Translation and translational regulation in chloroplasts

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Abstract

The translation mechanism of chloroplast mRNAs originated as prokaryotic-type, but has since evolved considerably. Chloroplast translation became, in large part, uncoupled from transcription, and turned into a highly regulated process. Concomitantly, chloroplast ribosomes, general translation factors, and transcripts changed substantially from their prokaryotic counterparts. A multitude of nucleus encoded regulatory proteins evolved that interact in a specific manner with elements in mRNAs to allow translation regulation in response to environmental and developmental cues. In this chapter, we sum up the current knowledge regarding the translation machinery in the chloroplast using examples of mechanisms utilized for chloroplast translation regulation.

1 Introduction

Chloroplasts are derived from endosymbiosis of oxygenic photosynthetic eubacteria in a non-photosynthetic eukaryotic host (Gray 1993). Hence, the translation mechanism of chloroplast mRNAs originated as prokaryotic-type. As summarized hereinafter, accumulating evidence suggests that chloroplast translation has evolved considerably from its prokaryotic origin. The chloroplast ribosomes, the translation factors, and the transcripts resemble their prokaryotic counterparts but also contain many changes that most likely evolved to facilitate the particular requirements of chloroplast gene expression. A better understanding of the unique features of chloroplast translation is likely to uncover these special requisites.

Gene expression regulation might occur at different points along the linear path from gene to functional protein. In its evolution, chloroplast gene expression underwent a shift from the mostly (but not entirely, Gold 1988) transcriptional regulation observed in prokaryotes to primarily posttranscriptional-based regulation, including regulation of transcript stability, translation, protein turnover, and protein activity (for reviews see Mullet 1988; Gruissem and Tonkyn 1993; Mayfield et al. 1995; Danon 1997; Stern et al. 1997; Zerges 2000; Rochaix 2001; Choquet and Wollman 2002; Eberhard et al. 2002; Nickelsen 2003). The shift to post-transcriptional regulation is also reflected by the mechanism of translation. Whereas translation of nascent transcripts, i.e., cotranscriptional translation, pre-

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vails in bacteria, the translation of chloroplast mRNAs is typically uncoupled to transcription, and is self-regulated in response to environmental and developmental cues (reviewed in Gillham et al. 1994; Mayfield et al. 1995; Danon 1997; Zerges 2000; Eberhard et al. 2002). The finding that transcription and translation are commonly uncoupled indicates the presence of new inhibitory steps that disrupt the constitutive course of prokaryotic-type translation and, thereby, converting it into a regulated mechanism. As will be discussed below, the regulation of the translatability of chloroplast messages entails the concerted action of RNA structures and sequence motifs, mostly in the untranslated region (UTR) of the mRNA, and of nucleus-encoded transacting proteins.

It is important to note that the shift to posttranscriptional-based regulation occurred concomitantly to the most dramatic change of chloroplast genome evolution, the retaining of only about 60 to 200 genes out of the several thousands of its progenitor genome (Martin and Herrmann 1998). Thus, the chloroplast underwent a drastic reduction in the number of transcripts encoded by its own genome. In contrast, hundreds of nucleus-encoded proteins are expected to be transported into the chloroplast and to be involved in RNA-binding activities (Martin and Herrmann 1998; Lorkovic and Barta 2002, Plasmid Proteome Data Bank http://ppdb.tc.cornell.edu; Lurin et al. 2004; van Wijk 2004), suggesting a high ratio of interacting proteins per single chloroplast transcript. Notably, accumulative results of genetic analyses in *Chlamydomonas reinhardtii* (Kuchka et al. 1988, 1989; Rochaix et al. 1989; Drapier et al. 1992; Girard-Bascou et al. 1992; Zerges and Rochaix 1994; Yohn et al. 1996; Stampacchia et al. 1997; Zerges et al. 1997; Cahoon and Timko 2000; Rochaix 2001; Wostrikoff et al. 2001; Dauvillee et al. 2003) and *Arabidopsis thaliana* (Meurer et al. 1996, 1998; Felder et al. 2001; Lennartz et al. 2001; Plucken et al. 2002; Nakamura et al. 2003; Sane et al. 2005; Barneche et al. 2006; Lennartz et al. 2006) have identified nucleus-encoded gene products that are required for the posttranscriptional regulation of chloroplast mRNAs. Interestingly, many of these mutations were each specific to a unique chloroplast mRNA. Moreover, mutational analysis identified three nuclear genes that are required for the translation of a single chloroplast mRNA (Zerges and Rochaix 1994; Zerges et al. 1997). Hence, it is possible that the translation of the small number of chloroplast transcripts itself underwent a shift towards a more transcript specific-type regulation, and that each transcript might be regulated by one, or more, distinct nucleus-encoded proteins. The possibility that the regulatory mechanisms of translation have diversified during chloroplast evolution is further implicated by the non-conserved position of the Shine-Dalgarno (SD) ribosome binding site in chloroplast transcripts and the finding that existing SD sequences are not always necessary for translation initiation (Fargo et al. 1998; Sugiura et al. 1998).

This review aims at summarizing the present understanding of translation and translation regulation mechanisms in the chloroplast. As described below, the most recurrent theme seems to be that a multitude of different strategies were adopted for the regulation of translation of small number of chloroplast mRNAs. The different regulatory schemes use an abundance of unique nucleus-encoded

factors acting together with structured and unstructured cis-elements located predominantly in the 5'UTR of the chloroplast mRNAs.

2 Chloroplast translation machinery

The translation machinery in the chloroplast generally resembles that found in prokaryotes; the chloroplast ribosomes are closely related to the eubacterial 70S-type ribosomes, chloroplast transcripts are not m^7G capped at their 5' end, and lack 3' poly(A) tails. Furthermore, the anti-Shine-Dalgarno (SD) sequences at the 3' ends of the 16S rRNAs of cyanobacteria and chloroplasts share high homology with the *E. coli* anti-SD sequence (Dron et al. 1982; Steege et al. 1982; Maidak et al. 1996). Yet, important differences, which will be presented in detail hereinafter, indicate that the translation and its regulation have evolved considerably.

The genes encoding the chloroplast translational machinery are distributed between the chloroplast and nuclear genomes. The rRNA and tRNA genes are located in the chloroplast genome, while the genes for tRNA synthetases, processing/modification enzymes and part of the ribosomal proteins are located in the nuclear genome. Proteomic studies identified all of the protein components of both the ribosomal 30S and 50S subunits in spinach and in the unicellular green alga *C. reinhardtii* (Yamaguchi and Subramanian 2000; Yamaguchi et al. 2000, 2002, 2003). Spinach plastid ribosome comprises 59 proteins (33 in 50S subunit and 25 in 30S subunit and a putative ribosome recycling factor in the 70S ribosome) of which 53 are *Escherichia coli* orthologues and six have no *E. coli* orthologues and are plastid-unique proteins. Two 50S subunit *E. coli* proteins have no orthologues in the spinach plastid. Similarly, the majority of the proteins that were identified in *C. reinhardtii* are *E. coli* orthologues. Only 20 proteins out of the 59 ribosomal proteins of spinach are encoded in the plastid genome, while the rest are encoded by the nuclear genome. Due to the plastid specific ribosomal proteins and to N- and C-terminal extensions added to some of the other ribosomal proteins, the protein mass of the plastid ribosome is bigger then *E. coli* ribosome in both spinach and *C. reinhardtii*, though the specifics differ between the two organisms. In contrast, only minor changes occur in chloroplast rRNA structure. It was proposed that the additional domains of 30S ribosomal proteins and the 30S plastid-specific proteins might be involved in the regulation of chloroplast translation by mediating the effect of nucleus-encoded factors and/or by assisting in positioning of mRNAs on the ribosome for translation initiation. One suggested role of the plastid-specific 50S ribosomal proteins might be associated with protein targeting to thylakoid membranes (Yamaguchi and Subramanian 2000; Yamaguchi et al. 2000; Manuell et al. 2004).

