similar to NifS than is Slr0387, but shows strong similarity to Slr0077. It will be interesting to see whether this protein is involved in the maturation of FeS proteins.

We found that under some conditions the *Synechocystis* NifU-like protein can release sulphur from Slr0387 after this enzyme has reacted with free cysteine, with concomitant oxidation of the cysteine side chains at NifU. The same reaction might have occurred in lysed chloroplasts in the work of Takahashi et al. [19]. This would explain the need for light or NADPH for sulphide production, because the oxidized NifU-like protein would have to be reduced, and the most likely reductase is ferredoxin–thioredoxin oxidoreductase. This enzyme itself becomes reduced in the light via ferredoxin, which, in turn, is reduced by photosystem I.

Concluding remarks

Significant progress has been made in recent years in terms of understanding FeS cluster assembly in various organisms. The mechanism of this process seems to be similar, but by no means identical, in various organisms. Cyanobacteria are apparently different from most other organisms in which FeS cluster assembly is currently under investigation, particularly concerning the role of the NifU/IscU-like protein and the presence of a cyst(e)ine desulphurase other than the NifS-like proteins.

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Assembly-controlled regulation of chloroplast gene translation

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Abstract

Studies of the biogenesis of the photosynthetic protein complexes in the unicellular green alga *Chlamydomonas reinhardtii* have pointed to the importance of the concerted expression of nuclear and chloroplast genomes. The accumulation of chloroplast- and nuclear-encoded subunits is concerted, most often as a result of the rapid proteolytic disposal of unassembled subunits, but the rate of synthesis of some chloroplast-encoded subunits from photosynthetic protein complexes, designed as CES proteins (Controlled by Epistasy

Key words: biogenesis, cytochrome b_6f, *C. reinhardtii*, thylakoid.
Abbreviations used: CES, control by epistasy of synthesis; SUIV, subunit IV; UTR, untranslated region; WT, wild type.
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of Synthesis), is regulated by the availability of their assembly partners from the same complex. Cytochrome *f*, a major subunit of the cytochrome b_6f complex is a model protein for the study of the CES process. In the absence of subunit IV, another subunit of the cytochrome b_6f complex, its synthesis is decreased by 90%. This results from a negative autoregulation of cytochrome *f* translation initiation, mediated by a regulatory motif carried by the C-terminal domain of the unassembled protein [Choquet, Stern, Wostrikoff, Kuras, Girard-Bascou and Wollman (1998) Proc. Natl. Acad. Sci. U.S.A. **95**, 4380–4385]. Using site-directed mutagenesis, we have characterized this regulatory motif. We discuss the possible implications regarding the mechanism of the CES process for cytochrome *f* expression. We have studied the possible generalization of this mechanism to other CES proteins.

Introduction

In higher plants and green algae, the four major multimeric protein complexes of the photosynthetic thylakoid membrane comprise subunits encoded either by the nucleus or by the chloroplast. Co-ordinated expression of the two genetic compartments is therefore needed to produce the various subunits of photosynthetic proteins in the stoichiometry required for their assembly into a functional complex. This is often achieved by an efficient post-translational degradation of unassembled subunits (reviewed in [1,2]).

However, the translation rate of some chloroplast-encoded proteins, rather than their stability, is controlled by their assembly into a complex, a phenomenon described as Control by Epistasy of Synthesis (CES). Thus there is a hierarchical organization in the expression of the subunits of a complex. The CES subunits are those whose rate of synthesis is dependent on the presence of some assembly partners, called 'dominant' proteins. Studies of mutants of the green alga *Chlamydomonas reinhardtii* have shown that each complex of the thylakoid membrane contains at least one CES subunit (reviewed in [1,2]).

The mechanism of this CES control has only been studied in detail for the cytochrome b_6f complex, whose cytochrome *f* subunit is a CES protein. In the absence of its assembly partners cytochrome b_6 or subunit IV (SUIV), its synthesis rate drops to approx. 10% of that observed in the wild type (WT) [3]. Cytochrome *f* synthesized under those conditions is inserted in the membrane and is as stable as in the WT, even though it is not assembled. We have previously shown that the CES control of cytochrome *f* expression corresponds to regulation of the initiation of cytochrome *f* translation, which requires the *petA* 5′ untranslated region (UTR) [4].

