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Intertwined translational regulations set uneven stoichiometry of chloroplast ATP synthase subunits

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The (C)F1 sector from H^+-ATP synthases comprises five subunits: α, β, γ, δ and ε, assembled in a 3:3:1:1:1 stoichiometry. Here, we describe the molecular mechanism ensuring this unique stoichiometry, required for the functional assembly of the chloroplast enzyme. It relies on a translational feedback loop operating in two steps along the assembly pathway of CF1. In Chlamydomonas, production of the nucleus-encoded subunit γ is required for sustained translation of the chloroplast-encoded subunit β, which in turn stimulates the expression of the chloroplast-encoded subunit α. Translational downregulation of subunits β or α, when not assembled, is born by the 5′UTRs of their own mRNAs, pointing to a regulation of translation initiation. We show that subunit γ, by assembling with $\alpha_3\beta_3$ hexamers, releases a negative feedback exerted by α/β assembly intermediates on translation of subunit β. Moreover, translation of subunit α is transactivated by subunit β, an observation unprecedented in the biogenesis of organelle proteins.

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Introduction

Energy transduction in mitochondria or chloroplasts is performed by membrane-associated oligomeric proteins that comprise subunits of dual genetic origin. Many subunits are expressed in the nucleo-cytosol before being imported into the organelle, but a subset of subunits is organelle-encoded and therefore translated next to the membranes, where functional assembly of the proteins takes place. Studies of chloroplast proteins in the unicellular green alga *Chlamydomonas reinhardtii*, or of mitochondrial proteins in the yeast *Saccharomyces cerevisiae*, have disclosed a unique trait in the biogenesis of these multimeric enzymes: the expression of several organelle-encoded subunits is regulated at the translational step in an assembly-dependent manner. We have termed this process *Control by Epistasy of Synthesis* (CES), whereby the presence of one subunit is required for sustained synthesis of another organelle-encoded subunit from the same protein complex (for reviews see Wollman *et al*, 1999; Choquet and Vallon, 2000; Choquet and Wollman, 2002). In all instances studied so far, the CES process involves transmembrane subunits that assemble in a 1 to 1 stoichiometry in the final protein. In these cases, the molecular basis for this regulation has been consistently identified as a negative feedback of the unassembled subunit on the initiation of its translation.

By contrast with the major subunits of photosystem I, photosystem II, cytochrome bc/b_6f complexes and cytochrome oxidase, which are integral membrane proteins, the catalytic sector of the proton ATP synthase, F1, which is responsible for the reversible synthesis of ATP is made of extrinsic subunits that assemble in a hydrophilic environment (Abrahams *et al*, 1994). The other sector, F0, that behaves as a selective proton channel, is membrane embedded and assembles within the lipid bilayer (Deckers-Hebestreit and Altendorf, 1996). A unique feature of the biogenesis of the ATP synthase is the requirement for an uneven stoichiometry between subunits in both F1 and F0. In F1, 3 copies each of the α and β subunits assemble with only one copy of the γ, δ and ε subunits, whereas 10–14 copies of suIII (named subunit c in bacteria) assemble with 1 copy of subunits I, II and IV in F0.

In bacteria and mitochondria, the two sectors may assemble independently. In *Escherichia coli*, a functional F0 is inserted into the membrane in the absence of F1 synthesis (Aris *et al*, 1985; Fillingame *et al*, 1986), while mutants lacking F0 accumulate a functional F1 sector, showing ATPase activity (Klionsky and Simoni, 1985). Mitochondrial F0 contains organelle-encoded subunits, whereas mitochondrial F1 is made exclusively of nuclear-encoded subunits that may assemble independently of the presence of F0 (for a review see Ackerman and Tzagoloff, 2005). In marked contrast, both CF0 and CF1 are made of subunits of mixed genetic origin in chloroplasts (Lemaire and Wollman, 1989a; for a review see Strotmann *et al*, 1998). SuI, III and IV from CF0 are chloroplast-encoded, but SuII is nucleus-encoded. CF1 subunits α, β and ε are expressed from the chloroplast genes *atpA*, *atpB* and *atpE*, respectively, while subunits γ and δ are expressed from the nuclear genes *ATPC* and *ATPD*. The biogenesis of the chloroplast ATP synthase thus requires control mechanisms to ensure not only these specific and uneven stoichiometries between its constitutive subunits, but also a cross-talk between two distinct genetic compartments. Studies of ATP synthase mutants of *C. reinhardtii* have shown that defects in the expression of any

