Translation of chloroplast psbD mRNA in Chlamydomonas is controlled by a secondary RNA structure blocking the AUG start codon

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ABSTRACT
Translation initiation represents a key step during regulation of gene expression in chloroplasts. Here, we report on the identification and characterization of three suppressor point mutations which overcome a translational defect caused by the deletion of a U-rich element in the 5′-untranslated region (5′-UTR) of the psbD mRNA in the green alga Chlamydomonas reinhardtii. All three suppressors mediate their secondary RNA structure encompassing the psbD AUG initiation codon within a double-stranded region as judged by the analysis of site-directed chloroplast mutants as well as in vitro RNA mapping experiments using RNase H. In conclusion, the data suggest that these new element serves as a negative regulator which mediates a rapid shut-down of D2 synthesis.

INTRODUCTION
The control of mRNA translation represents a key regulatory step for gene expression in both prokaryotes and eukaryotes. Despite the fundamental differences between their translation machineries, in both types of organisms the initial steps of translation are known to play an essential role (1). These early events of protein synthesis depend on a variety of cis- and trans-acting determinants which usually mediate their functions via the 5′- and 3′-untranslated regions (5′ and 3′-UTR) of protein-coding transcripts (2–6). In chloroplasts, the typical endosymbiotic organelles of photosynthetic plants and algae—a special situation developed during evolution. A basic translational system, which is of prokaryotic phylogenetic origin, is embedded in a eukaryotic cellular context. This results in a novel hybrid apparatus for protein synthesis which is controlled by nucleus-encoded eukaryotic-type factors (7–9). During the last years, substantial work has been invested to identify these factors and to elucidate the molecular mechanisms which underlie regulatory translational processes within this specialized cellular compartment.

Genetic analyses in the green alga Chlamydomonas reinhardtii and vascular plants have recently led to the identification and cloning of some of the trans-acting translation factors involved (10–14). Moreover, the genes for RNA-binding proteins which associate with chloroplast 5′-UTRs in vitro (for a review see (15,16) and in vivo (17,18)) were identified by biochemical means. However, apart from the target regions on chloroplast mRNAs, relatively little is known about the precise molecular working mode of the respective factors.

Initially, the cis-acting RNA targets were mapped by analyzing reporter gene constructs carrying mutated versions of distinct chloroplast 5′-UTRs (19–22) or by site-directed mutagenesis of the endogenous chloroplast gene regions (16,23,24). Alternatively, a chloroplast in vitro translation system from tobacco was used to define cis-acting translational elements within various plastid 5′-UTRs in higher plants (25,26). These analyses revealed that the typical prokaryotic signal for translation initiation, i.e. a Shine–Dalgarno element located 4–12 nt upstream of the AUG start codon, is functional in some but not all chloroplast mRNAs (25,27–29). Furthermore, sequences surrounding the AUG start codon were shown to significantly affect translational efficiency (30–32).

More systematic mutagenesis approaches then identified additional regions within 5′-UTRs as well as downstream regions (33,34) which affect chloroplast protein synthesis. In both C.reinhardtii and tobacco, stem–loop structures within the psbA 5′-UTR have been shown to be critical for determining translational efficiency (27,35). RNA secondary structure elements within 5′-UTRs were also found to affect protein synthesis from the psbC, petD and rps7 mRNAs in C.reinhardtii (20,22,24,36) and the atpB mRNA in tobacco (25).

We have previously demonstrated that the psbD 5′-UTR in C.reinhardtii contains the target site for the nucleus-encoded RNA stability factor Nac2 (3,37) which connects processes of psbD RNA stabilization and translation initiation.
Analysis of nucleic acids and proteins

Total DNA from *C. reinhardtii* was isolated using the DNeasy Plant Kit (Qiagen, Hilden). Algal RNA was prepared with hot phenol (42). RNA secondary structures were calculated by using the RNAdraw software (43). Northern analysis, primer extension assays and western analysis were performed exactly as described (44). Radioactive labelling of RNAs and UV cross-linking with proteins were also performed as described (39).