Materials and methods

Media, strains, crosses, transformation experiments and phenotypic characterizations

WT and mutant strains were grown at 25 °C on TAP medium under dim light. We used the nuclear mutants *tab*1-F15,*mt*− [5] and *mdb*1-thm24,*mt*− [6], the deletion strain Δ*petA*,*mt*+ [7], the chloroplast mutant C3, lacking PsaB accumulation [8,9], and the chloroplast-transformed strain AFFF [4]. Crosses were performed as described in [10]. Cells were transformed by tungsten-particle bombardment as described previously [3] and transformants were selected on TAP-spectinomycin-containing plates (60 μg/ml). At least three independent transformants were analysed for each construct. The characterization of photosystem I and cytochrome b_6f mutant phenotypes was performed with their fluorescence induction kinetics as described in [11]. Pulse-labelling experiments, protein and RNA isolation, separation and analysis were performed as described in [3].

Manipulations of nucleic acids

Mutagenesis of the C-terminal region of cytochrome *f* was performed by PCR amplification with appropriate oligonucleotide primers and the *Acc*I–*Eco*RI 600 bp fragment, cloned in vector pBSKS−, as a template. After mutagenesis we replaced the WT AE600 fragment of plasmids pWFK and pWFΔpetD [12] by the mutated one. A DNA fragment containing the *psaA* 5′ UTR, flanked by the restriction sites *Cla*I and *Nco*I on the promoter and ATG sides respectively, was amplified by PCR with appropriate oligonucleotide primers, then cloned into the plasmid pWF [3], in frame with the *petA* coding region, in accordance with the strategy described in [4]. The *aadA* cassette conferring spectinomycin resistance [13] was then inserted into the single *Hin*cII restriction site, located upstream of the *petA* gene, to allow the selection of spectinomycin-resistant transformants.

Results and discussion

Characterization of a regulatory motif, carried by unassembled cytochrome *f*, that mediates the regulation

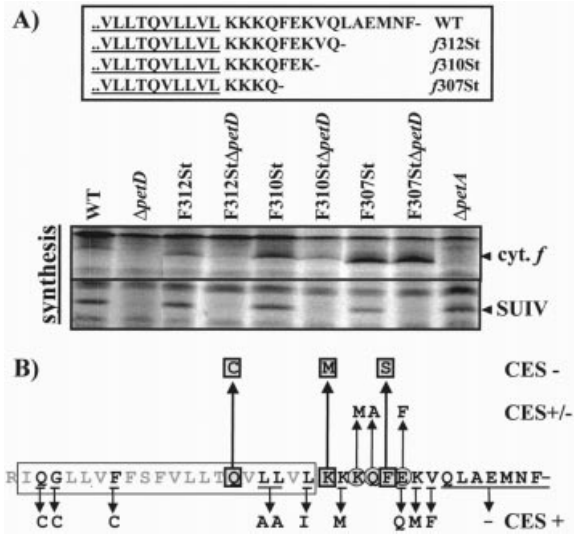
The rate of synthesis of a soluble form of cytochrome *f* accumulated in the thylakoid lumen, generated by site-directed mutagenesis, that lacks the transmembrane helix anchor and the C-terminal stromal extension (F298ST [14]), is no longer repressed in the absence of SUIV. This led us to propose that the regulation of cytochrome *f* translation was mediated by a protein motif, shielded during assembly, carried by the C-terminal domain of the unassembled protein [4,14,15].

To confirm that hypothesis and to characterize this regulatory motif, in an attempt to understand better the interaction of *petA* 5′ UTR with unassembled cytochrome *f*, we undertook an extensive mutagenesis analysis of the C-terminal domain.

Figure 1

Characterization of the regulatory motif carried by the C-terminal domain of cytochrome *f*

(**A**) Phenotypic characterization of three transformed strains expressing cytochromes *f* truncated at the C-terminus. Upper panel: amino acid sequences of cytochrome *f* in WT and transformed strains. Lower panel: synthesis rates of cytochromes *f* and SUIV were determined by pulse-labelling with [^{14}C]acetate for 5 min, in strains carrying the mutations either alone or associated with a deletion of the *petD* gene encoding SUIV. (**B**) Analysis by site-directed mutagenesis of the residues contributing to the formation of the regulatory motif. The sequence of the WT cytochrome *f* is indicated, with the transmembrane helix boxed. Substitutions indicated below the sequence (underlined residues) do not affect the regulation of cytochrome *f* synthesis in the absence of SUIV. Substitutions indicated just above the sequence decrease, but do not abolish, the CES process (circled residues). Substitutions shown in the upper line completely impair the CES process (residues in squares). Residues shown in grey were not tested.