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of its constitutive subunits lead to a pleiotropic loss in most polypeptides, from both CF0 or CF1 (Lemaire and Wollman, 1989b). Thus, assembly of the chloroplast ATP synthase is a concerted process. These studies also pointed to a tight coupling between the rates of synthesis of several subunits: mutants lacking subunit β display a markedly decreased synthesis of subunit α, whereas mutants defective in the expression of subunit α show some increase in synthesis of subunit β (Lemaire and Wollman, 1989b; Drapier *et al*, 1992). Whether interactions between rates of synthesis of the two major CF1 subunits α and β play a role in their three time accumulation over that of their assembly partners in CF1 has not been investigated.

In the present work, we used reporter genes translated under the control of the 5′ untranslated regions (UTRs) of *atpA* and *atpB* to demonstrate that the final $\alpha_3\beta_3\gamma$ stoichiometry required for functional assembly of CF1 indeed results from an assembly-dependent control of translation initiation of the α and β subunits. In addition we report here the first instance where one subunit (subunit β) activates in *trans* the expression of its assembly partner, subunit α.

Results

Coordinated synthesis of CF1 subunits

A convenient way to assess the possible effect of the state of assembly of CF1 on the rates of synthesis of its constitutive subunits is to examine how mutations specifically preventing the expression of one of the main CF1 subunits in *C. reinhardtii* affect the expression of other subunits. To this end, we generated a Δ*atpA* deletion strain (Table I; Figure 1). We also used the Fud50 chloroplast mutant strain, hereafter

Table I Transformation experiments

Transformants[a]	Recipient strains[a]	Transforming plasmid
Δ*atpA*[b]	WT	pΔ*atpA*::K^r
β_{Tr}[b]	WT	p*atpB*$_{335}$StK^r
dBf	WT	pK*dBf*
{β_{Tr}, *dAf*}[b]	*dAf*	p*atpB*$_{335}$StK^r
atpC1 {*dBf*}	*atpC1*	pK*dBf*
atpC1 {Δ*atpA*}	*atpC1*	pΔ*atpA*::K^r
atpC1 {β_{Tr}}[b]	*atpC1*	p*atpB*$_{335}$StK^r
{β_{Tr}, *dBf*}	β_{Tr}[c]	pK*dBf*
atpC1 {β_{Tr}, *dBf*}	*atpC1* {β_{Tr}}[c]	pK*dBf*
{Δ*atpA*, *dAf*}	*dAf*	pΔ*atpA*::K^r
{Δ*atpA*, β_{Tr}, *dAf*}	{β_{Tr}, *dAf*}[c]	pΔ*atpA*::K^r
α_{Tr}	WT	pK^r*atpA*$_{300}$St
{α_{Tr}, *dAf*}[d]	*dAf*	pK^r*atpA*$_{300}$St
{α_{Tr}, β_{Tr}, *dAf*}[d]	{β_{Tr}, *dAf*}[c]	pK^r*atpA*$_{300}$St
*aA*α[e]	WT	pK*aA*α
{β_{Tr}, *aA*α}[e]	β_{Tr}[c]	pK*aA*α

All recipient strains were sensitive to spectinomycin. Transformants were selected for resistance to spectinomycin (100 μg ml^{-1}).
[a]Strains are named by their genotype. By convention, the chloroplast genotype is indicated between accolades for strains containing more than one mutation and follows, when required, the nuclear genotype.
[b]These strains were initially selected for spectinomycin resistance due to the presence of the recycling spectinomycin resistance cassette (K^r). Once homoplasmic with respect to the ATP synthase mutation, they were grown on TAP medium for several generations to allow the spontaneous loss of the recycling cassette, according to Fischer *et al* (1996), but not that of the mutated ATP synthase allele.
[c]They, therefore, became spectinomycin sensitive again and could be used as a recipient strain in a new round of transformation experiments based on selection for spectinomycin resistance.
[d]These strains are shown in the right panel in Figure 7C and D, but are described in Supplementary Figure S1 and related text.
[e]These strains were used to obtain the results presented in Table II, but they are described in Supplementary Figure S2 and related text.

