64 mutants than in wild type [93]. This clearly shows that translatability is not the only determinant of mRNA stability. Other mutants in which the mRNA levels are increased include mutants probably affected at the level of translation elongation of *psbA* RNA [47], mutants deficient in *atpA* translation [17] and a strain with a frameshift mutation within *psaB* [92]. Taken together, these data indicate that there is no simple relationship between mRNA stability on the one hand, and ribosome association and translation on the other.

Trans-*acting factors involved in translation*

Although *C. reinhardtii* cells are able to synthesize and assemble photosynthetic complexes in the dark, there are considerable differences in the rate of synthesis of several chloroplast encoded subunits of these complexes between dark- and light-grown cells [55]. In particular, synthesis of the photosystem II reaction center polypeptides D1 and D2 increases considerably during dark-light transitions in both wild type and the y-1 mutant strain which is unable to synthesize chlorophyll in the dark [55]. Since the corresponding mRNA levels remain unchanged in the light, the observed increase of D1 and D2 synthesis must occur at a post-transcriptional level. It has indeed been shown that translation is a key step in this process [10, 55].

A protein complex has been isolated and shown to bind to the 5′-untranslated region of *psbA* mRNA [10]. This complex includes polypeptides of 60 and 47 kDa. The latter binds to a 36 base stem-loop structure located upstream of a potential SD sequence of the *psbA* mRNA. A good correlation was found between the RNA binding activity of the complex and the level of translation of the *psbA* mRNA under light and dark growth conditions [10]. In contrast, only minor differences in the amount of 47 and 60 kDa protein could be detected in light- and dark-grown wild-type cells. These observations suggest that modulation of the binding of the protein complex to the 5′-untranslated region of *psbA* mRNA controls its translation.

To test the function of the stem-loop structure of *psbA* RNA in translation, several mutations were generated in this region and tested *in vivo* [59]. Deletion of the terminal loop or disruption of base pairing in the stem significantly reduced D1 synthesis to levels less that 5% of wild type. These mutants were however still able to accumulate D1 to 20% of wild-type levels when grown under non-saturating light. Translation of *psbA* mRNA in dark-grown cells was found to be the same for these mutants and wild type. Surprisingly, deletion of the entire stem-loop structure did not affect appreciably D1 synthesis and accumulation. Whether light activation of D1 translation occurs in this mutant was not determined. Taken together, these data suggest that the *psbA* stem loop region could act as a translational attenuator that is overcome in wild-type cells to induce translation [59].

Recent work has identified some of the molecular mechanisms controlling the binding of the protein complex to the 5′-untranslated region of *psbA* mRNA. A serine/threonine protein phosphotransferase associated with the *psbA* mRNA-binding complex has been identified that utilizes the β-phosphate of ADP to phosphorylate the 60 kDa protein and to inactivate *psbA* mRNA binding *in vitro* [11]. These data raise the possibility that translation of *psbA* mRNA is decreased by phosphorylation of the *psbA* mRNA complex in response to an increase in the stromal ADP levels upon transfer of cells to the dark.

It has been proposed recently that light-regulated translation of chloroplast *psbA* mRNA may occur through photosynthesis-generated redox potential [12]. The evidence is based mostly on *in vitro* experiments showing that oxidized *psbA* protein complex no longer binds to *psbA* RNA and that binding can be restored upon incubation of the protein complex with dithiol-containing reducing agents. These results raise the interesting possibility that thioredoxin, which is known to activate the enzymes of the reductive pentose phosphate cycle [4], could also modulate binding of the *psbA* protein complex to *psbA* RNA and, hence, D1 translation in response to changes in the reducing power generated by photosynthesis. The *in vivo* effect of chloroplast redox potential on D1 expression was examined in a photosystem I-deficient mutant strain