First we compared the expression of mutated cytochromes *f* generated by site-directed mutagenesis: f312St, f310St and f307St, respectively truncated for the last 6, 8 and 11 cytochrome *f* residues, in the presence or absence of the dominant SUIV (Figure 1A). The three truncated cytochromes *f* were able to assemble into a fully functional cytochrome b_6f complex. The synthesis of cytochrome *f* truncated by six residues (f312St) was similar to that of WT cytochrome *f* and was repressed in the absence of SUIV. In contrast, the cytochrome *f* truncated by 11 residues (f307St) was overexpressed 3-fold with regard to WT cytochrome *f*, irrespective of the presence or absence of SUIV, as is soluble cytochrome *f* [14]. The eight-residue truncation (f310St) yielded a partial phenotype (a limited overexpression in the presence of SUIV when compared with the WT cytochrome *f*, and a partial repression of cytochrome *f* synthesis when SUIV was lacking). This proved that a negative regulatory motif was indeed present in the C-terminal domain of the protein. Positions 307–311 of cytochrome *f* contribute essential residues to this motif, whereas the last six residues (positions 312–317) of the protein are not required.

Next we studied the contribution of individual amino acids to the formation of the regulatory motif by substituting each residue from the stromal extension (residues 303–311) as well as some from the transmembrane helix (residues 283–302). Results from this study are summarized in Figure 1(B). Most substitutions (indicated below the amino acid sequence of the C-terminal region of WT cytochrome *f*) do not affect the CES process, still allowing the proper folding of the regulatory motif. In particular, most charged residues (Lys-304, Glu-308, Lys-309) from the stromal extension can be replaced by uncharged residues with little, if any, effect on CES control of cytochrome *f* translation. Only three residues (Gln-298, Lys-303 and Phe-307, indicated on the top line of Figure 1B) were crucial for the integrity of the regulatory motif: these substitutions completely abolished the CES control. One of them, Gln-298, is part of the transmembrane helix. Substitutions at three other positions (Lys-305, Gln-306 and Glu-308), clustered around the residue Phe-307, resulted in decreased CES regulation, as in the f310St strain.

Taken together, these results do not support a direct interaction between the target of the CES

process, the *petA* 5′ UTR, and the regulatory signal, carried by the C-terminal region of unassembled cytochrome *f*. Positively charged amino acids take little part in the formation of the regulatory motif, whereas a residue buried in the thylakoid membrane is required.

Identification of TCA1, the probable effector of the CES process

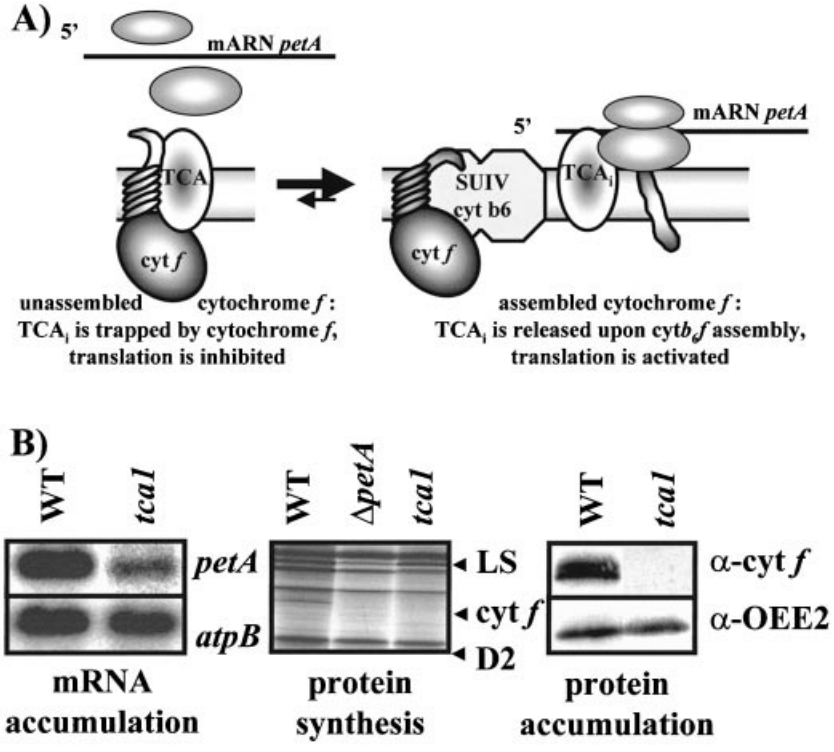
Instead of direct regulation, the interaction is likely to be indirect and to rely on a ternary effector capable of competitive binding to the regulatory motif of unassembled cytochrome *f* and to the *petA* 5′ UTR. This effector would be trapped by the regulatory motif of unassembled cytochrome *f* and be unavailable for the initiation of *petA* mRNA translation. On assembly, the regulatory motif would be shielded by the other subunits, leading to the release of the effector, which would then be available again for translation (Figure 2A). This effector would then behave as a translational activator. In an extensive search for such a factor, we screened more than 80 mutant strains lacking cytochrome b_6f activity. We found seven mutants that were specifically deficient in cytochrome *f* translation. After genetic analysis they were found to carry different mutated alleles of a single nuclear gene that we called *TCA1* (for deficiency in translation of cytochrome b_6f *petA* mRNA). In an attempt to identify partner proteins that would interact with the TCA1 factor, we isolated 21 suppressed strains from three different *tca1* alleles that recovered cytochrome *f* expression and phototrophic growth. All except one were nuclear suppressors; genetic analysis showed that the 14 strains that were further characterized resulted from intragenic suppression events. Thus it seems likely that TCA1 is the only nuclear factor required for the translation of cytochrome *f*, making it a likely candidate for the epistatic effector.