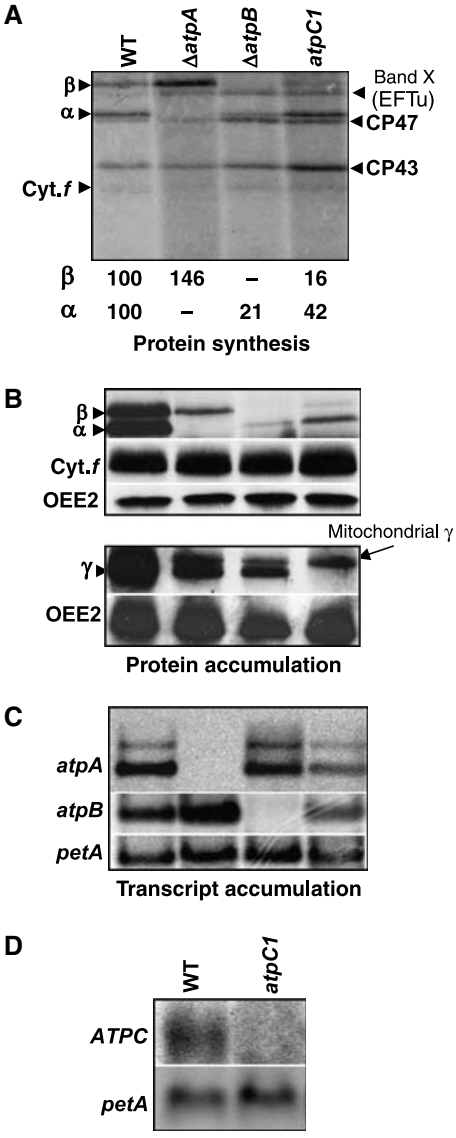

Figure 1 Impaired CF1 assembly feeds back on the expression of subunits α and β. (**A**) Synthesis of subunits α and β, as determined in a representative 5 min pulse-labelling experiment in the wild type and in mutant strains defective for CF1 assembly. Quantification of the levels of synthesis—normalised to that of the four major PSII subunits (apoCP47, apo-CP43, D1 and D2) to correct from variations in ^{14}C uptake and incorporation—are shown below. Note that, at variance with previous reports (Lemaire and Wollman, 1989b; Drapier *et al*, 1992), cycloheximide and ^{14}C-acetate were added simultaneously. Positions of subunits α, β and cytochrome *f* are shown, as well as those of two subunits from PSII: apo-CP47, -CP43, and that of EFTu. (**B**) Accumulation of subunits α, β and γ in the same strains, detected with specific antibodies. In the bottom panel, immunoblots reacted with antibody against subunit γ were over-exposed in order to detect residual γ in strains Δ*atpA* and Δ*atpB*; these conditions allow detection of a cross-reaction with mitochondrial subunit γ. Cytochrome *f*, subsequently used as a reporter, displays similar expression levels in all strains. OEE2, loading control. (**C**) Accumulation of *atpA* and *atpB* transcripts in the same strains. *petA* transcript, loading control. (**D**) Accumulation of transcripts for *ATPC* and *petA* (as a loading control) in the wild-type and *atpC1* strains.

referred to as Δ*atpB*, lacking expression of subunit β because of a partial deletion of the *atpB* gene (Woessner *et al*, 1984; Figure 1), and the nuclear mutant strain *atpC1* defective for the expression of subunit γ as it lacks *ATPC* mRNA (Smart and Selman, 1991; Figure 1B and D).

Translation of subunits α and β was assessed in wild-type and mutant strains by 5 min pulse-labelling experiments by adding ^{14}C-acetate simultaneously with cycloheximide, an inhibitor of cytosolic translation (Figure 1A). In the wild-type strain, the rates of synthesis of subunits α and β were similar. In strain Δ*atpA*, synthesis of subunit β showed some upregulation. In strain Δ*atpB*, synthesis of subunit α was considerably decreased, as previously reported (Lemaire and Wollman, 1989b; Drapier *et al*, 1992), and its accumulation was barely detectable. This behaviour qualifies subunit α as a CES subunit (Wollman *et al*, 1999; Choquet and Wollman, 2002) requiring the presence of subunit β to be expressed at a significant rate. In strain *atpC1*, translation and accumulation of subunit β were drastically decreased. Subunit β, therefore, is also a CES protein whose rate of synthesis depends on the presence of subunit γ. We note that subunit α, despite its inability assemble with subunit γ, resisted proteolytic degradation, as did subunit β in the absence of subunit α: both accumulated to significant amounts in exponentially growing cells from strains *atpC1* and Δ*atpA*, respectively (Figure 1B).