RNase H mapping of RNA secondary structure

Templates comprising 134 bp (wt) or 127 bp (suΔU+10, suΔU+9 and suΔU−3) for *in vitro* synthesis of the various *psbD* RNA probes were PCR-amplified from appropriate DNAs with the oligonucleotide su3131 : 5′-tgctgctgttca-3′, complementary to the coding region of *psbD* from position +39 to +15 relative to the ATG and oligonucleotide 2126: 5′-taatactgactaatagggcaagttataaatataa-3′ spanning the *psbD* 5′ region from position −74, as well as the T7 promoter sequence (39). *In vitro* transcription reactions and radioactive labelling of the RNAs were performed as described (23). RNA probes (15 fmol) were diluted in cacodylate buffer (50 mM Na-cacodylate, 20 mM CaCl₂ and 10 mM KCl) and incubated with 10 pmol of the oligonucleotide RH-1: 5′-aatgtcattgctgtatc-3′ which is complementary to position −11 to +9 relatively to the AUG start codon. The samples were heated to 60°C for 5 min and cooled down with a rate of 1°C per min to 25°C. After addition of one volume 2x cacodylate buffer with MgCl₂ (50 mM Na-cacodylate, 10 mM KCl, 20 mM CaCl₂ and 20 mM MgCl₂), samples were incubated with 0.5 U RNase H (Ambion, Cambridgeshire) for 2 min or 5 min and loaded on 12% Polyacrylamide TBE gels (45). After electrophoresis, gels were sealed in plastic bags and exposed to Fuji X-ray films at −20°C.

Pulse labelling of membrane proteins

For pulse labelling of proteins, cells were grown as described (23). After measurement of chlorophyll content, cells were harvested and resuspended to 80 μg chlorophyll/ml. A total of 500 μl of the cells were incubated with cycloheximide (10 μg/ml) for 10 min. Subsequently, cells were fed with 50 μCi ³⁵S-sulphate (Amersham, Freiburg) for 20 min in bright light (100 μE m⁻² s⁻¹). After centrifugation, sedimented cells were frozen in liquid nitrogen. Broken-cell preparation and gel electrophoresis were carried out as described (44). Signals were quantitated by using the Scion Image software and standardized to the internal Cytf control.

RESULTS

Three independent point mutations suppress the AU mutation

Previously, it was shown that a striking U-rich element within the 5′-UTR of the chloroplast *psbD* mRNA is involved in its translation (Figure 1). This element is recognized by the RNA-binding protein RBP40, a process that is dependent on the presence of the nucleus-encoded RNA stability factor Nac2 (39). Replacement of the U-track by a BamHI restriction

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site in the mutant ΔU resulted in complete loss of both D2 synthesis as well as RBP40-binding (23). Furthermore, the photosynthetic revertant suΔU containing a 5 bp duplication immediately upstream of the BamHI site restored both D2 synthesis and RBP40-binding to almost wild-type levels. These findings together with a more comprehensive mutational analysis underlined the significance of the U-element/RBP40 interaction for translation of the psbD message (39).

We have now isolated three novel, independent phenotypic revertants of the ΔU mutation, namely suΔU+10, suΔU+9 and suΔU−3. Genetic crosses revealed that each suppressor mutation resides within the chloroplast genome (see Materials and Methods). However, in contrast to the previously characterized suΔU strain, sequencing of the psbD region revealed that in the suppressors the immediate vicinity of the mutated U-tract is not altered. Instead, each suppressor strain harbours a single point mutation which is localized downstream of the U-stretch at positions +10 (suΔU+10), +9 (suΔU+9) and −3 (suΔU−3) with regard to the AUG start codon (Figure 1). Biotic back-transformations of chloroplasts with constructs containing the psbD leader region of either suΔU+10, suΔU+9 or suΔU−3 showed that all three constructs were able to complement the ΔU mutant verifying that indeed each mutation is sufficient to cause the suppressor phenotype on its own (Figure 1).

All three suppressors accumulated almost wild-type levels of psbD mRNA (Figure 2A; Table 1) indicating that the respective point mutations have no effect on RNA stabilization. The psbD mRNA has previously been shown to exist in two forms. The difference lies in the length of their 5′-UTR and is most likely due to a 5′ processing event (23,38). In neither of the three suppressor strains, this 5′ maturation was compromised (Figure 2B). However, D2 protein accumulation was found to be restored to varying extent. Whilst the suppressor suΔU+9 accumulated almost 50% of D2 protein as compared to the wild-type, suΔU+10 and suΔU−3 reached only levels of 20 and 15%, respectively (Figure 2C; Table 1). This was in agreement with reduced photoautotrophic doubling times of 37 h for suΔU+9, 38 h for suΔU+10 and 45 h for suΔU−3 as compared to 35 h for the wild-type (Table 1).