As detailed below, CES control relies extensively on a regulation of translation initiation. Because it seems unlikely that all those CES proteins that are regulated at the level of trans-

Figure 2

TCA1, the putative effector of the CES process

(**A**) Model of the CES process for cytochrome *f* (see the text). (**B**) Phenotypic characterization of a TCA1 mutant. The accumulation and translation rate of *petA* mRNA and product accumulation were compared in the WT and in a representative strain carrying a mutant *tca1* allele, as well as the accumulation of OEE2, as a loading control. Abbreviations: α-cyt *f*, anti-cytochrome *f*; α-OEE2, anti-(oxygen-evolving enhancer protein 2).

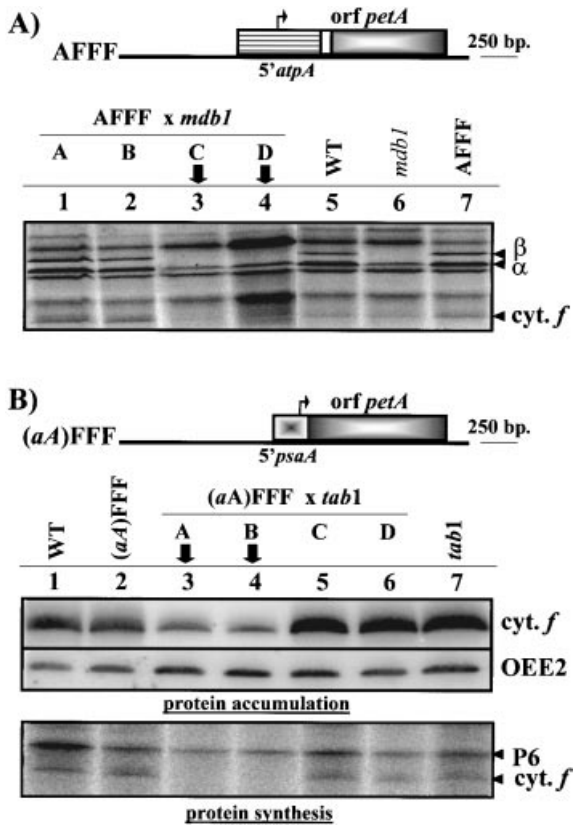


lation initiation have evolved a specific RNA-binding motif able to bind their own messenger 5′ UTR, the nuclear-encoded translation factors, a knowledge of whose regulatory function is still pending, could be important in the assembly of complexes through the translational autoregulation of CES polypeptides.

Figure 3

atpA and *psaA* 5′ UTRs confer CES behaviour on cytochrome *f* translated under their control

