Update on Chloroplast Molecular Biology

Translational Regulation in the Chloroplast[1]

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TRANSLATIONAL REGULATION IN THE CHLOROPLAST

Gene expression is potentially amenable to regulation at different points along the linear path from a gene to a functional protein. To control gene expression the regulatory event has to occur at a limiting rate. Chloroplast gene expression has been shown to be primarily regulated by posttranscriptional events, including transcript stability, translation, protein turnover, and protein activity. An increasing body of data has demonstrated the significant role of translational control in regulation of gene expression in mature chloroplasts (for review, see Gillham et al., 1994; Mayfield et al., 1995; Rochaix, 1996).

What might be the special requirements in the mature chloroplast that made translational control a favored mechanism for regulating gene expression? Translational regulation provides a capacity to rapidly induce or reduce massive levels 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 diminish translation in a short time. Is there a requirement for rapid adjustment of gene expression in mature chloroplasts? Light, the primary energy source of plants, is captured in the chloroplast and converted into chemical energy by a set of protein complexes localized in the thylakoid membranes. The chemical energy produced by the thylakoid protein complexes in the form of ATP and reducing power drives the different stromal reactions, such as fixation of carbon, fixation of nitrogen, biosynthesis of amino acids, and biosynthesis of lipids. Consequently, the availability and intensity of light have pronounced effects on chloroplast metabolism and hence on chloroplast gene expression. In addition, light absorbance and conversion into beneficial chemical energy is accompanied by deleterious side effects that result in protein turnover that parallels light intensity (Barber and Anderson, 1992). Light intensity and availability are not controlled by plants, and fluctuate during the course of the day and from day to day. Therefore, dynamic adjustment of gene expression is required.

The chloroplast, which probably evolved from endosymbiotic photosynthetic cyanobacteria (Whatley, 1993), lost most of its self-encoded functions to the nuclear genome during its evolution and underwent a shift from the mostly, but not entirely (Gold, 1988), transcriptional regulation observed in prokaryotes. The mitochondrion, the other semiautonomous prokaryotic-like organelle, shows evolutionary parallelism in its evolution to the chloroplast, including the transfer of many of its self-encoded functions to the nucleus and an increase in translationally regulated gene expression (Fox, 1996). It is possible that the similar situation of the chloroplast and mitochondrion, as semiautonomous endosymbiotic organelles, dictates this common path of evolution.

REGULATION OF TRANSCRIPT TRANSLATABILITY

A transcript may have to undergo both nonreversible modifications, such as editing, splicing, and processing, and reversible modifications, such as association with specific proteins, to be competent for translation. Regulation of transcript translatability by nonreversible modifications may allow rapid induction of protein synthesis but should be coupled with decreased mRNA stability to enable rapid decline of translation. Conversely, regulation by reversible modifications can be used for rapid and massive changes of both induction and repression of translation. There is evidence for both types of modifications in the chloroplast. Editing of an ACG codon to the initiator codon AUG creates translatable *psbL* mRNA in tobacco (*Nicotiana tabacum* L.) plastids. This editing activity appears to be mediated by a *psbL*-specific factor that is present in limiting amounts, suggesting that its activity controls the translatability of *psbL* mRNA (Chaudhuri et al., 1995). Processing of *petD* mRNA in maize requires a specific factor *crp1* whose absence results in lack of the monocistronic form of *petD* and a large decrease in translation of *petD* mRNA. This implies that *crp1* may affect the translatability of *petD* by controlling its processing (Barkan et al., 1994). Alternative processing of the 5'-UTR of *rbcL* transcript in barley was proposed to modulate the translatability of *rbcL* in response to exogenous methyl jasmonate (Reinbothe et al., 1993).