The 30S ternary complex with mRNA in prokaryotes includes three initiation factors, IF1, IF2, and IF3 (for review see Laursen et al. 2005). Homologues of IF2 have been identified in the chloroplast of *Euglena gracilis* (Ma and Spremulli 1990) and bean (Campos et al. 2001). IF3 homologue has been identified in *Euglena gracilis* (Wang and Spremulli 1991; Lin et al. 1996). Despite the homology,

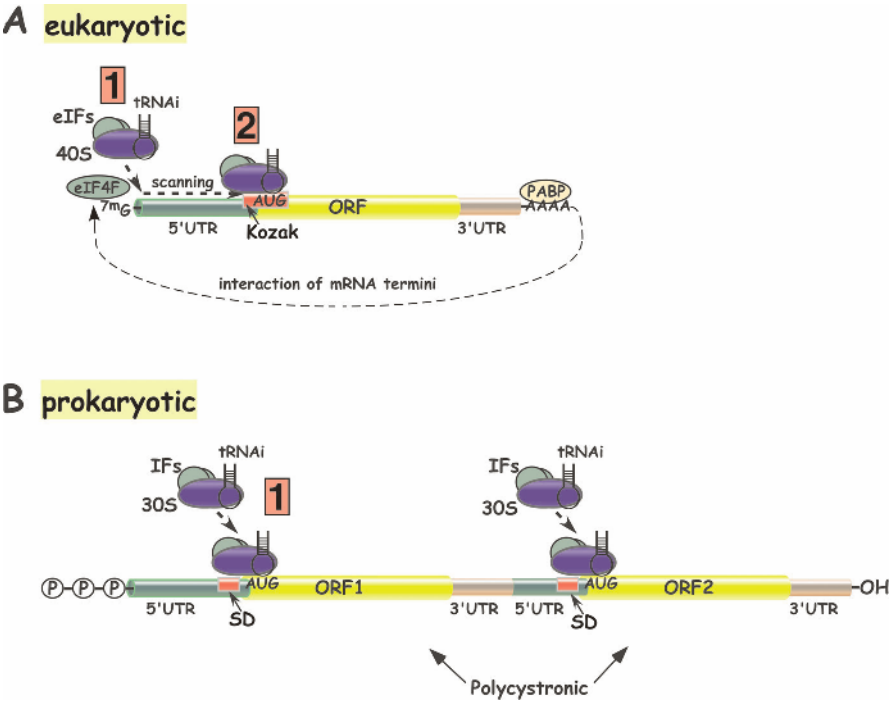

Fig. 1. Translation initiation in prokaryotes and eukaryotes. a. In eukaryotes translation initiation occurs through the scanning mechanism of initiation, which consists of two separate steps. First, the 40S small ribosomal subunit is loaded on the mRNA immediately downstream of the 5'-cap through an interaction between the cap-binding eIF4F complex and the 40S bound eIFs. The eIF4F complex also interacts with the poly(A) binding protein (PABP) bound to the 3' poly(A) tail, creating a 'closed loop' that promotes the recruitment of the 40S ribosomal subunit. In the second step of initiation, the 40S subunit, with the aid of an RNA helicase, which is also a component of the eIF4F complex, scans the RNA in the 5'-->3' direction for the first AUG codon that is embedded in the proper sequence context. b. In prokaryotes the initiation complex binds directly to the initiation codon. The binding of the 30S small ribosomal subunit to the mRNA is facilitated by base pairing between the SD ribosome binding site on the 5' UTR and sequences in the 3' end of the 16S rRNA of the 30S subunit, and this binding localizes the initiation complex to the correct initiation codon. This mechanism allows for the simultaneous translation of several ORFs in a polycistronic transcript. IFs, initiation factors; eIFs, eukaryotic initiation factors; SD, Shine-Dalgarno ribosome binding site.

the chloroplast initiation factors also seems to differ from their prokaryotic homologues. The algal IF3 contains NH_2- and COOH-terminal extensions that are not found in *E. coli* IF3. Sequences in these extensions reduce the activity of IF3 in promoting initiation complex formation with chloroplast mRNAs and 30S ribosomal subunits. It was proposed that these regions allow for a chloroplast-specific regulatory mechanism of initiation, and that their inhibitory effect might be allevi-

ated in response to developmental or environmental conditions such as light (Yu and Spremulli 1998).

3 Mechanisms of translation initiation

Translation initiation of both prokaryotes and eukaryotes begins with two key steps: The *first step* involves the binding of the mRNA by the ribosomal small subunit, and the *second step* is the selection of the proper initiation codon. While in eukaryotic translation these are two well-separated steps, in prokaryotes the two steps are generally combined, such that the binding of the ribosomal small subunit to the mRNA concurrently positions it on top of the selected initiation codon. In eukaryotes, initiation of translation of the vast majority of mRNAs occurs through the following intermediate steps, each of which might be subjected to regulation, of the scanning mechanism (Fig. 1a); (i) The preinitiation complex, comprised of the 40S ribosomal subunit, $tRNA_i^{Met}$ and initiation factors, interacts with the cap-binding initiation factor eIF4F, and as a result binds the mRNA immediately downstream of the 5'-cap; (ii) After exchanging several translation initiation factors and acquiring helicase activity the preinitiation complex scans the mRNA in the 5'-->3' direction for the first AUG codon that is embedded in a consensus sequence that promotes initiation; (iii) On recognition of the proper AUG triplet, which in most cases is the closest to the mRNA cap, base-pairing with the $tRNA_i^{Met}$ anticodon takes place, triggering GTP hydrolysis, and release of several initiation factors. The efficiency of recognition is determined by the sequence surrounding the initiation codon. Energy is needed for the scanning process, and secondary structures in the 5'UTR, or the association of RNA-binding proteins can regulate the rate of translation initiation. A few mammalian cellular mRNAs and several RNA viruses utilize a different translation initiation mechanism that recruits the preinitiation complex to the mRNA by a structured stem-loop RNA motif called the internal ribosome entry element (IRES) in the 5' untranslated region (UTR) of the mRNA (for reviews see Jackson 2005; Kozak 2005).

In prokaryotes, the initiation complex does not bind to the 5' end of the mRNA but rather directly to the SD site, a purine rich sequence typically found approximately 5 to 9 bases upstream of the initiation codon (Fig. 1b). The recognition of and binding to the SD is facilitated by complementary base pairing between the SD and sequences in the 3' end of the 16S rRNA of the small ribosomal subunit. The binding of the initiation complex is further augmented by the interaction of the S1 protein of the 30S ribosomal small subunit with a pyrimidine rich sequence in the 5'UTR of the mRNA (Subramanian 1983; Boni et al. 1991; Sorensen et al. 1998; Sengupta et al. 2001). The binding of the initiation complex to the SD sequence localizes the initiation complex to the proper initiation codon. The *exact distance* between the SD sequence and the initiation codon is therefore *critical* for bona fide translation initiation. This process does not require energy, is usually independent of upstream sequences, and therefore allows for the simultaneous translation of several ORFs in a polycistronic transcript (Gold 1988). The combined

steps of mRNA binding and initiation codon selection in prokaryotic-type translation, thereby, result in the typical cotranscriptional translation. In the rare cases of regulated translation, it occurs via the occlusion of the SD by either RNA structure or RNA-binding protein.

Alternative translation initiation pathways exit in prokaryotes as well, as leaderless mRNAs, lacking an SD sequence, are efficiently translated *in vitro* (Moll et al. 2002). The binding of leaderless mRNA to the ribosome differs from that of canonical mRNAs, but the exact mechanism is not clear yet (for review see Laursen 2005). The faithful translation of leaderless mRNAs in heterologous systems indicated that the ability to translate leaderless mRNAs might be an evolutionary conserved function of the translational apparatus (Moll et al. 2002).