Suppressor mutations do not restore RBP40-binding

As mentioned above, in the previously described cis-acting suppressor suΔU, a 5 bp duplication immediately upstream of the U-rich translation element resulted in restored binding of RBP40 to the psbD 5′-UTR thereby explaining the observed suppressor phenotype (39). Therefore, we tested whether the suppressor mutations suΔU+10, +9 and −3 also affect RBP40-binding in vitro. Radiolabelled RNA probes from each 5′-UTR were incubated with stromal protein extract from the wild-type, and the RBP40 signal was monitored after UV cross-linking of RNAs and proteins. As expected, a strong binding signal was observed with a wild-type psbD.
probe but not with a mutant ΔU probe. The same held for the three suppressor RNAs, none of which led to a RBP40-binding signal (Figure 3A). To further confirm the different RNA-binding properties of RBP40, competition binding experiments were carried out by using radiolabelled wild-type RNA and increasing excess of now unlabelled probes. As shown in Figure 3B, only the homologous wild-type 5'-UTR efficiently competed with the wild-type probe while excess of the suppressor RNAs had only a minor effect on RBP40-binding. Taken together, these data strongly suggest that in contrast to the known suppressor suΔU+, the suppressor mutations suΔU+10, suΔU+9 and suΔU−3 do not restore the binding of RBP40 to the psbD U-track but instead appear to act via a RBP40-independent mechanism.

Table 1. Molecular characteristics of analyzed strains

| Strains       | WT | ΔU | suΔU+10 | suΔU+9 | suΔU−3 | mutsu | suwt+10 | suwt+9 | suwt−3 | stabIR |
|---------------|----|----|---------|--------|--------|-------|---------|--------|--------|--------|
| Photoautotrophic growth* | 35 ± 2 | -- | 38 ± 1.5 | 37 ± 1 | 45 ± 2 | 40 ± 2 | 31 ± 1.5 | 30 ± 2 | 32 ± 1 | 39 ± 2 |
| RNA           | 100 | 80 ± 5 | 108 ± 6 | 112 ± 4 | 115 ± 3 | 114 ± 5 | 104 ± 3 | 99 ± 4 | 105 ± 4 | 112 ± 5 |
| Protein       | 100 | 0  | 20 ± 6  | 46 ± 5 | 15 ± 7 | 42 ± 6 | 117 ± 8 | 116 ± 9 | 118 ± 9 | 61 ± 6  |

Values display the mean of three independent experiments except for b.
*Doubling times in HS medium at 100 µE m−2 s−1.
*bValues display the mean of 10 independent experiments.

The psbD AUG start codon is part of a secondary RNA structure

Bioinformatic inspection of the psbD 5' RNA secondary structure revealed that all three suppressor mutations are located in a putative stem-structure of a RNA hairpin which partly encompasses also the AUG start codon (Figure 4). To test whether this RNA secondary structure or the RNA sequence context close to the AUG codon is important for mediating suppression, compensatory mutations were introduced into the psbD leader by site-directed mutagenesis (23). The mutations revsu+10, revsu+9 and revsu−3 each restored the putative stem-region in the respective suppressor backgrounds by an appropriate exchange of the mismatching nucleotides on the opposite strand of the stem (Figure 4). When mutant ΔU
chloroplasts were transformed with these constructs, no complementations were observed for neither of them (Figure 1). This suggested that indeed the restoration of the RNA stem–loop structure inhibited the suppression effect independent of the sequence context.

To further substantiate that the suppressor mutations affect a critical structural rather than a sequence element, the secondary structure of the wild-type and the suppressor psbD 5′-UTRs were determined by comparative RNase H mapping (45). Radiolabelled RNA probes were hybridized with oligonucleotide RH-1 which is complementary to the respective AUG regions. RNA/DNA hybrid formation is assumed to be possible only in unpaired RNA stretches which then become a substrate for RNase H. Consequently, unstructured RNA regions will cause an efficient cleavage of the RNA into defined smaller products. As shown in Figure 5, the wild-type psbD 5′-UTR was almost resistant against RNase H digestion over a time period of 5 min suggesting that a double-stranded RNA structure prevents stable base pairing with the oligonucleotide. In contrast, all three suppressor RNAs were efficiently cleaved into expected major fragments of 59 and 30 nt being indicative of a more or less unstructured RNA region in the vicinity of the start codon.