Regulation of message translatability by specific protein-mRNA interactions has been implicated mostly by genetic analysis in the unicellular green algae *Chlamydomonas reinhardtii*. Several nuclear mutations in *C. reinhardtii* and *Arabidopsis thaliana* have identified gene products that are required for the translation of chloroplast mRNAs (Meurer et

Abbreviations: ORF, open reading frame; RBS, ribosome-binding site; SD, Shine-Dalgarno sequence; UTR, untranslated region.

al., 1996; Rochaix, 1996). It is interesting that many of these mutations were each specific to a unique chloroplast mRNA, suggesting that specific nuclear-encoded proteins are transported into the chloroplast and that each protein regulates the translation of a specific mRNA (Rochaix, 1996). Chloroplast mutations that suppress the phenotype of the nuclear mutations can be used to identify the site in the organelle acted upon by the nuclear-encoded factors. Chloroplast suppressors of the nuclear mutations F34 and F15 have genetically identified the 5'-UTR of psbC and psaB mRNAs as the site for interaction with the respective nuclear-encoded gene product (Rochaix, 1996). Taken together, these results suggest that nuclear-encoded proteins, targeted to the chloroplast, each interact with the 5'-UTR of a specific chloroplast mRNA and activate the translation of the downstream ORF (Fig. 1). As seen above, many of the nonreversible and reversible modifications that affect transcript translatability affect or interact with the 5'-UTR of the transcript. The biological role of the 5'-UTR has to be discussed to better understand the mechanisms by which translation is regulated.

THE ROLE OF THE 5'-UTR IN INITIATION OF TRANSLATION IN PROKARYOTES AND EUKARYOTES

Ribosomes must recognize and bind the initiator codon of the correct ORF to initiate translation. Several steps preceding and facilitating this event begin with the binding of the translation-initiation complex containing the small ribosomal subunit and several initiation factors to the 5'-UTR of the mRNA. These early steps of initiation of translation differ significantly between prokaryotic and eukaryotic translation (Table I). In eukaryotes the m7G-capped 5' terminus of the mRNA generally serves as the binding site of the translation-initiation complex. Binding of the translation-initiation complex is facilitated by the initiation factor eIF-4F (Mader and Sonenberg, 1995). After binding to the 5' terminus of the mRNA, the initiation complex scans the 5'-UTR for the correct initiator codon. The sequence surrounding the initiator codon determines the efficiency of the recognition by the initiation complex. The scanning process is dependent on the initiation complex

Figure 1. Model of function of nuclear-encoded factors involved in gene-specific translational regulation in the chloroplast.

intrinsic helicase activity and requires energy in the form of ATP. Secondary structures in the 5'-UTR and binding of specific proteins to the 5'-UTR affect the rate of initiation of translation (Kozak, 1992). A small class of cellular eukaryotic mRNAs and several RNA viruses seem to use the internal ribosome entry segment, and not the m7G-cap, as the binding site of the initiation complex (Jackson and Kaminski, 1995).

In contrast, the initiation complex in prokaryotes directly binds to a consensus RBS known as the SD (Gold, 1988). Binding of the initiation complex is facilitated by complementary base pairing of the SD-RBS and sequences in the 3' terminus of 16S rRNA of the 30S small ribosomal subunit. The SD-RBS is typically located about five to nine nucleotides upstream of the initiator codon. Because of the close distance between the SD-RBS and the initiator codon, binding to the RBS localizes the initiation complex to the correct initiator codon and usually determines the rate of initiation of translation. In contrast to initiation of translation in eukaryotes, this process in prokaryotes is not dependent on helicase activity and does not require ATP hydrolysis. The direct RBS binding and internal initiation of translation in prokaryotes is usually independent of upstream sequence and allows for the simultaneous translation of several ORFs in a polycistronic transcript (Gold, 1988).