The decreased synthesis of the CES subunits, β in the absence of γ, or α in the absence of β, could not be accounted for by the limited changes in the amount of *atpA* or *atpB* mRNAs (Figure 1C), suggesting a translational or early post-translational regulation.

The atpB-5′UTR confers a γ-dependent translation to a reporter protein

We investigated the mechanism by which the nucleus-encoded γ subunit regulates synthesis of the chloroplast-encoded β subunit, by testing whether it resulted from a regulation of translation initiation. To this end, we constructed a chimeric gene, made of the *atpB* promoter and 5′UTR regions fused to the *petA* coding sequence for cytochrome *f*, previously shown to be a convenient reporter gene (Wostrikoff *et al*, 2004; Minai *et al*, 2006). This chimeric gene (5′*atpB-petA*) was associated with an *aadA* spectinomycin resistance cassette (Goldschmidt-Clermont, 1991) to allow

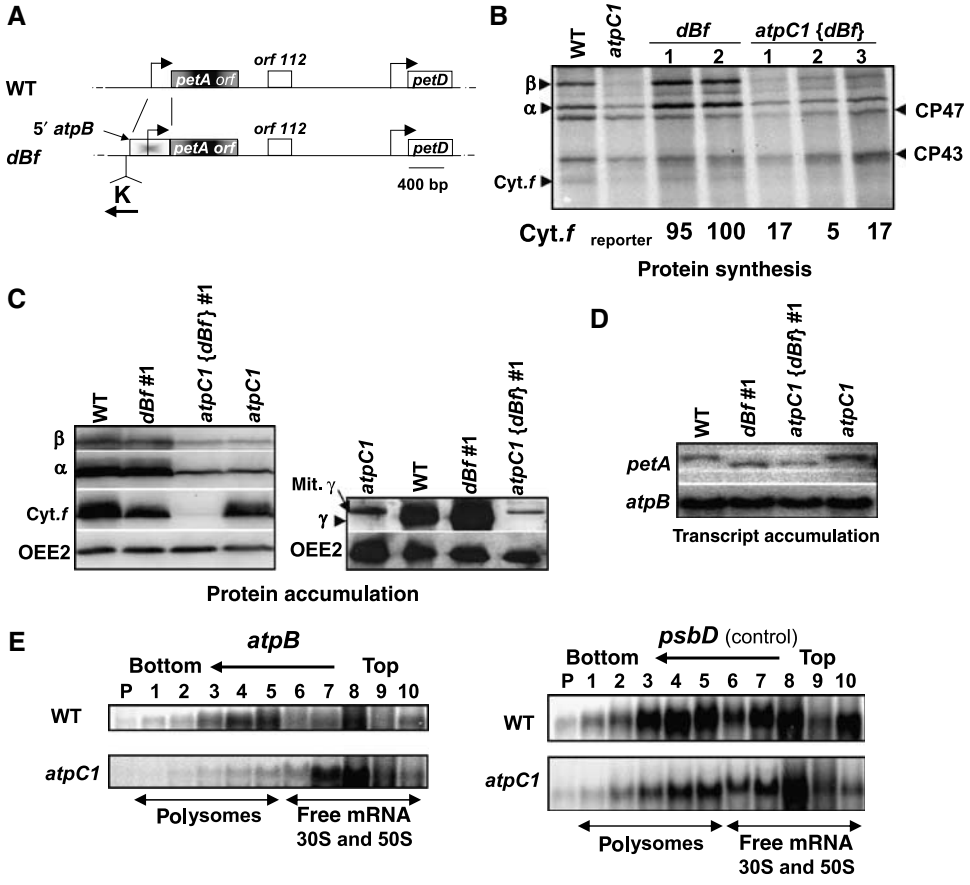

Figure 2 The CES behaviour of subunit β corresponds to a translational regulation mediated by the *atpB* 5′UTR. (**A**) Map of the chloroplast *petA* gene in wild-type and *dBf* strains. Arrows indicate transcription start sites. K stands for the *aadA* cassette, in opposite orientation with respect to *petA*. (**B**) Chloroplast translates in strains *dBf* and *atpC1* {*dBf*} (2 and 3 independent transformed strains are shown, respectively) that express the reporter cytochrome *f*. WT and *atpC1* strains expressing the regular *petA* gene are shown as control. The positions of cytochrome *f*, subunits α and β, CP43 and CP47 are indicated. Levels of synthesis of the cytochrome *f* reporter, normalised to those of apo-CP43, are indicated. (**C**) Accumulation of α, β, γ subunits, cytochrome *f* and OEE2 (loading control). (**D**) Accumulation of transcripts for *petA*, either endogenous or chimeric (slightly shorter) and *atpB* (loading control). (**E**) Distribution of *psbD* and *atpB* mRNAs on sucrose gradients in WT and *atpC1* strains. Polysomes are recovered in the pellet and fractions 1–5, while free mRNAs and dissociated 50S and 30S ribosome subunits are recovered in fractions 6–10, as described in (Minai *et al*, 2006). *psbD* mRNA serves as a control for a chloroplast transcript unrelated to CF1 biogenesis.