The common uncoupling of transcription and translation in the chloroplast suggests that additional levels of regulation are needed to prevent constitutive translation initiation of mRNAs translation through SD-16S rRNA interactions. Indeed, chloroplast translation initiation seems to deviate from the "classical" prokaryotic translation initiation in several key issues, and there might be more than one way for initiating translation in the chloroplast. A key difference is the role of SD sequences in translation initiation in the chloroplast. Whereas, the exact distance between the SD sequence and the initiation codon is critical for bona fide translation initiation in prokaryotes, the precise role of the SD in chloroplast mRNAs is yet unclear. Though many of the plant chloroplast genes have SD-like sequences in their 5'UTR, the distance of this sequence from the initiation codon is often variable, and deviates from its conserved position in *E. coli*. In the tobacco chloroplast genome, 30 of 79 chloroplast protein-coding genes have no SD-like sequence located within 20 nt upstream from the initiation codon. The remaining 49 genes have SD-like sequences, but not necessarily at a conserved position, and overall two thirds of chloroplast mRNAs do not contain SD sequences at the correct position (Sugiura et al. 1998). Bearing in mind that in prokaryotes the distance between the SD sequence and the initiation codon is critical to the *positioning* of the small ribosomal subunit on top of the authentic initiation codon, the absence of SD sequence at the correct position in approximately two thirds of chloroplast mRNAs suggests that either the binding of mRNA by the ribosomal small subunit and selection of initiation codon are *two separated steps* in the translation initiation of these mRNAs, or that the binding and positioning is achieved by alternative factors, perhaps by regulatory nucleus-encoded proteins that interact with structural RNA elements. Hence, though for some chloroplast mRNAs translation initiation might occur similarly to prokaryotic-type translation initiation (for graphical illustration see Fig. 2a), translation initiation of the majority of mRNAs require additional elements to facilitate recognition of the proper initiation codon by the small ribosomal subunit and efficient translation, allowing for additional levels of regulation. Thus, alternative pathways for the identification of the correct initiation codon in the chloroplast may coexist (Fig. 2b, 2c).

This notion is reinforced by a series of experiments utilizing different species of plants and different methods (for summary see Table 1). First, chloroplast transformation in *C. reinhardtii* was used to look at mutants of chloroplast SD-like sequences. The results demonstrated differing levels of effect on translation

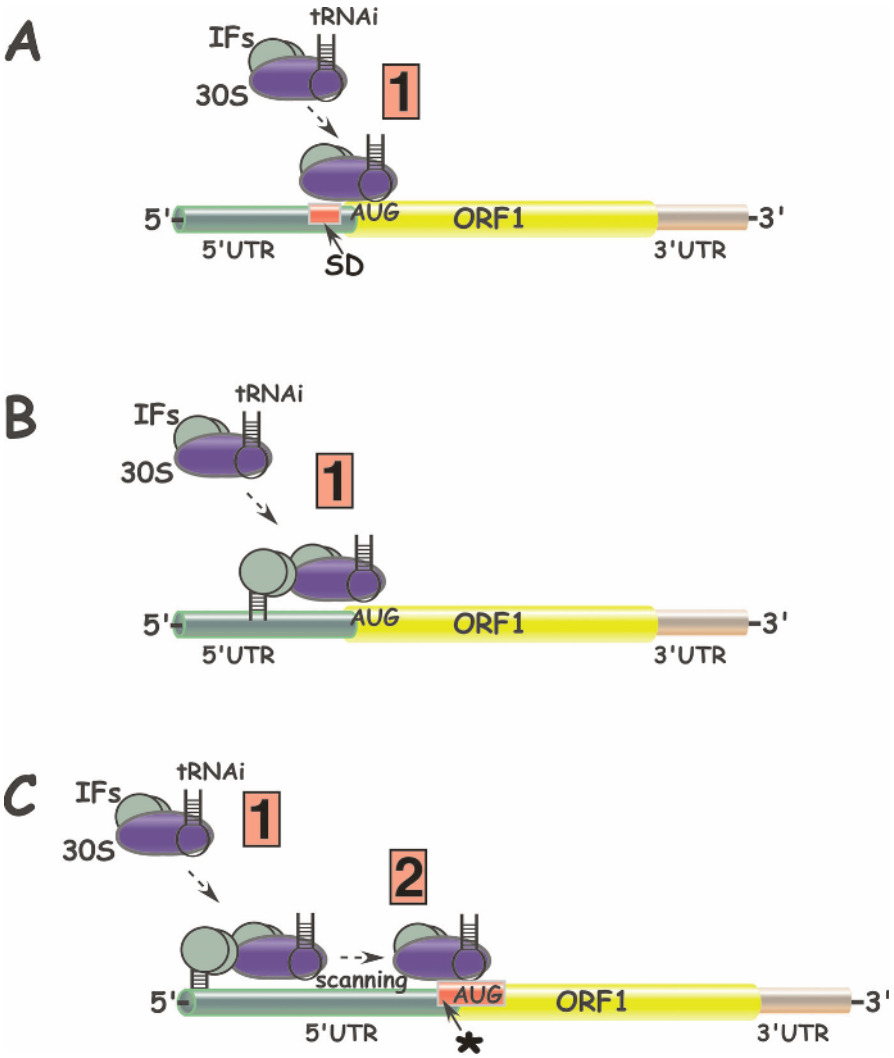

Fig. 2. Models of chloroplast initiation of translation. a. For some mRNAs, translation initiation might occur in the "classical" prokaryotic mechanism. The SD sequence is situated in a conserved position on the 5' UTR, and can recruit the 30S small ribosomal subunit to the correct initiation codon. b. In 5' UTR that lack a conserved ribosome binding site, secondary structure elements within the 5'UTR aided by transacting factors can correctly position the initiation complex to the initiation codon. **c**. Alternatively, cis- and trans-acting elements might bind the initiation complex upstream of the initiation codon, and a mechanism reminiscent of the eukaryotic scanning mechanism might be needed to direct the initiation complex to the correct initiation codon. Notably, a scanning mechanism is likely to require a signature sequence, functionally analogous to the eukaryotic Kozak consensus sequence, stimulating the recognition of the authentic initiation codon. IFs, initiation factors; SD, Shine-Dalgarno ribosome binding site; ∗ initiation codon recognition signature sequence.

Table 1. SD and regulatory cis-elements in the 5'UTR of chloroplast mRNAs

Species	essential SD	nonessential/no SD	alternative cis-elements
Algae			
C. reinhardtii	*psbA*[a], *psbD*[b]	*petD*[c], *atpB*[d], *atpE*[d], *rps4*[d], *rps7*[d]	*petD*[c,e], *atpB*[f], *rps7*[f,g], *psaB*[h], *psbC*[i,j], *psbA*[a], *psbD*[b]
E. gracilis	*atpH*[k]	*rbcL*[l]	*rbcL*[l]
Higher plants			
N. tabacum	*atpE*[m], *rbcL*[m], *rps14*[n]	*rps12*[m], *petB*[m], *psbA*[o], *atpB*[p]	*psbA*[o], *atpB*[p]
Z. mays			*petA*[q], *psaC*[q],
S. oleracea			*psbA*[r], *atpI*[s]

[a]Mayfield et al. 1994, [b]Nickelsen 1999, [c] Sakamoto et al. 1994, [d]Fargo et al. 1998, [e]Higgs et al. 1999, [f]Hauser et al. 1996, [g]Fargo et al. 1999, [h]Stampacchia et al. 1997, [i]Rochaix et al. 1989, [j]Zerges et al. 1997, [k]Betts and Spremulli 1994, [l]Koo and Spremulli 1994, [m] Hirose and Sugiura 2004a, [n] Hirose et al. 1998, [o] Hirose and Sugiura 1996, [p] Hirose and Sugiura 2004b, [q] Schmitz-Linneweber et al. 2005, [r] Boni et al. 1991, [s]Merhige et al. 2005.

depending on the mRNA. Deletion of the SD-like sequence from *psbA* 5'UTR abolished translation, and reduced the level of mRNA (Mayfield et al. 1994). On the other hand, replacement mutations of the SD-like sequences in the 5'UTR of five mRNAs, *petD, atpB, atpE, rps4*, and *rps7* had little or no effect on their translation *in vivo* (Sakamoto et al. 1994; Fargo et al. 1998). In another experiment, replacement mutagenesis of the SD-like sequence in the *psbD* 5'UTR reduced synthesis of the polypeptide to 25% of the wild type level (Nickelsen et al. 1999). Hence, the function of the SD sequence appears to be dependent on the identity of the mRNA.

In a different approach, the 'toe-printing' method was used to examine the role of SD-like sequences in the 5'UTRs of chloroplast mRNAs in *E. gracilis*. In the 'toe-printing' method the position of an initiation complex on the mRNA is determined by its hindering effect of primer extension reaction. Even though mutations of the SD-like sequence of the *E. gracilis* chloroplast *atpH* mRNA resulted in two to fivefold reductions in the efficiency of initiation complex formation, the *rbcL* mRNA was found to be translated independently of the SD like sequence (Betts and Spremulli 1994; Koo and Spremulli 1994).