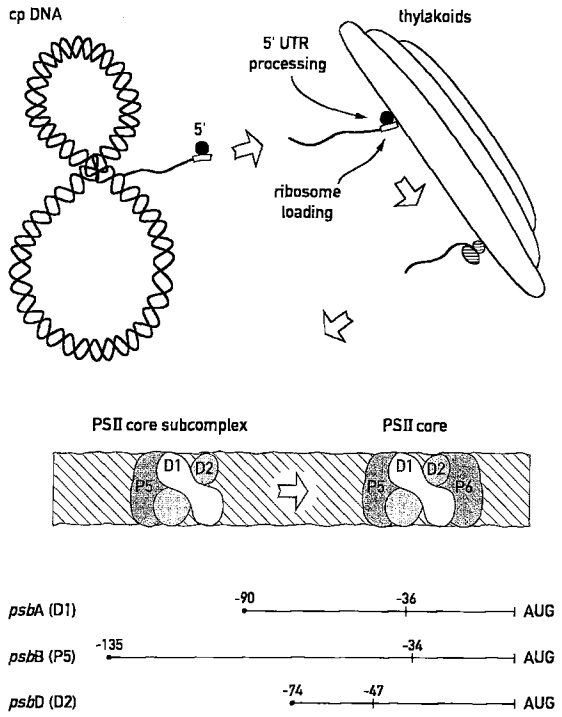

Figure 4. Model for synthesis of the photosystem II core polypeptides. The mRNAs of D1 (*psbA*), D2 (*psbD*) and P5 (*psbB*) are synthesized as precursors. A protein complex binds to the 5′ end of the leader, thereby targeting and/or docking the RNA to the thylakoid membrane. Cleavage of the leader is required to induce a conformational change allowing ribosome binding and initiation of translation. Synthesis of D1, D2 and P5 which form an intermediate photosystem II core subcomplex may proceed by a similar mechanism, including the same maturation pathway for the 5′ leader. The *psbC* product P6 is integrated in a subsequent step to form the photosystem II core complex. The size of the 5′-untranslated regions of *psbA*, *psbB* and *psbD* mRNA with the cleavage site are indicated in the lower part of the figure. Similar models have been proposed by others [22, 60].

in which thioredoxin is no longer reduced [4]. The observed decreased binding of the complex to *psbA* RNA and the reduced synthesis of D1 and the other photosystem II core polypeptides in light-grown cells are difficult to interpret because photosystem I-deficient mutants are highly sensitive to light. In contrast to this report, earlier pulse-labeling studies with photosystem I deficient mutants did not reveal a diminished synthesis of the core photosystem II polypeptides [23, 86].

A puzzling observation is that both S1 nuclease mapping and primer extension studies have revealed that the *psbA* mRNA is made as a precursor with its 5′ end at position −90 relative to the initiation codon and that this RNA is cleaved at −36 to give rise to the more abundant mature *psbA* RNA [21, 66]. The presumed processing site is located within the stem loop structure shown to constitute the binding site of the *psbA* protein complex [10]. Cleavage at this site would most probably abolish the RNA binding activity of the protein complex. This case resembles the processing of the *psbD* precursor RNA described above which abolishes binding of the 47 kDa protein [66]. It is possible that binding of the protein complex to the *psbA* mRNA is required for its targeting or docking to the thylakoid membrane, the site of translation of *psbA* mRNA [34]. Proper docking would be followed by cleavage of the precursor RNA which would make the *psbA* RNA competent for ribosome loading and translation initiation (Fig. 4).

Besides the mRNAs of *psbA* and *psbD*, the *psbB* RNA is also synthesized as a precursor and cleaved within its 5′-untranslated region to generate the mature mRNA (J. Nickelsen, unpublished results). Previous work has revealed the concerted expression of the *psbA*, *psbD* and *psbB* genes [13, 20, 42]. Their corresponding products, D1, D2 and P5, form a protein complex intermediate during the biosynthesis of the photosystem core [13]. Possibly, synthesis of these three hydrophobic polypeptides might follow a common pathway as outlined above for *psbA* mRNA translation (Fig. 4).

The analysis of proteins binding to the 5′-untranslated regions of several chloroplast mRNAs (*psbA*, *atpB*, *rbcL*, *rps7*, *rps12*) by UV crosslinking revealed at least seven polypeptides of 81, 62, 56, 47, 38, 36 and 15 kDa [31]. These RNA-binding proteins were detectable in cells grown under mixotrophic, heterotrophic and phototrophic conditions. Competition experiments demonstrated the binding specificity of some of these *trans*-acting proteins for the chloroplast 5′-untranslated regions [31]. These regions are highly AU-rich (70–84%) in *C. reinhardtii* with no significant sequence homology to one another. However, most of these regions appear to have a secondary structure, in particular stem-loops, which might be required for the binding. The RNA binding activity of the 36 kDa protein was undetectable in mutant cells with reduced chloroplast protein synthesis capacity. Such cells are known to preferentially translate mRNAs for chloroplast-encoded ribosomal proteins and to severely reduce the translation of photosynthetic proteins [54]. Whether the 36 kDa protein is synthesized on chloroplast ribosomes or is required for translation of mRNAs of photosynthetic proteins remains to be determined.