INITIATION OF TRANSLATION IN THE CHLOROPLAST

The elucidation of the mechanism of initiation of translation in the chloroplast is crucial for an understanding of regulation of protein synthesis in the chloroplast. Unfortunately, the information provided up to now has been inconclusive and may support the hypothesis that there is more than one pathway for initiating translation functions in the chloroplast. The major constituents of the translation machinery in chloroplasts resemble their prokaryotic counterparts, but several differences can be found as well. Similar to prokaryotes, the chloroplast contains 70S-like ribosomes, chloroplast transcripts are not m7G-capped at their 5' end, and chloroplast transcripts can be transcribed as polycistronic units. However, a close inspection of chloroplast ribosomes has suggested that they have deviated from the bacterial ribosomes (Harris et al., 1994). It is conceivable that the chloroplast ribosomes may have undergone these changes to accommodate the translational regulatory pathways that are unique to chloroplasts.

What is the binding site of the chloroplast translation initiation complex? Studies of expression of polycistronic transcripts have indicated that internal ORFs can be translated in polycistronic transcripts (Barkan, 1988), suggesting that, similar to prokaryotes, the chloroplast translation-initiation complex binds internally to the chloroplast transcript and not to its 5' terminus. Analysis of the 5'-UTR of chloroplast mRNAs has demonstrated that only about 40% of the chloroplast mRNAs contain SD-like sequences in close proximity to the initiator codon, and that the spacing between the SD-like domains and the initiator codon is not as strict as in the prokaryotes (Harris et al., 1994). Determination of the functionality of the SD-like domains by site-directed mutations analysis has yielded inconclusive
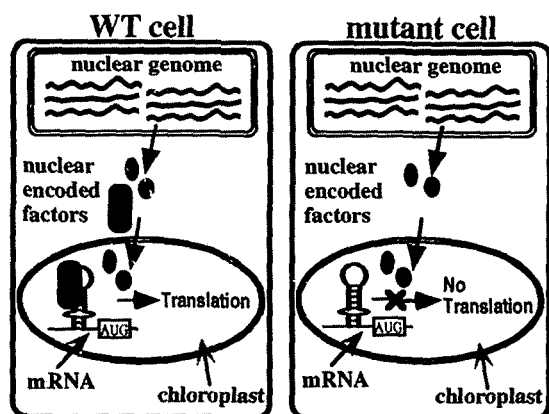
Table I. *Comparison of initiation of translation in prokaryotes, eukaryotes, and chloroplasts*

Property	Prokaryotes	Eukaryotes	Chloroplasts
Initiation complex binding	Internal	Cap-binding	Internal?
RBS	Consensus-SD	m7G-cap	Consensus-SD and others?
Initiation complex binding facilitated by:	Base pairing of SD-RBS and 16S rRNA	Initiation factor eIF-4F	?[a]
Scanning	No	Yes	?
Helicase activity	No	Yes	?
Energy requirement	No	Requires ATP	?
Initiator codon	AUG, GUG, UUG	AUG	AUG, GUG
Recognition of initiator codon	Orientation by RBS-binding	By sequence surrounding the initiator codon	?

[a] ?, As yet unknown.

results. Deletion of the SD-like domain in *psbA* mRNA in *C. reinhardtii* diminished translation and accumulation of the D1 protein, concomitant with decreased stability of *psbA* mRNA in vivo (Mayfield et al., 1994). In contrast, base changes of this site in a chimeric mRNA composed of 5'-UTR of tobacco *psbA* and *lacZ* ORF did not significantly affect its translation in the chloroplast in vitro translation system. However, mutations of additional sites that do not resemble SD domains, but are complementary to the 3' terminus of 16S rRNA, resulted in a significant reduction of translation (Hirose and Sugiura, 1996).

Analysis of the binding of *Escherichia coli* 30S ribosomal subunits to barley chloroplast mRNAs in vitro has shown that it varies among different messages (Kim and Mullet, 1994). In a message containing an SD-like sequence located in close proximity to the initiator codon, the *E. coli* ribosomal 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 initiator codon, their patterns of association differed (Kim and Mullet, 1994). These results suggest that binding of the initiation complex is mediated by the SD-like sequence in messages in which the SD-like sequence is located in close proximity to the initiator codon, and that additional factors unique to the chloroplast are required in the remainder of chloroplast messages. The identity of the *cis*-elements and *trans*-acting factors required for binding of the initiation complex to chloroplast mRNAs that do not contain an SD-like sequence in close proximity to the initiator codon is yet to be determined. Finding of additional 5'-UTR *cis*-elements that are required for translation may help to identify these alternative binding sites for the chloroplast initiation complex and elucidate their mode of function.