Biochemical assays utilizing an *in vitro* translation system from tobacco chloroplast proved useful in looking at different regulatory elements of translation initiation (Hirose and Sugiura 1996). Using this system to look at SD-like sequences in tobacco chloroplast mRNAs it was demonstrated that the position of the SD sequence relative to the initiation codon determines its necessity for translation initiation (Hirose et al. 1998; Hirose and Sugiura 2004a). The *atpE*, *rps14*, and *rbcL* mRNAs have SD-like sequences at a position similar to the conserved SD region in *E. coli*, and these sequences were found to be essential for transla-

tion. On the other hand, SD-like sequences in the *rps12* mRNA and in *petB* mRNA are located far from and too close to the initiation codon, respectively, and these sequences are not essential for translation of the corresponding message. The same *in vitro* translation system yielded two very informative observations. First, it was shown that although the tobacco *rps2* mRNA possesses an SD-like sequence at a proper position from the initiation codon, this sequence functions as a negative regulatory element for translation (Plader and Sugiura 2003), suggesting, at least for *rps2* mRNA, a deviation from the prokaryotic function of the SD sequence. Second, an unstructured sequence containing the initiation codon in the proper sequence context was shown to be required for the translation of the tobacco *atpB* mRNA, which does not contain an SD-like sequence (Hirose and Sugiura 2004b), suggesting an additional divergent mechanism for translation initiation. In both cases, trans-acting factors were implied in translation regulation.

Analysis of the binding of *E. coli* 30S ribosomal subunits to barley chloroplast mRNAs *in vitro* has shown that it varies among different messages depending on the existence of a conserved SD sequence. In a message containing an SD-like sequence located in close proximity to the initiation codon, the *E. coli* ribosoma1 subunits associated with the same region as chloroplast ribosomes. Conversely, in a message that does not contain an SD-like sequence located in close proximity to the initiation codon, their patterns of association differed (Kim and Mullet 1994). These results support the notion that mRNA binding by plastid ribosomes seems to have evolved distinct features, and that it may require interactions with trans-acting proteins that are unique to plastid ribosomes.

It is interesting to note that the SD sequence is necessary for the translation of the *psbA* mRNA in *C. reinhardtii*, but is not necessary for its translation in tobacco (Mayfield et al. 1994; Hirose and Sugiura 1996). Similarly, the SD sequence is necessary for the translation of the *rbcL* mRNA in tobacco (Hirose and Sugiura 2004a), but not in *E. gracilis* (Koo and Spremulli 1994). Although the differences might stem from the different assays used to dissect the importance of the SD sequence, the data suggest that the role of the SD sequence in the translation of a specific protein is not necessarily conserved across species.

The role of the prokaryotic S1 protein in binding of ribosomes to mRNAs may suggest a similar function in the chloroplast, especially in messages lacking SD in the correct position. Chloroplast homologs of bacterial S1 were identified in cyanobacteria (Sugita et al. 1995), spinach (Franzetti et al. 1992; Shteiman-Kotler and Schuster 2000), and *C. reinhardtii* (Merendino et al. 2003). The chloroplast S1 protein is a nuclear-encoded protein and is much shorter than the bacterial protein. Different RNA-binding specificities were reported for the chloroplast S1 protein with preference to AU-rich RNA sequences that are common in the 5'UTR of chloroplast genes (Franzetti et al. 1992; Alexander et al. 1998; Shteiman-Kotler and Schuster 2000; Merendino et al. 2003). Thus, further work is needed to determine the authentic binding site of the chloroplast S1 and its possible contribution to the positioning of the ribosome in translation initiation.

Extended interactions between the mRNA and the initiator tRNA might also contribute to the efficiency of translation initiation. In prokaryotes, a uridine at position –1 upstream of the initiation codon was proposed to allow a fourth base pair

with the adenine immediately downstream of the initiator tRNA anticodon. Similarly to *E. coli*, *C. reinhardtii* chloroplast genes preferentially have a U at the -1 position. Indeed, *in vitro* and *in vivo* experiments support a 5' extended codon– anticodon interaction in *C. reinhardtii petA* mRNA translation initiation (Esposito et al. 2001, 2003). It will be interesting to check the relative importance of this extended base-pairing in mRNAs that lack a conserved SD sequence.

Taken together, the above results strongly suggest that: 1) consistently with the expanded role of translational control in the chloroplast, the mechanism of translation initiation of a large portion of chloroplast mRNAs deviates from the classical prokaryotic mechanism of translation initiation; 2) alternative divergent translation initiation pathways exist; 3) trans-acting factors are probably involved in the translational control.

4 Translation initiation regulation – intricate interplay between cis- and trans-acting elements

The findings that SD-like sequences are not always necessary for translation initiation in the chloroplast suggest that the binding and/or the positioning of the initiation complex along the mRNA is mediated by alternative cis-element and trans-acting factors. Though detailed data on the mechanisms that allow for efficient translation initiation in these alternative pathways is still missing, several repeating themes seem to emerge from existing information, and those will be summarized below.

4.1 Cis-elements in chloroplast 5'UTRs

A hallmark of regulated translation in the chloroplast seems to be an abundance of cis-acting elements in the 5'UTRs of chloroplast mRNAs. The mechanisms that are involved in translation attenuation by these cis-elements vary as well. The chloroplast 5'UTR cis-elements can presumably participate in translation initiation pathways in mRNAs that do not utilize a SD sequence. Alternatively, they might complement the SD sequence, or confer specific regulation of translation in response to environmental or developmental cues. In support of this notion, a considerable portion of the cis-elements, that were found up to date in the 5'UTRs of chloroplast mRNAs, appear to promote translation. Examples include elements in the 5'UTRs of the mRNAs of *psbC, petD, rps7, psbD, psbA, psaB*, and *atpB* in *C. reinhardtii* and in several mRNAs from higher plants (for review see Danon 1997; Zerges 2000, and Table 1 and below). There are at least two examples, the *psbD* and *psbA* mRNAs of *C. reinhardtii*, in which both an SD-like sequence and additional cis-elements in the 5' UTR appear to be required for efficient translation initiation, though the mechanisms particulars seem to differ (Mayfield et al. 1994; Nickelsen et al. 1999). The prevalence of positive regulation of translation is higher in chloroplasts relative to prokaryotes, where secondary structure of cis-

elements or protein binding to the mRNA, usually repress translation by blocking access to the initiation site (Gold 1988, Kozak 2005). On the other hand, there are examples of translation regulation that are reminiscent of prokaryotic type translation regulation. The Control by Epistasy of Synthesis (CES) mechanism (Choquet and Vallon 2000, see below) is similar to negative feedback loops found in prokaryotes (Kozak 2005). Another example is the *C. reinhardtii psbD* gene, encoding the D2 protein of photosystem II, which exhibits both positive and negative translation regulation by cis-elements. One *cis*-acting element comprises a stretch of multiple U residues whose deletion completely abolishes the synthesis of the *psbD* gene product (Nickelsen et al. 1999). A second negative regulator element is a double-stranded RNA region encompassing the initiation codon, whose conformation needs to be changed before translation initiation (Klinkert et al. 2006). An interesting example of translation regulation by cis-elements, that demonstrate the divergence of chloroplast translation mechanisms from the "classical" prokaryotic type translation initiation, is the translation regulation of the chloroplast *rps2* gene. The tobacco *rps2* mRNA, which encodes ribosomal protein S2 of the 30S ribosomal subunit, has an SD-like sequence at a proper position relative to the initiation codon. Hence, it was expected that this sequence would play important role in translation initiation of the *rps2* mRNA. Unexpectedly, using *in vitro* translation assay the SD-like sequence of tobacco *rps2* mRNA was found to act as a negative regulator of translation. A trans-acting factor was implicated in the process (Plader and Sugiura 2003).