The observation that six of the RNA-binding proteins bind to five distinct chloroplast leaders under a wide variety of environmental conditions suggests that at least some of them, in particular the 81 and 47 kDa proteins, are general leader binding proteins. However, it is likely that there is a whole class of RNA-binding proteins in the 47 kDa range, some of which may be message-specific while others may bind ubiquitously to all chloroplast leaders.

Assembly of the photosynthetic complexes

The numerous mutants of *C. reinhardtii* deficient in photosynthesis have provided unique opportunities to dissect the assembly process of photosynthetic complexes and, in particular, to study how the absence of certain polypeptides alters the assembly of the other subunits. These studies have revealed that impaired synthesis of one subunit usually leads to the destabilization of the corresponding complex through increased turnover of the other subunits [13, 20, 42, 72, 81a]. It is striking that the complexes of *C. reinhardtii* are considerably more sensitive to small alterations than their homologues in cyanobacteria. In *C. reinhardtii*, loss of the *psbK* subunit of photosystem II leads to the destabilization of the complex which accumulates at vastly reduced levels relative to wild type [87]. In cyanobacteria, however, a mutant lacking *psbK* is still able to grow photoautotrophically [41]. Similarly, disruption of the *psaC* gene prevents stable accumulation of the photosystem II complex in *C. reinhardtii* [86], but not in cyanobacteria [56]. These observations point to the existence of a clearing system in *C. reinhardtii* which recognizes and degrades misfolded polypeptides and protein complexes, and that is more efficient than its cyanobacterial counterpart. The molecular components of this clearing system remain to be identified.

Assembly of the cytochrome *b6f* compex of *C. reinhardtii* has been examined by using mutants deficient in cytochrome *b6f* activity which were unable to synthesize the chloroplast-encoded subunits cytochrome *f* or cytochrome *b6*. In these mutants, the other subunits of the complex do not accumulate [53]. In contrast, in the absence of the nuclear-encoded Rieske protein, the chloroplast-encoded subunits accumulate to over 50% of wild-type levels [53]. This concerted accumulation of the chloroplast-encoded subunits has been studied further by constructing mutants with deletions of *petA*, *petB* or *petD*, which do not synthesize cytochrome *f*, cytochrome *b6* and subunit IV, respectively [51]. Analysis of these mutants revealed that the rate of synthesis of cytochrome *b6* and subunit IV are unaffected by the absence of the other subunits of the complex, although the accumulation of either of these two subunits is considerably reduced. In marked contrast, the rate of cytochrome *f* synthesis was severely decreased in the absence of either subunit IV or cytochrome *b6*, but cytochrome *f* was stable under these conditions [51]. Hence, two distinct mechanisms operate to achieve stoichiometric accumulation of the chloroplast-encoded subunits of the cytochrome *b6f* complex: proteolytic degradation of unassembled subunits and a regulatory process in which the production of cytochrome *f* depends on its immediate interaction with cytochrome *b6* and subunit IV.

The stability of cytochrome *f* observed in the absence of the other subunits suggests that it is the core subunit through which the other subunits of the complex are stabilized. One would therefore expect that the stoichiometric accumulation of cytochrome *f* with the other subunits is controlled to prevent overproduction of the protein. The C-terminal region of cytochrome *f* which includes a transmembrane anchoring domain appears to be involved in the control based on the observation that its removal leads to a 3-fold increase in the rate of synthesis of the truncated soluble form of cytochrome *f*, which is redox-active and accumulates in the thylakoid lumen [52]. It has been proposed that the C-terminal region of cytochrome *f* down-regulates synthesis of the protein when it is not properly assembled within the complex. Thus, mutants lacking the C-terminal region or expressing unstable cytochrome *f* lack the feedback control [90]. The nature of this feedback contol remains to be determined.