These findings strongly suggested that the psbD 5′-UTR forms a stable RNA stem–loop structure which partially includes the AUG codon in its stem-region. Furthermore, this structure appears to act as a negatively regulating element for D2 synthesis at least in a ΔU genetic background (Figure 1) probably by preventing access of the small ribosomal subunit to the initiation codon. If this scenario were true, then, a suppression of the ΔU mutation should be achievable by altering other nucleotides of the stem-region. Thus, an A to C mutation at position 1 relative to the start codon was introduced into the ΔU 5′-UTR to test whether this stem destabilizing mutation, similar to the genetically selected suppressors suΔU+10, +9 and −3, would also lead to a suppression effect. A corresponding construct indeed was capable of complementing the ΔU mutant (Figure 1). In the resulting transformant mutsu, D2 accumulation was found to be restored to 42% of the wild-type level (Figure 6B; Table 1) further supporting the idea of a repressive RNA structure located at the psbD translation initiation codon.

Analysis of suppressor mutations in the presence of the psbD U-rich element

The above mentioned data provided good evidence that the psbD stem–loop structure is a negative determinant for psbD gene expression in the absence of the U-rich translation element and, as a consequence, the absence of RBP40 activity. Thus, the next question concerned the phenotype of mutants which contain both the U-element and any of the suΔU+10, +9 and −3 mutations. Adequate site-directed mutant versions, named suwt+10, suwt+9 and suwt−3, were constructed and transformed into ΔU chloroplasts. As expected, all three
constructs complemented the U-stretch mutation (Figure 1). Interestingly, resulting transformants were shown to accumulate slightly increased levels of D2 protein (Figure 6B; Table 1). This was in agreement with a moderate enhancement of D2 synthesis in $suwt+10$, $suwt+9$ and $suwt/C0$ as compared to the wild-type in protein pulse labelling experiments using radioactive sulphate (Figure 6C). This increase in D2 accumulation in $suwt+10$, $suwt+9$ and $suwt/C0$ suggested that also other subunits of the PS II complex accumulated to enhanced levels because unassembled PS II proteins are rapidly degraded by a chloroplast proteolytic ‘clearing system’ (23). Consistently, all three strains exhibited a slightly accelerated photoautotrophic growth rate as compared to the wild-type (Table 1). These findings supported the idea that the RNA secondary structure serves as a negative element for $psbD$ gene expression and, similar to the situation in cyanobacteria, D2 protein accumulation represents a key regulatory step for PS II assembly (46).

Figure 5. In vitro mapping of RNA secondary structure. 100 fmol of indicated radiolabelled RNA probes of 116 nt (wt) and 109 nt ($suΔU+10$, $suΔU+9$ and $suΔU−3$) were incubated with oligonucleotide RH-1 complementary to the AUG regions and 0.5 U RNase H for 2 or 5 min. Major RNase H cleavage products of 59 and 30 nt (arrows) were visualized on autoradiograms after separation by denaturing PAGE. Computer-predicted secondary RNA structures with a boxed AUG initiation codon are given at the top. Position of oligonucleotide RH-1 is indicated by bold characters.

Figure 6. Molecular characterization of site-directed $psbD$ mutants. For northern (A) and western (B) analyses see legend of Figure 2. (C) Proteins from indicated strains were pulse labelled with $^{35}$S-sulphate for 20 min and separated by SDS–PAGE.
In conclusion, these results suggested that the RNA stem–loop structure containing the psbD AUG start codon negatively regulates synthesis of the D2 protein and as a consequence PS II accumulation. As an additional control for this hypothesis, the site-directed mutant stabIR was constructed by introducing a U residue at position −5 of the wild-type psbD leader (Figure 4). This alteration led to a stem-region which was extended by 1 bp and thus, a RNA structure of enhanced stability should be formed. Although this construct was still able to complement the ΔU mutant (Figure 1), the subsequent molecular characterization of the resulting transformant stabIR revealed a significant reduction of D2 accumulation to nearly 60% compared to the wild-type level (Figure 6B; Table 1). This finding provided an additional piece of evidence for the RNA conformation at the translation initiation codon playing a critical role for psbD gene expression.