4.2 Structural elements in 5'UTRs

Stem-loop structures in the 5'UTRs of chloroplast mRNA seem to play an important role in the regulation of translation initiation. One example is the translation of the *C. reinhardtii psbC* mRNA, encoding the 51-kDa chlorophyll-binding PSII reaction center subunit P6, which requires the central 100 nt of its 547 nt 5'UTR (Zerges et al. 1997). This region has the potential to form a stable stem-loop secondary structure. Two bulges in the stem, caused by two sites of noncomplementarity between the strands, are essential for translation. Stem-loop mutations that increased the structure stability resulted in inhibited translation of *psbC* mRNA *in vivo*, whereas point mutation that weakened the structure suppressed the effect of nuclear mutation (Rochaix et al. 1989).

Stem-loop structures were shown as well to be critical to the translation initiation of *C. reinhardtii psbA* (Mayfield et al. 1994, see below) and *petD* mRNAs. Site-directed and linker-scanning mutagenesis identified three distinct elements within the 5'UTR of *petD* mRNA (encoding subunit IV of the cytochrome b6/complex) (Sakamoto et al. 1994; Higgs et al. 1999). Element I appears to form a small stem-loop and is located at the 5' end of the mRNA. It is required for both stability and translation of the mRNA, and may interact with a protein factor to block 5' to 3' exoribonucleolytic degradation of the mRNA (Higgs et al. 1999). The two other elements, II and III, are required for translation, but not mRNA stability. Element II is an unstructured region of 16 nt located in the center of the

UTR and appears to bind proteins that protect it from dimethyl sulfate modification. Element III spans a region of 14 nt close to the AUG initiation codon. This sequence appears to form a stem-loop *in vivo* (Higgs et al. 1999).

Comparison of the 5'UTR sequences of orthologous *petD* mRNAs among four *Chlamydomonas* species demonstrated that although the overall sequence conservation across these species is low, the sequences of the three regulatory elements present in the 5'UTR of the *petD* mRNA and their relative positions appear partially conserved (Kramzar et al. 2006). Functionality of the divergent 5'UTRs was tested in *C. reinhardtii* chloroplasts using reporter genes. Only the nearly identical *C. incerta petD* 5'UTR retained its translational control in *C. reinhardtii* chloroplasts (Kramzar et al. 2006).

Thus, the work on both the *psbC* and *petD* mRNAs suggests that the regulatory interactions between 5'UTR elements and nucleus-encoded factors are highly specific and very sensitive to minor sequence changes (Rochaix et al. 1989; Kramzar et al. 2006).

An interesting case is the translation of *rps7* mRNA encoding the chloroplast ribosomal protein S7. Several mutations isolated in the 5'UTR of the chloroplast *rps7* gene in *C. reinhardtii* reduce expression of reporter genes. These mutations altered the predicted secondary structure of the 5'UTR by weakening the stability of stem structures. Second site mutations that restored the predicted secondary structure suppressed the loss of reporter activity caused by the original mutations, suggesting that a *stable* RNA structure is required for translation (Fargo et al. 1999). The translational negative mutations failed to bind a 20 kDa protein that turned out to be S7 itself (Fargo et al. 2001).

Translation initiation that involves stem-loop 5'UTR elements that interact with protein factors is reminiscent of the translation initiation mechanism demonstrated in a few mammalian cellular mRNAs and several RNA viruses that recruit the 40S ribosomal subunit to the mRNA by a structured stem-loop RNA motif in the 5'UTR called the internal ribosome entry element (IRES) (Jackson 2005).

4.3 General and specific translation factors

A multitude of genetic and biochemical approaches were used to identify nuclear genes that participate in chloroplast gene expression (reviewed in Barkan and Goldschmidt-Clermont 2000). Very little information emerged concerning general factors that promote translation of multiple mRNAs. Using UV crosslinking, at least seven binding proteins of 81, 62, 56, 47, 38, 36, and 15 kDa were detected that bind several different *C. reinhardtii* chloroplast mRNAs. The 81, 47, and 38 kDa proteins were shown to associate with all tested 5'UTRs (Hauser et al. 1996). The identity and function of the different proteins is not yet clear. The level of the 36 kDa protein was diminished in cells that preferentially translate chloroplast-encoded ribosomal proteins, suggesting that it may be required for translation of a class of proteins encoding photosynthetic proteins (Hauser et al. 1996). In another set of experiment the S7 ribosomal protein was shown to bind several different mRNAs including the *rps12, rbcL, atpB, and psbA* mRNAs, raising the intriguing

hypothesis that S7 might have a role in the translation initiation of a subset of chloroplast mRNAs (Fargo et al. 2001). Competition assays in spinach demonstrated that four ATP synthase 5'UTRs were able to compete with each other for binding by proteins in a chloroplast extract. Thus, at least some of the binding proteins recognized all four of those 5'UTRs (Hotchkiss and Hollingsworth 1999). Furthermore, competition-binding assays between an ATP synthase 5'UTR and 5'UTRs from several other chloroplast genes revealed that the ATP synthase-binding proteins can bind the majority of the 5'UTRs examined (Robida et al. 2002). Though the function of these binding proteins is not known, these findings suggest that some RNA-binding proteins have a more general role in the regulation of either mRNA stability or translation.

In contrast to the paucity of data regarding general translation initiation factors, a growing body of data supports the importance of mRNA specific protein factors. Thus, a large number of nucleus-encoded proteins were found, each needed for the translation of only one or few chloroplast mRNAs. This became evident at first with the discovery of several nuclear mutations in *C. reinhardtii* that cause reduction or elimination of translation of specific chloroplast mRNAs. Examples include nuclear mutations that disrupt the translation of the *psbC* (Rochaix et al. 1989; Zerges and Rochaix 1994; Zerges et al. 1997), *psbA* (Girard-Bascou et al. 1992; Yohn et al. 1996), *psaB* (Stampacchia et al. 1997; Dauvillee et al. 2003), *atpA* (Drapier et al. 1992), *petA* (Wostrikoff et al. 2001), and *psbD* mRNAs (Kuchka et al. 1988, 1989). Though less common, there are also examples of nuclear mutations in higher plants that cause decrease in synthesis of specific chloroplast proteins. In maize, mutants of the nuclear gene *crp1* are defective in the translation of the chloroplast *petA* and *petD* mRNAs, and also fail to process a monocistronic *petD* mRNA from its polycistronic precursor (Barkan et al. 1994). The maize nuclear *atp1* gene is required for translation of the *atpB* mRNA (McCormac and Barkan 1999).

4.4 Multiple proteins interact with single mRNA

As exemplified in the translation of *psbA* mRNA, that is discussed in detail further on, another emerging theme in the translation regulation of chloroplast mRNAs is the involvement of several protein factors in the translation regulation of a single mRNA. UV crosslinking experiments identified at least seven proteins that bind to several different *C. reinhardtii* chloroplast mRNAs (Hauser et al. 1996), and five RNA binding proteins ranging from 16 to 80 kDa were shown to bind to the *rps7* 5'UTR (Fargo et al. 2001). The different protein factors can interact with each other and the 5'UTR or can function independently of each other. For example, the products of three nuclear loci were shown to interact with the *psbC* 5'UTR of *C. reinhardtii*. Two of them, Tbc1 and Tbc3, interact with each other and sequence elements in the 5'UTR to activate translation initiation at the GUG initiation codon of the mRNA (Zerges et al. 1997). Another nuclear gene, Tbc2, appears to function in *psbC* translation independently of Tbc1 and Tbc3 (Zerges et al. 1997, 2003).

The 5'UTR of the chloroplast *psbD* gene of *C. reinhardtii* encoding the D2 protein of photosystem II contains several distinct RNA elements, which are involved in the translational control of its expression. One of these elements is an SD-like sequence. A second is a stretch of eleven consecutive U residues, interrupted by a single A residue. Deletion of this sequence abolishes translation of the *psbD* mRNA (Nickelsen et al. 1999). A 40 kDa RNA binding protein (RBP40) was shown to interact specifically with the U-rich element, and is needed for translation of the *psbD* mRNA. Furthermore, interaction of RBP40 with the *psbD* 5'UTR was found to be dependent on the Nac2 factor, which is required for the stabilization of the *psbD* mRNA (Nickelsen et al. 1994; Boudreau et al. 2000; Ossenbuhl and Nickelsen 2000).