(**A**) Top panel: schematic representation of the reporter gene *AFFF* (the bent arrow indicates the position of the transcription start; the white box represents the first 25 residues of the α subunit, translated in-frame with the cytochrome *f* presequence). Lower panel: translation rates of cytochrome *f* and CF1 subunits α and *β*, determined in a pulse-labelling experiment, are compared in WT, in parental strains AFFF or *mdb1*-thm24 and in a representative tetrad progeny from the cross *mdb1*-thm24,*mt*− × AFFF,*mt*+. Progeny that inherited the mutant *mdb1* allele are indicated by a filled arrow. (**B**) Upper panel: schematic representation of the reporter gene *(aA)FFF* (the bent arrow indicates the position of the transcription start). Lower panel: cytochrome *f* expression is compared in the WT, in parental strains (*aA*)FFF and *tab1*-F15 and in representative tetrad progeny from the cross *tab1*-F15,*mt*− × (*aA*)FFF,*mt*+. The progeny that inherited the nuclear mutation *tab1*-F15, as determined by fluorescence induction analysis and immunoblotting with an anti-PsaA antibody (results not shown), are indicated by a dark arrow. Cytochrome *f* accumulation was detected with a specific antibody; the accumulation of oxygen-evolving enhancer protein 2 (OEE2) is presented as a loading control. The rates of synthesis of cytochrome *f* in the same strains were analysed by pulse-labelling experiments.



5′ UTRs of *atpA* and *psaA* are similarly involved in the CES behaviour of the α subunit of CF1 and the RC1 subunit PsaA

We wondered whether some aspects of the mechanism of the CES process, determined for cytochrome *f*, could be generalized to other CES proteins.

The α subunit of the ATP synthase CF1 complex is a CES subunit whose rate of synthesis drops in the absence of the *β* subunit from the same complex [6]. We introduced, by transformation, the chimaeric gene *AFFF* described previously [4], made from the coding sequence of the *petA* gene translated under the control of the 5′ UTR of the *atpA* gene encoding subunit α, in the chloroplast genome of *C. reinhardtii*, in place of the resident *petA* gene. We compared the expression of the reporter gene in various genetic contexts, permitting or preventing the expression of the dominant *β* subunit, by crossing the transformed AFFF strain with the nuclear mutant *mdb*1-thm24, lacking stable accumulation of the *atpB* mRNA [5]. In the resulting tetrads all progeny inherited the chloroplast chimaeric gene, which was transmitted uniparentally. Progeny that inherited the nuclear mutation lacked the dominant *β* subunit and displayed a strongly decreased rate of translation of the chimaeric cytochrome *f* (Figure 3A, lanes 3 and 4) in comparison with the WT (lane 5), the parental strains *mdb*1 and AFFF (lanes 6 and 7) and progeny with a WT nuclear genome (lanes 1 and 2). Thus the CES control of *atpA* expression corresponds to a regulation of translational initiation, mediated by the *atpA* 5′ UTR.

We also studied the expression of PsaA, one of the major subunits of RCI. PsaA is a CES protein because the translation rate of *psaA* is strongly decreased in mutant strains (either chloroplast or nuclear) lacking the expression of PsaB, the other major RCI subunit, which thus behave as a dominant subunit [5,8]. Similarly, we selected transformed strains that expressed a chimaeric gene (*aA*)*FFF* composed of the *psaA* 5′ UTR fused in-frame to the *petA* coding sequence (Figure 3B). Because the transformants (*aA*)FFF recovered photosynthetic growth, the chimaeric gene was translated efficiently. We compared its expression in various genetic contexts, permitting or preventing the expression of PsaB, by crossing

the (*aA*)FFF strain with the nuclear mutant *tab*1-F15, *mt*−, lacking translation of the *psaB* mRNA [5]. The two members of the progeny lacking the dominant PsaB subunit, because they inherited the nuclear mutation, showed little accumulation of the chimaeric cytochrome *f*, which was poorly translated (Figure 3B, lanes 3 and 4), compared with the members with a WT nuclear genome (lanes 5 and 6) and to the parental strain (lanes 2 and 7). The expression of the chimaeric gene was similarly decreased in a strain lacking PsaB because of a chloroplast mutation in the *psaB* structural gene (results not shown). The *psaA* 5′ UTR is therefore sufficient to confer the CES behaviour on a reporter gene translated under its control, demonstrating that the CES process also relies on a regulation of translation initiation in the case of *psaA*.

The large subunit of ribulose bisphosphate carboxylase/oxygenase is a CES subunit, both in *C. reinhardtii* and in higher plants [16,17]. The mechanism of *rbcL* translation attenuation in the absence of the small subunit RbcS has not been investigated in *C. reinhardtii*; however, available results for tobacco point to a control of translation initiation. Anti-sense plants with reduced expression of the small subunit showed a decreased proportion of *rbcL* mRNA species associated with polysomes [17]. Thus we have now overwhelming evidence that chloroplast CES subunits result from an assembly-dependent control of translation initiation. It remains to be determined whether such a regulation of translation initiation also operates in the CES process at work during the biogenesis of photosystem II (where D2 is dominant over D1, which is itself dominant over the apoprotein of CP47).

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