ALTERNATIVE TRANSLATIONAL *cis*-ELEMENTS IN THE 5'-UTR OF CHLOROPLAST mRNAs

The absence of SD-RBS in the large fraction of chloroplast mRNAs suggests that binding of the initiation complex to the mRNA is mediated by an alternative type of cap-independent-binding domain. Since sequence complementarity with a small ribosomal subunit may not be required for binding, the binding of the initiation complex could be facilitated by proteins interacting with this do-

main. The finding of upstream 5'-UTR *cis*-elements that are required for translation may identify these putative initiation complex binding domains. In vivo expression of truncated forms of the 363-nucleotide-long 5'-UTR of *petD* in *C. reinhardtii* has demonstrated the presence of two such domains. One is located from 210 to 161 and the other from 51 to 33 nucleotides upstream from the initiator codon (Sakamoto et al., 1994). Mutation analysis of the 5'-UTR of tobacco *psbA* mRNA fused to a reporter ORF has identified an additional translationally active *cis*-element that does not resemble SD-RBS and interacts with specific proteins (Hirose and Sugiura, 1996). Genetic analyses in *C. reinhardtii* have identified a 97-nucleotide region located 236 nucleotides upstream of the initiator codon of *psbC* mRNA (Zerges et al., 1997) and a 130-nucleotide region located 12 nucleotides upstream of *psaB* mRNA (Stampacchia et al., 1997); both have the capacity to fold into stem-loop structures. The interaction of each of these *cis*-elements with its mRNA-specific nuclear-encoded factor is crucial for mRNA translation. This is independent of which ORF is fused downstream of the 5'-UTR, suggesting that initiation is the checkpoint for regulating translation.

Mutations predicted to decrease structural stability of the 5'-UTR *cis*-elements of *psbC* and *psaB* mRNAs increased the translatability of the mRNA in the absence of a functional, interacting, nuclear-encoded factor (Stampacchia et al., 1997; Zerges et al., 1997). Mutations that increased the structural stability of the 97-nucleotide *cis*-element decreased the level of translation of *psbC* mRNA. Taken together, these results suggest that nuclear-encoded proteins interact with the alternative *cis*-elements in an mRNA-specific manner to control the initiation of translation of the downstream ORF, and that the structure of these *cis*-elements is crucial for their translational regulatory function. Strengthening this hypothesis is the finding that both the association of chloroplast *psbA* mRNA with ribosomes and its association with a 5'-UTR-binding protein complex are aberrant in the presence of the nuclear mutation *F35*, which reduces translation of *psbA* mRNA (Yohn et al., 1996).

The isolation of a nuclear mutation (TBC3) that partially suppresses both the phenotype of the nuclear mutation F34 and the deletion of the 97-nucleotide *cis*-element of the chloroplast *psbC* mRNA has identified an additional nuclear-encoded factor required for regulation of transla-

tion of *psbC* mRNA (Zerges et al., 1997). The genetic interaction of TBC3 and F34 gene products and the 97-nucleotide *cis*-element suggests that several proteins may form a translational regulatory complex that interacts specifically with the 97-nucleotide stem loop in the 5'-UTR of *psbC* mRNA. The regulation of yeast mitochondria and chloroplast gene expression shows many parallels. Expression of mitochondrial mRNAs in yeast is dependent on the interaction of several nuclear-encoded factors, with specific structural elements located in the 5'-UTR of the mitochondrial mRNA. These nuclear-encoded factors combine to form a protein complex that was found to interact with small subunit ribosomal proteins, suggesting that the protein complex may mediate the association of the 30S ribosomal subunit with the 5'-UTR of the mRNA (Fox, 1996).