the selection of transformed cells (Figure 2A and Table I). In order to compare the expression of this chimera in strains expressing, or not, the γ subunit, this construct was introduced by biolistic transformation at the *petA* locus on the chloroplast genome of wild-type and *atpC1* strains. Spectinomycin resistant transformants, hereafter referred to as *dBf* or *atpC1* {*dBf*}—because they express a complex **d** (ATP synthase) 5′*atpB* driven cytochrome *f* -, were recovered from both strains. Transformants *dBf* were capable of phototrophic growth and expressed the cytochrome *f* reporter at a similar level as endogenous cytochrome *f* in the wild type (Figure 2B and C). By contrast, transformants *atpC1* {*dBf*}, lacking subunit γ, displayed a strong decrease in synthesis of the cytochrome *f* reporter (Figure 2B) leading to the accumulation of only 5% of cytochrome *f* in strain *atpC1* {*dBf*} as compared to strain *dBf* (Figure 2C).

The reduced expression of the cytochrome *f* reporter did not result from a reduced accumulation of its chimeric mRNA in strain *atpC1*{*dBf*} (Figure 2D). Thus, the decreased synthesis of subunit β in the absence of its assembly partner—subunit γ- is governed by the *atpB* 5′UTR and is most likely controlled at the level of translation initiation. This conclusion was further substantiated by the markedly decreased binding of *atpB* mRNA to polysomes (fractions P-5) in the *atpC1* mutant: less than 10% of total *atpB* mRNA compared to 40% in the wild type (Figure 2E).

Subunit γ does not act as a transactivator in translation of subunit β

Down-regulation of *atpB* translation in the absence of subunit γ could be explained in either of two ways: the presence of subunit γ could contribute to activate translation of subunit β (transactivation hypothesis) or the failure to assemble subunit β within CF1 in absence of subunit γ could lead to a translational repression of subunit β (autoregulation hypothesis). Since the *atpB*-5′UTR conferred the γ-sensitivity to the translation of chimeric genes, these two hypotheses could be discriminated by looking at the expression of a reporter, here the 5′*atpB*-driven cytochrome *f*, in the absence of both the β and γ subunits. In the trans-activation hypothesis, the absence of the positive regulator should result in a poor expression of the chimeric construct, irrespective of the presence of subunit β. By contrast, in the autoregulation hypothesis, a strain lacking subunit β should show a high expression of the chimera, even in absence of subunit γ. Still, strains lacking subunit β because of the absence of the endogenous *atpB* mRNA—such as Δ*atpB*—may also show higher expression of 5′*atpB*-driven reporter genes because of an increased availability in specific translational activators normally bound to the regular *atpB* transcript in the wild type (Barkan and Goldschmidt-Clermont, 2000). To avoid such an ambiguity, we substituted the *atpB* gene from wild-type and *atpC1* strains by a mutated version encoding a truncated β