DISCUSSION

In this report, we describe the identification and characterization of a structural RNA element which serves as a negative regulatory determinant for the synthesis of the D2 protein in C. reinhardtii chloroplasts. The analysis of genetically selected suppressors, various site-directed chloroplast mutants as well as in vitro mapping studies using RNase H showed that the AUG start codon of the psbD mRNA is located in a double-stranded RNA region which has to be resolved before translation initiation. It should be noted, however, that in suppressor strains suΔU+9 and suΔU+10 the amino acid sequence of the D2 protein was changed at position 3 from isoleucine to methionine and at position 4 from alanine to valine, respectively (Figure 4). These alterations might have influenced overall D2 protein stability and thus D2 accumulation. For instance, it was reported previously that the replacement of the threonine at position 2 of D2 with acidic—but not neutral—amino acid residues abolished D2 synthesis/accumulation at a post-initiation level in C. reinhardtii (47,48). However, similar effects are not likely to play a significant role for the D2 accumulation rates in suppressors suΔU+9 and suΔU+10 since they represent gain of function mutants. Additionally, when placed in an otherwise wild-type background, both point mutations apparently had no negative effect on PSII activity as documented by the fitness of the strains suwt+9 and suwt+10 (Table 1). Hence, it seems rather unlikely that the amino acid changes in suΔU+9 and suΔU+10 add a substantial effect to the suppression mechanism.

The influences of RNA secondary structure elements on chloroplast protein synthesis rates have been noticed before. In principle these structures can have two different functions (49). First, they might serve as recognition sites for trans-acting, translation-activating factors as has been suggested for several chloroplast 5′-UTRs (21,22,24,27,35,36). Secondly and like the element described here, secondary RNA structures might serve as negative regulatory elements which block access of the small ribosomal subunit to the translation initiation region resembling the situation which is frequently found in prokaryotes (1). In chloroplasts for instance, it has been proposed that the processing of petD precursor RNAs in maize results in the release of the initiation codon from base pairing within a secondary structure and only thereby allows efficient synthesis of the petD gene product (50). Also in barley chloroplasts, methyl jasmonate-dependent processing of the rbcL 5′-UTR was proposed to influence translation initiation rates (51). By using a chloroplast in vitro translation system from tobacco, it was recently shown that the RNA conformation around the AUG codon of the atpB mRNA negatively affects translation efficiency (25). Moreover, bioinformatic inspection of the close vicinity of translational initiation regions (each ranging from position −15 to +18) from all chloroplast genes in C. reinhardtii revealed that, potentially, nine of the respective initiation codons might be parts of double-stranded RNA structures with a thermodynamic stability in the range of the psbD mRNA one (ΔG = −8.53 kcal at 25°C). These regions include that of the psbN (−9.31 kcal), atpB (−7.53 kcal), psbC (−7.32 kcal), ORF2971 (−6.77 kcal), rpoA (−6.71 kcal), rpl2 (−6.4 kcal), psaA (−6.06 kcal) and petG (−5.3 kcal) genes. However, only additional experimental data will provide an answer to the question whether such ‘prokaryotic-like’, negative principles of regulation represent a more common theme of chloroplast gene expression.

Whilst the psbD stem–loop structure plays a crucial role for gene expression in the absence of the U-rich region and thus RBP40 activity, its destabilization in an otherwise wild-type background did not result in a dramatic but only moderate increase in D2 synthesis. This suggests that under optimal conditions the inhibition of D2 synthesis via the stem–loop is not strictly rate-limiting. This is reminiscent of the situation found in chloroplast mutants of the petA- or atpB-1 nt (31,52). In both cases nucleotide changes in the respective −1 triplets did not show any measurable effect on protein accumulation. Only if the AUG start codon was changed into the sub-optimal AUU codon, differences in protein accumulation became detectable (31,52). Under optimal conditions, i.e. the presence of the U-element, most probably the availability of positively
regulating trans-acting factors such as the Nac2 protein and the Nac2-dependent RNA-binding protein RBP40 mainly determine D2 synthesis rates as has recently been proposed (39,44). In the absence or reduction of Nac2/RBP40 activity however, this element might be involved in a rapid shut-down of psbD translation thereby allowing a fast adjustment of protein synthesis rates to changing environmental conditions.

Figure 7 summarizes the main psbD gene expression modes which were characterized during the course of this work. In conclusion, the fact that the isolated suppressor mutations overcome a defect in RBP40-binding without restoring the interaction between this factor and the U-rich element strongly suggests that at least one function of RBP40 is to resolve the secondary structure in the wild-type situation. We have recently identified the RBP40 gene but its putative structure does not reveal a typical helicase-like activity (I. Elles and J. Nickelsen, unpublished data). Hence, it appears more likely that the binding of RBP40 alters the RNA conformation thereby enabling access of the small ribosomal subunit to the initiation codon. Future work is focussing on the interactions between the involved trans-acting factors and the characterized RNA elements and, thus, their precise molecular working modes.

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