Synthesis of cytochrome *f* involves translation of the *petA* mRNA, N-terminal processing of the precursor protein and heme attachment. To test whether the latter two events are interdependent, chloroplast cytochrome *f* mutants were constructed whose heme-binding site or the processing site was inactivated [50]. The mutants affected in heme binding were non-phototrophic, accumulated reduced levels of cytochrome *f* and revealed that heme binding is not required for cytochrome *f* processing. Similar conclusions were reached from the analysis of mutants defective in heme attachment to apocytochromes *c6* and *f* [39, 40]. The analysis of the processing site mutants showed that pre-apocytochrome *f* can bind heme and that it is competent for assembly in cytochrome *b6f* complexes [50]. Incorporation of these mutations in the truncated form of cytochrome *f* lacking the C-terminal membrane anchor

led in all cases to a stimulation of synthesis of cytochrome *f*. Hence, the two maturation events of cytochrome *f* do not appear to be rate-limiting for its synthesis [50].

Other cases are known in *C. reinhardtii* in which some chloroplast-encoded subunits display decreased rates of synthesis when another subunit of the same complex is no longer synthesized. These epistatic effects in protein synthesis lead to the stoichiometric accumulation of the various subunits of photosynthetic complexes and ensure that some subunits, such as cytochrome *f*, are produced at a rate which is governed by the assembly process.

In mutants unable to synthesize the PsaB reaction center subunit of photosystem I, the PsaA subunit is no longer produced [23]. However, the opposite is not true: mutants unable to synthesize the PsaA subunit still synthesize the PsaB subunit [23]. Similarly, analysis of several photosystem II-deficient mutants has revealed a dependence of D1 synthesis on the presence of D2 and of P5 synthesis on D1 [13, 20, 42]. Further, for the chloroplast ATP synthase, the β-subunit appears to control the rate of synthesis of the α-subunit [17]. As the chloroplast genes of these subunits are transcribed independently, the observed coordinated synthesis cannot be explained by translational coupling as described for bacterial polycistronic RNAs, in which translation of an upstream open reading frame facilitates translation of a downstream sequence (cf. [25]).

Conclusions and perspectives

Analysis of numerous nuclear mutants of *C. reinhardtii* deficient in photosynthetic activity has revealed that the interactions between the nuclear and chloroplast genetic systems are highly complex. A surprisingly large number of nuclear loci are involved in the expression of chloroplast genes. These loci define factors that are most likely imported into the chloroplast, where they act in a gene-specific manner, mostly at post-transcriptional steps such as RNA stabilization, RNA processing and splicing and translation. Several recent studies based on the use of the powerful chloroplast transformation technology and molecular-biochemical methods have identified the targets on the chloroplast messages of several of these *trans*-acting factors. The picture which emerges from this work is that the 5′- and 3′-untranslated regions of the plastid mRNA play a key role in RNA metabolism and several *cis* elements, in particular the stem-loop structures of the 5′ leaders of *psbC* and *psbA*, appear to be especially important. Although gel mobility shift assays and UV crosslinking experiments have revealed the existence of several RNA-binding reactions, little is known on how these proteins interact with their targets and how they modulate the expression of the chloroplast genes. It is likely that several of these RNA-binding proteins form protein complexes with other factors that could be involved in various functions such as RNA targeting or docking to the thylakoid membrane, RNA stability and activation of translation, and whose action may be environmentally controlled. With the improved nuclear transformation efficiency in *C. reinhardtii* and the availability of cosmid libraries, genomic complementation of specific nuclear photosynthetic mutants has been achieved. Gene tagging has also been used successfully to isolate nuclear genes [88]. It is therefore likely that several mutant loci will be cloned in the next years and the corresponding products identified and characterized.

An important task will be to complement these studies with *in vitro* systems. While RNA stability can be studied *in vitro* with suitable chloroplast extracts, processing of RNA precursors has met with limited success and no functional *in vitro* splicing system has been reported, except for the self-splicing of the group I introns. The recent development of a faithful plastid *in vitro* translation system in tobacco [37] opens promising possibilities for a biochemical dissection of the mechanisms of plastid translation and its relation to RNA stability and processing.

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