The involvement of multiple proteins in the translation regulation of a single mRNA was demonstrated in higher plants as well. In spinach chloroplasts, two conserved regions in the 5'UTR of *atpI* mRNA were shown to bind at least two different proteins, though the exact function of the proteins remains to be clarified (Merhige et al. 2005).

The findings of both general and transcript specific RNA-binding proteins may implicate a more eukaryotic type regulation. The emergence of systems biology has effectively demonstrated that RNA-binding proteins that regulate eukaryotic gene expression tend to bind specific mRNA subpopulations ranging from tens to hundreds different mRNAs (Hieronymus and Silver 2004; Keene and Lager 2005), and that the final level of synthesized protein is influenced by the combinatorial effect of several regulatory circuits at the same level. Gene expression regulation in the chloroplast might represent a similar but much smaller regulatory network. Nucleus-encoded RNA-binding proteins might bind specifically only one or a few chloroplast mRNAs, and the final protein level might depend on the combined effect of several smaller regulatory circuits on any specific mRNA.

5 Translation regulation examples

The complex array of general and mRNA specific cis- and trans-regulatory elements creates a network that allows for the dynamic and coordinated chloroplast translation regulation necessary to respond to developmental and environmental cues. There are only a few cases for which detailed information regarding this intricate regulation exists. Some examples are given below.

5.1 Translation regulation of D1 synthesis

Photodamage to the D1 protein of photosystem II necessitates rapid turnover and replacement with newly synthesized D1 for continuation of efficient photosynthesis. Light induces a 50 to 100-fold enhancement of synthesis of D1 without an equivalent increase in *psbA* mRNA levels in higher plants and algae cells, suggesting that translation is the regulated step (Fromm et al. 1985; Klein et al. 1988;

Malnoe et al. 1988; Krupinska and Apel 1989). The mechanism of light-regulated translation of *C. reinhardtii psbA* mRNA, encoding the D1 protein, was thoroughly studied over the last few years, and therefore provides a good case study to look at the way the repeating themes of translation regulation in the chloroplast converge to create an orchestrated response to changing environmental conditions.

The 5'UTR of *C. reinhardtii psbA* gene contains a stem-loop element immediately upstream of a putative SD sequence. The SD sequence is located 27 nucleotides upstream of the initiation codon, i.e., in a non-conserved position. Both elements play a role in protein synthesis. Deletion of the SD-like sequence abolished translation, and reduced the level of mRNA, while site-directed mutations that disrupt the stem-loop element reduce D1 protein synthesis without affecting *psbA* mRNA accumulation (Mayfield et al. 1994).

A set of mRNA binding proteins which bind the *psbA* 5'UTR with high affinity and specificity has been identified and purified from *C. reinhardtii* cells by RNA-affinity chromatography, capable of isolating both proteins that bind directly to the RNA and proteins that are associated through protein-protein interactions (Danon and Mayfield 1991). *psbA* 5'UTR-binding proteins are composed of four proteins: RB38, RB47, RB55, and RB60. These form a complex (*psbA* 5'PC), which binds the mRNA through the RB47 protein. The level of binding of *psbA* 5'PC to the mRNA parallels the level of *psbA* mRNA translation and association with polyribosomes in light- and dark-grown wild type *C. reinhardtii* and in several mutants lacking translation of *psbA* mRNA (Danon and Mayfield 1991; Trebitsh and Danon 2001; Zou et al. 2003). This suggests that light regulates polyribosome association and translation of *psbA* mRNA by modulating the binding of *psbA* 5'PC to the 5'UTR.

In contrast to most of the nucleus-encoded translation regulators of chloroplast mRNAs, two of the four proteins that constitutes the regulatory *psbA* 5'PC, namely RB47 and RB60, have been cloned and characterized. Both proteins are nucleus-encoded proteins that are targeted to the chloroplast of *C. reinhardtii* (Yohn et al. 1998b; Trebitsh et al. 2001), where they associate with both the full length 5'UTR of *psbA* mRNA and its mature processed form (Danon and Mayfield 1991; Bruick and Mayfield 1998) and regulate the expression of the message (Yohn et al. 1996, 1998a; Trebitsh et al. 2000; Trebitsh and Danon 2001). RB47 is a member of the eukaryotic poly(A)-binding protein (PABP) family, and like all members of the family, contains four conserved RNA recognition motifs (RRMs) (Yohn et al. 1998a). PABPs are involved in polyadenylation of mRNA, but also in different aspects of translation initiation and termination, and mRNA decay (Mangus et al. 2003). In *Chlamydomonas*, the cytoplasmic PABP, a 69 kDa polypeptide, is imported from the cytoplasm into the chloroplast, where it is processed to the 47 kDa form (Yohn et al. 1998a). The RB60 protein shows high homology to protein disulfide isomerase (PDI) (Trebitsh et al. 2001), an oxidoreductase that was identified first as a highly abundant, essential protein in the lumen of the ER where it catalyzes the formation, reduction and isomerization of disulfide bridges of nascent proteins during their folding in the ER. RB47 binds directly to the mRNA (Danon and Mayfield 1991), whereas, RB60 is thought to bind to RB47 and to modulate its activity, probably by oxidoreducing specific thiol groups of

RB47 (Danon and Mayfield 1994b; Kim and Mayfield 1997; Fong et al. 2000; Trebitsh et al. 2000; Alergand et al. 2006).

Two complementary regulatory mechanisms have been proposed for RB60 control of RB47 activity. In the first, the binding of *psbA* mRNA is regulated by the reduction and oxidation of disulfide groups in RB60 (Danon and Mayfield 1994b; Trebitsh et al. 2000). Because the pool of RB60-thiols in the chloroplast becomes proportionally reduced with increasing light intensity it was suggested that the purpose of this regulatory mechanism is to modulate *psbA* mRNA translation in parallel to incident light (Trebitsh et al. 2000). In the second mechanism, ADP-dependent phosphorylation of RB60 inactivates the binding to *psbA* mRNA. As the inactivation by phosphorylation of RB60 requires high ADP concentrations, normally attained only in chloroplasts in the dark, the role of this mechanism is thought to diminish *psbA* mRNA translation in darkness (Danon and Mayfield 1994a). The mechanism by which the phosphorylation, or redox state, of RB60 activates or inactivates the translation of the *psbA* mRNA is still unknown.

Recently, a new player in the translation regulation of *psbA* mRNA was cloned and characterized. Tba1 is a novel protein, whose expression is needed for *psbA* mRNA/ribosome association and D1 translation. Tba1 is also needed for RB47 RNA binding activity, but its exact role in the mechanism described above is still unknown (Somanchi et al. 2005).

Whether a similar mechanism of light-regulated translation of *psbA* mRNA exists in higher plants is unclear to date. Yet, light-regulated translation in higher plants exhibits several similar characteristics to the mechanism identified in *C. reinhardtii* and some differences as well. In *A. thaliana,* two proteins of 43- and 30-kDa were shown to bind the *psbA* 5'UTR. Oxidizing conditions abolished the association of the proteins with the 5'UTR, while RNA-binding activity was recovered upon incubation with a reductant. Thus, it was hypothesized that similarly to *C. reinhardtii,* redox-dependent interactions play a role in the posttranscriptional regulation of *psbA* gene expression in *A. thaliana* (Shen et al. 2001).

Heterologous genes fused to tobacco *psbA* 5'UTR are enhanced by light, suggesting that similarly to *C. reinhardtii*, initiation of D1 translation in tobacco plastids is controlled via the *psbA* 5'UTR (Staub and Maliga 1994). But, unlike *C. reinhardtii psbA* mRNA, the SD-like sequence in the 5'UTR of tobacco *psbA* mRNA has little influence on translation. Translation requires three other elements within the 5'UTR. Two of them are complementary to the 3'-terminus of chloroplast 16S rRNA (termed RBS1 and RBS2) and the other is an AU-rich sequence located between RBS1 and RBS2 and is termed the AU box (Hirose and Sugiura 1996). The AU box was shown to be recognized by a protein factor(s) and a model was proposed for the initiation of *psbA* translation whereby RBS1 and RBS2 bind cooperatively to the 3'-end of 16S rRNA resulting in looping out of the AU box, which facilitates the interaction of a transacting factor(s) (Hirose and Sugiura 1996).