5'-UTR-BINDING PROTEINS

The expanding number of nuclear mutations, each responsible for the lack of translation of a unique chloroplast mRNA, identifies several different factors, with each regulating the synthesis of one protein in the chloroplast. A simple model would predict that one nuclear-encoded protein, unique to one chloroplast mRNA, is interacting with the 5'-UTR and regulating the translation of downstream ORF (Fig. 1). However, genetic studies of chloroplasts and mitochondria have suggested that more than one protein is required for the regulation of a single organellar mRNA (Zerges et al., 1997). Comparisons of 5'-UTR interacting proteins of *rps12*, *rbcL*, and *atpB* have detected at least seven binding proteins of 15, 36, 38, 47, 56, 62, and 81 kD. The 38-, 47-, and 81-kD proteins were shown to associate with all tested 5'-UTRs, and multiple forms of the 47- and 81-kD proteins have been identified (Hauser et al., 1996). These results, obtained by UV cross-linking, which mostly detects proteins that have direct contact with the RNA, show that several proteins may associate with the 5'-UTR of chloroplast mRNAs. This requires the determination of the significance of each protein to translational regulation. The level of the 36-kD protein was diminished in cells that preferentially translate chloroplast-encoded ribosomal proteins, suggesting that it may be required for translation of a class of mRNAs encoding photosynthetic proteins (Hauser et al., 1996). Binding of a 46-kD protein to the 5'-UTR of *psbC* was detected only in cells carrying the nuclear mutation *F64*, which is responsible for the translational arrest of the chloroplast *psbC* mRNA. This implies that the binding of 46-kD protein may inhibit translation of *psbC* (Zerges and Rochaix, 1994).

RNA-affinity chromatography, capable of isolating both proteins that bind directly to the RNA and proteins that are associated through protein-protein interactions, was used to identify a set of four proteins (38, 47, 55, and 60 kD) specific to the 5'-UTR of *psbA*. The binding activity of the 38- and 47-kD proteins to the RNA was lower in protein lysates isolated from dark-grown cells than from light-grown cells, yet the amount of the 47-kD protein did not vary between light and dark (Danon and Mayfield, 1991). Lower binding activity of these proteins was also detected in cells bearing the nuclear mutation *F35*, which causes

diminished translation of *psbA* (Yohn et al., 1996), as well as in cells aberrant in photosynthetic electron transport (Danon and Mayfield, 1994a). These results and the modulation of RNA-binding activity of these proteins in vitro in response to redox and ADP concentration indicate that the capacity to respond to chloroplast-generated signals is intrinsic to the *psbA* 5'-UTR-binding proteins (Danon and Mayfield, 1994a, 1994b). These results suggest that variation in illumination controls the activity of *psbA* 5'-UTR-binding proteins, which in turn controls the translation of *psbA* mRNA. The capacity of these proteins to alter their RNA-binding activity with no apparent change in their amount in vivo and in vitro implies that these proteins may function semiautonomously, altering chloroplast gene expression by responding locally to chloroplast-generated signals.