In spinach, a 43 kDa protein homologous to the *E. coli* ribosomal S1 protein has been shown to bind an element in the 5'UTR of *psbA* mRNA that comprises an SD-like sequence. Binding activity of this protein can be detected only after plants have been illuminated (Alexander et al. 1998). There is evidence suggesting

that *E. coli* ribosomal S1 protein can mediate association of mRNA with the 30S ribosomal subunit by binding pyrimidine rich sequences upstream of the SD sequence in the mRNA (Boni et al. 1991). Whether or not the 43 KDa protein might play a similar role in spinach is unclear.

5.2 Negative feedback loops: assembly-controlled regulation of translation

A second well-studied example of cis-acting elements in the 5'UTR of mRNA, which regulate translation initiation through protein binding, is the CES mechanism. The four major multimeric complexes in the thylakoid membrane (PSI, PSII, ATP synthase and cytochrome b_6f) comprise subunits encoded by the chloroplast genome side by side with nucleus-encoded subunits. Thus, a regulated coordinated expression of proteins from the two genomes is essential for an energy efficient and functional assembly of the complexes. This is achieved by post-translational degradation of unassembled subunits (for review see Choquet and Vallon 2000), but also by an assembly regulated translation of some chloroplast-encoded proteins, a phenomenon called CES. The CES process was first studied in the cytochorome b_6f complex in *C. reinhardtii* (Choquet and Wollman 2002). Cytochrome f shows a reduced rate of synthesis in the absence of its assembly partners, cytochrome b_6 or subunit IV, but there is no change in the stability of the protein that is synthesized (Kuras and Wollman 1994). This assembly-dependant regulation of cytochrome f synthesis stems from autoregulation of translation initiation of its own *petA* mRNA. Two components which are required for the reduced translation initiation were identified: the 5'UTR of *petA* mRNA which is sufficient to confer the CES behavior to a reporter gene (Choquet et al. 1998), and a repressor motif on the C-terminal of the unassembled cytochrome f protein that is able to inhibit further translation of its own mRNA (Kuras and Wollman 1994; Choquet et al. 2001, 2003). As there is no evidence for direct binding of cytochrome f to its mRNA, the involvement of a ternary effector was suggested (Choquet et al. 2003). Thus, a negative feedback mechanism insures translation arrest following accumulation of unassembled cytochrome f.

In *C. reinhardtii*, similar negative feedback loops, controlling translation via the 5'UTRs of relevant mRNAs, were shown to exist for the three other multicomplexes in the thylakoid membrane, PSI, PSII, and ATP synthase (Choquet and Wollman 2002; Wostrikoff et al. 2004; Minai et al. 2006). Furthermore, though the molecular details are different, assembly controlled translation of cytochrome b_6f exists also in tobacco (Monde et al. 2000b), and there is evidence for a similar regulation of PSII components in barley (Gamble and Mullet 1989; Kim et al. 1994b).

Ribulose1,5-bisphosphate carboxylase/oxygenase (Rubisco), the enzyme responsible for CO_2 fixation during photosynthesis, is another example of a multimeric complex containing subunits encoded by the chloroplast, side by side with nucleus-encoded subunits. It is composed of a nucleus-encoded small subunit and a chloroplast encoded large subunit. It was shown that when accumulation of the

large subunit is limiting (as in some *rbcL* nonsense and missense mutants), the small subunit levels are adjusted to those of the large subunit at the level of protein degradation (for review see Rodermel 1999). On the other hand, limiting the amounts of small subunits by expression of an *rbcS* anti-sense RNA in tobacco (Rodermel et al. 1988, 1996) or deletion of the gene by insertional mutagenesis in *C. reinhardtii* (Khrebtukova and Spreitzer 1996) resulted in decrease in the translation of the chloroplast *rbcL* mRNA. Thus, it was suggested that the level of small subunits regulates large subunits accumulation at the level of *rbcL* translation. The decrease in large subunit translation might be mediated by inhibition of translation by free large subunits, similarly to CES, or by lack of positive regulation by small subunits. A recent work in tobacco suggests that indeed the underlying mechanism is CES-like, and unassembled large subunit autoregulates its own translation (Wostrikoff 2007), though the identity of the cis-elements involved are not yet known. Interestingly, it was suggested that the light-induced oxidative stress inhibition of Rubisco large subunit translation is caused by structural changes that result in exposure of an RNA recognition motif (RRM) at the N-terminal of the large subunit. It was suggested that the exposed RRM will then bind any RNA in its vicinity including its own transcript, resulting in the translational arrest of the large subunit (Cohen et al. 2005).

6 Regulation of translation elongation

Most of the aforementioned discussion has focused on translation regulation at the level of initiation, which is most commonly the rate-limiting step in translation, and has gained most of the attention of researchers. Yet, evidence for regulation at the level of elongation was found as well. Conceivably, regulation of translation elongation might be beneficial for processes such as cotranslational membrane insertion or assembly.

The activities of the *E. gracilis* chloroplast elongation factors EF-Tu, EF-G, and EF-Ts, as well as the activities of the pea chloroplast EF-G and EF-Tu were shown to be regulated by light (Breitenberger et al. 1979; Fox et al. 1980; Sreedharan et al. 1985; Akkaya and Breitenberger 1992; Singh et al. 2004), suggesting a possible role for these factors in regulation of translation in response to environmental cues. Furthermore, translation elongation of the *psbA* mRNA was demonstrated to be light-regulated in higher plants (Kim et al. 1991; Taniguchi et al. 1993; Kim et al. 1994a; Edhofer et al. 1998; Muhlbauer and Eichacker 1998). Toe print analysis in barley showed that ribosomes indeed pause at distinct sites during the elongation phase of *psbA* mRNA translation (Kim et al. 1991, 1994b; Kim and Mullet 1994). Taken together with extensive work demonstrating that chlorophyll stimulates the accumulation of Dl and other chlorophyll proteins by increasing chlorophyll apoprotein stability, it was suggested that ribosome pausing during elongation improves the efficiency of Dl synthesis by providing additional time for nascent chains to bind cofactors such as chlorophyll prior to polypeptide

release from the ribosomes (Klein et al. 1988; Mullet et al. 1990; Kim et al. 1994a; Kim and Mullet 1994; Zhang et al. 1999, 2000).

Regulation of elongation might occur in additional chloroplast mRNAs. For example, ribosome pausing was also suggested to play a role in the expression of the large ATP synthase gene cluster in spinach chloroplasts (Stollar et al. 1994). Additionally, it was shown that translation initiation complexes for *rbcL* mRNA (encoding the large subunit of Rubisco) are normally formed in the dark, but the elongating step right after formation of translation initiation complexes might be blocked. The release of this translational elongation block upon illumination may contribute to light-activated translation of the *rbcL* mRNA (Kim and Mullet 2003).

Recently, it was reported that that the *C. reinhardtii* plastid-specific ribosomal protein PSRP-7, which contains two S1 domains, is encoded by a gene whose complete ORF codes for a 110 kDa polyprotein that also contains two EF-Ts domains on its carboxy end. The 110 kDa protein containing the S1 domains and the EF-Ts was identified in cell extracts, as well as proteins containing only the S1 or the EF-Ts domains. It was suggested that the structure of this gene implicates coordinated expression of the S1 like protein and EF-Ts, but that the stable expression of the full 110 kDa protein implies that this protein plays a novel, yet unknown, role in translation (Beligni et al. 2004).