MODELS OF FUNCTION OF THE ALTERNATIVE TRANSLATIONAL *cis*-ELEMENTS AND INTERACTING PROTEINS

The proposed function of the alternative translational *cis*-elements is summarized in Figure 2. Two major steps must be facilitated for initiation to occur. The first (Fig. 2A) is the association of the initiation complex with the 5'-UTR, and the second (Fig. 2B) is the recognition of the correct initiator codon. The 5'-UTR *cis*-element can fold into a stem-loop structure that is required for the translational function of the *cis*-element. A protein complex comprised of nuclear-encoded proteins interacts specifically with its unique *cis*-element. The association of the protein complex and the RNA is controlled by modulating its RNA-binding activity, which is sensitive to chloroplast-generated signals, such as redox and ADP concentrations. The binding of the protein complex to the *cis*-element mediates the association of the mRNA and the small ribosomal subunit, and regu-
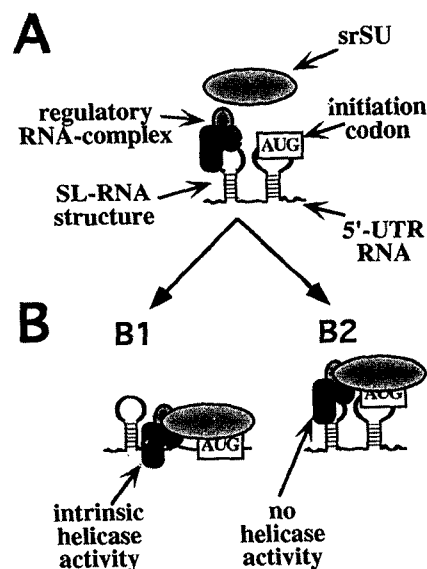

Figure 2. Models of initiation of translation mediated by the alternative translational *cis*-elements in the 5'-UTR.

lates initiation of translation. Because of the relaxed spacing between the alternative cis-elements and the initiator codon, it is not clear how binding of the initiation complex to the putative RBSs may facilitate initiator codon recognition.

Two main alternatives can be predicted. The first is B1 in Figure 2. Recognition of the initiator codon is mediated in a manner similar to eukaryotic initiation of translation by scanning of the 5'-UTR by the initiation complex and recognition of promoting sequences surrounding the correct initiator codon. This alternative predicts that translation initiation should be sensitive to the insertion of short ORFs upstream of the correct initiator codon. The step of scanning in this model requires helicase activity, which has yet to be identified in the chloroplast. The second is B2 in Figure 2. According to the second alternative, the structure of the 5'-UTR adjoins the upstream cis-elements and the initiator codon such that the association of the small ribosomal subunit with the translational regulatory protein complex, bound to the upstream cis-element, leads the small ribosomal subunit to the correct initiator codon and facilitates its recognition. This model is consistent with the observations that mutating the initiator codon of petA affected the efficiency but not the site of initiation and that insertion of a stop codon immediately upstream of the initiator codon did not compromise translation (Chen et al., 1995). According to this model, regulation of initiation could be mediated by either the activity of the translational regulatory protein complex or by changes in the structure of the 5'-UTR. Because of the primary role of the structure of the 5'-UTR, this model predicts that initiation of translation will be highly sensitive to deletions in the 5'-UTR.

PERSPECTIVES

Regulation of chloroplast gene expression was predicted to mimic regulation in prokaryotes because of its presumed prokaryotic origin. However, it is becoming clear that during chloroplast evolution it has undergone major changes in its genome composition and regulation. One of the consequences of the transfer of the large fraction of the chloroplast genome to the nucleus is the requirement for coordination of the nuclear and chloroplast gene expression. This coordination is especially crucial for regulating the amounts of chloroplast protein complexes that are comprised of both nuclear- and chloroplast-encoded subunits. The coordination of nucleus and chloroplast gene expression may require a communication capacity (signal transduction) that would mirror the demand for alteration in gene expression in one compartment to the other, and regulatory factors to control the expression of the other gene compartment. The nuclear-encoded factors, identified by genetic analysis as being required for regulation of chloroplast gene expression, are ideal candidates for factors participating in the nuclear control over the chloroplast genome. The nature of these regulatory factors is particularly intriguing, as they interface between the eukaryotic nuclear genome and the prokaryotic-like chloroplast genome. It is becoming apparent that regulation of chloroplast gene expression has deviated from the mostly transcriptional regulated gene expression in prokaryotes.

However, the mechanism of the particular posttranscriptional steps of chloroplast gene expression such as initiation of translation are far from being understood. The elucidation of the nature and mode of function of the nuclear encoded regulatory factors should help to unravel both the mechanisms of posttransriptional regulation in the chloroplast and the pathways by which the nuclear genome controls chloroplast gene expression.

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