7 Interactions of 5’ and 3’ ends of chloroplast mRNA

In eukaryotes, interactions between the two termini of cytoplasmic mRNAs stimulate the initiation of translation. The poly(A) binding protein (PABP) bound to the 3’ poly(A) tail interacts with initiation factors bound to the 5’UTR, thus creating a ‘closed loop’ that promotes the recruitment of the 40S ribosomal subunit. It is generally thought that the ‘closed loop’ role is a quality control mechanism to promote translation of full-length mRNAs rather than truncated forms (Gallie 1998). Translatable chloroplast mRNAs do not contain poly(A) tails. Most of them, similarly to prokaryotic mRNAs, contain an AU-rich 3’UTR with a terminal inverted repeat. The 3’UTR inverted repeat has been shown to play a role in the processing and stabilization of the mRNA (for review see Monde et al. 2000a). Examples of modulation of translation initiation by interactions between the two termini of mRNA in prokaryotes (Lindahl and Hinnebusch 1992; Franch and Gerdes 1996; Voorma 1996) raise the possibility that such interactions might also exist in chloroplast mRNAs and influence their expression. Indeed, there are several reports that support a role for the 3’UTR in translation initiation of several mRNAs. Correct processing of the 3’UTR was suggested to be required for high levels of translation initiation and polysomal association in *C. reinhardtii* cells (Rott et al. 1998). Recent results from tobacco transformants in which the influence of the *psbA* UTRs on translation of a reporter gene were studied indicated that including the *psbA* 3’UTR resulted in a three to fourfold enhancement of translation (Eibl et al. 1999). Furthermore, though high affinity binding of regula-

tor proteins to *C. reinhardtii psbA* mRNA is primarily via its 5'UTR, the 3'UTR was shown to increase the affinity of binding of the 5'UTR-binding protein complex (Katz and Danon 2002). In another study, deletion of the inverted repeat of the 3'UTR of tobacco *petD* mRNA led to a reduction in *petD* expression beyond that expected by the decrease in mRNA accumulation alone, indicating that the 3'UTR might also contribute to efficient translation (Monde et al. 2000a). Further research is needed to establish the generality of this phenomena and its importance for translation efficiency.

8 Subchloroplast location of translation

The chloroplast consists of several different subcompartments (such as the soluble stroma, thylakoids, and the chloroplast envelope), each requiring its own set of proteins as well as other molecules such as pigments, cofactors, and lipids. Furthermore, the assembly of functional complexes within the different subcompartments requires the coordinated assembly of components synthesized within the chloroplasts and components imported from the cytosol. Thus, the location of protein translation within the chloroplast is not self-evident, and several subcompartments were suggested to be involved in the process. Early sedimentation studies in extracts of *C. reinhardtii* demonstrated that a significant percentage of polyribosomes are attached to thylakoid membranes, and this attachment is light dependant (Chua et al. 1973; Margulies and Michaels 1974, 1975; Chua et al. 1976; Bolli et al. 1981). Furthermore, many thylakoid proteins were shown to be synthesized on thylakoid-attached polyribosomes of *C. reinhardtii* and higher plants (Margulies 1983; Minami and Watanabe 1984; Bhaya and Jagendorf 1985; Margulies et al. 1987; Breidenbach et al. 1988; Klein et al. 1988; Shinohara et al. 1988). It was thus suggested that an evolved function of the plastid-specific 50S ribosomal proteins might be associated with protein targeting to thylakoid membranes (Yamaguchi and Subramanian 2000; Yamaguchi et al. 2000; Manuell et al. 2004). Recently, proteomic analysis of *A. thaliana* identified components of the translation machinery in the chloroplast thylakoid membranes thus supporting the notion that thylakoid membranes play a role in chloroplast translation (Friso et al. 2004). Translation of thylakoid proteins on thylakoid-bound polyribosomes makes sense as the proper assembly of thylakoid complexes necessitates coordinated and stepwise assembly of the different components of the complex. Indeed, D1 is synthesized on membrane-bound polyribosomes, and assembled cotranslationally into the membrane (for review see Zhang and Aro 2002). The picture is complicated by the finding that the stromal large subunit of Rubisco is also synthesized on membrane-associated polyribosomes (Hattori and Margulies 1986; Breidenbach et al. 1988; Klein et al. 1988). It was suggested that the translation of the large subunit of Rubisco by thylakoid-attached polyribosomes allows for regulation of translation by the photosynthetic proton gradient without the need for signal transduction to stromal ribosomes (Muhlbauer and Eichacker 1999).

There is also suggestive evidence that translation is associated with the inner membrane of chloroplast envelope (for review see Sato et al. 1999). Furthermore, there is data that imply that thylakoid proteins might be translated in the chloroplast envelope. A set of RNA-binding proteins, including RB47, which was reported to be a specific activator of *psbA* mRNA translation, were found to be associated with chloroplast membranes whose buoyant density and acyl lipid composition imply that their origin is the inner chloroplast envelope membrane. These membranes were found to be associated with thylakoid membranes (Zerges and Rochaix 1998). An earlier report also found polyribosomes attached to a membrane fraction, which differed from thylakoid membranes in polypeptide composition and the amount of chlorophyll it contained (Margulies and Weistrop 1980). It is not entirely clear whether these membranes are a subfraction of the thylakoids or inner envelope membrane or a previously uncharacterized intrachloroplast compartment.

9 Concluding remarks

An increasing body of evidence suggests that chloroplast translation has evolved considerably from its prokaryotic origin. Chloroplast translation became, in large part, uncoupled from transcription, and turned into a highly regulated process. Concomitantly, chloroplast ribosomes, general translation factors and transcripts changed substantially from their prokaryotic counterparts. Accumulating evidence based on genetic, biochemical and proteomic approaches imply that a plethora of nucleus-encoded regulatory proteins that interact in a specific manner with structured and unstructured cis-elements located predominantly in the 5'UTR of chloroplast transcripts have evolved. The dramatic reduction in the number of chloroplast genes, and the expanded number of nucleus-encoded RNA-binding proteins, indicate that the translation of the small number of chloroplast transcripts underwent a shift towards a more transcript specific-type regulation.

While the importance of the chloroplast unique trans-acting proteins and 5'UTR elements to the regulation of translation has been demonstrated repeatedly, a most intriguing question is yet to be resolved; how the initiation complex is positioned onto the bona fide initiation codon? The variable location of transcript-unique translational cis-elements in the 5'UTR relative to the initiation codon, some located far upstream, indicate that the mechanism of positioning diversified from the prokaryotic type. Two likely scenarios might be envisioned; 1) the gap between the binding site of the initiation complex and the initiation codon is bridged by structural elements in the 5'UTR (Fig. 2b); 2) helicase activity associated with the initiation complex promotes, in a similar fashion to eukaryotic translation, scanning for the initiation codon (Fig. 2c). Such a mechanism is likely to require a signature sequence that will enhance the binding of the 30S subunit to the correct initiation codon, similarly to the function of the Kozak consensus sequence in eukaryotes.

Is there a biological gain in the convoluted evolution of chloroplast gene expression or is it a mere outcome of random selection? Interestingly, the mitochondrion, the other endosymbiotic prokaryotic-like organelle, shows many evolutionary parallelism in its evolution to the chloroplast including the transfer of most of its self-encoded functions to the nucleus and an increase in translationally regulated gene expression (Fox 1996). The similar evolution of chloroplast and mitochondrion gene expression indicates a high selection pressure for this type of regulation. The biological advantage of this type of system organization is yet unclear, but its clarification is important to our understanding of the primary principles governing organellar functions.

What might be the special requirements in the chloroplast that made translational control a favored mechanism for regulating gene expression? Examining the type of genes that were retained in the chloroplast and mitochondrion genomes might suggest a possible explanation. In addition to components of gene expression system, i.e., tRNAs, ribosomal RNAs, and proteins, most of the retained organellar genes encode proteins involved in electron transport and energy coupling. Thus, perhaps to counteract the potentially harmful side effects of electron transport chain reactions, structural proteins that maintain redox balance within bioenergetic membranes must be synthesized when and where they are needed (Race et al. 1999). The requirement for dynamic and tight regulation is further accentuated for photosynthetic gene expression in the chloroplast as rapid adjustment is critical to ensure efficient energy production and prevention of deleterious side effects in response to changing light intensity and availability. Translational regulation of gene expression allows for rapid on and off adjustment of rates of protein synthesis from an existing pool of transcripts. In contrast, transcriptional regulation is relatively slow to induce protein synthesis and has to be accompanied by mRNA instability to enable turning off translation in a short time. Why such a complex network of RNA-binding proteins and RNA cis-elements is required for the regulation of chloroplast gene expression? It is possible that the small number of pivotal chloroplast genes is subject to multiple regulatory circuits, including the coordination with nucleus expression, developmental regulation of plastid-type specific expression, metabolism switches between light and dark, and adjustments to changes in light intensity.

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