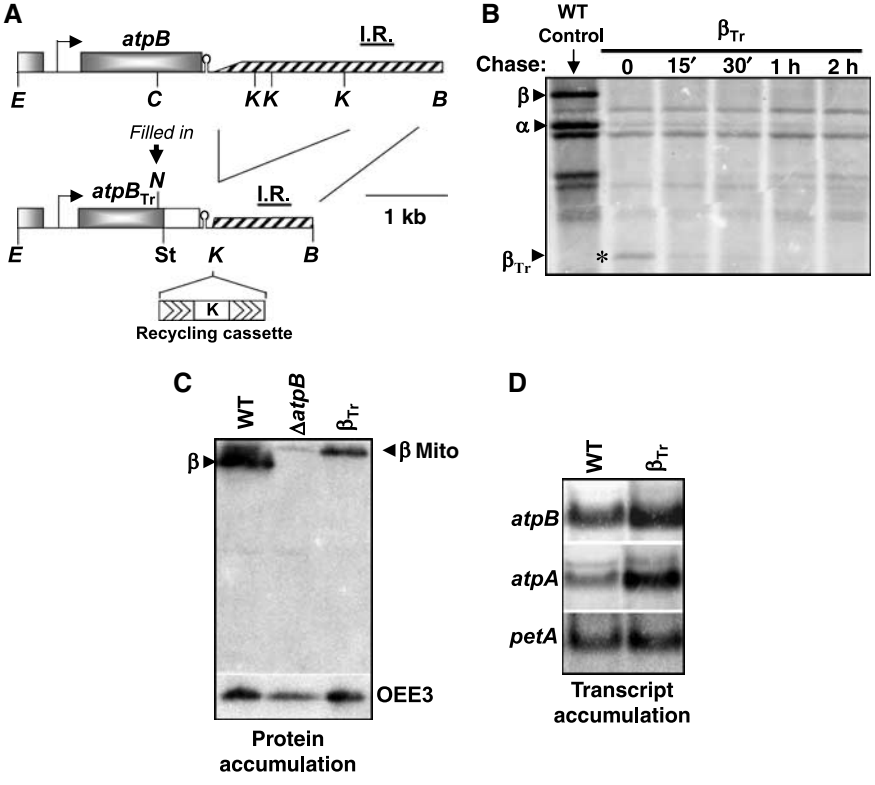

Figure 3 The {β_{Tr}} strain expresses a truncated and short-lived subunit β. (**A**) Strategy used to introduce a mutation (St) in the *atpB* gene, associated with the recycling spectinomycin resistance cassette, schematically depicted by K (not to scale). Arrow indicates transcription start site. Relevant restriction sites are shown (K: *Kpn*I, C: *Cla*I, N: *Nru*I), as well as the position of the chloroplast inverted repeat (hatched arrow). Coding sequences are indicated in grey. Due to the St mutation, about one third of *atpB* mRNA is not translated in the mutant (white rectangle). (**B**) Expression of truncated subunit β: *atpB* translation products in strain β_{Tr} were analysed by pulse labelling followed by a chase for the indicated times in the presence of an excess of unlabelled acetate. Pulse-labelled wild type is shown for comparison. (**C**) Accumulation of subunit β in wild-type, β_{Tr} and Δ*atpB* (as a control) strains. OEE3, loading control. (**D**) Accumulation of transcripts for *atpB*, *atpA* and *petA* (loading control) in wild-type and β_{Tr} strains.

subunit, that allows accumulation and translation of the *atpB* mRNA but prevents accumulation of the β subunit. We introduced a stop codon in the *atpB* coding sequence, at position + 335 with respect to the initiation codon (Figure 3A and M&M section), associated with the recycling spectinomycin resistance cassette (Fischer *et al*, 1996) to enable the selection of transformants on TAP-spectinomycin plates. In pulse-labelling experiments, the resulting β_{Tr} and *atpC1* {β_{Tr}} strains (Table I) show no neosynthesised subunit β, but the presence of a very short-lived ($t_{1/2}$: < 10 min) truncation product with an apparent molecular weight of ~25 kDa (see Figure 3B for strain β_{Tr}). Consequently, its accumulation remained below detection (Figure 3C), although a translatable *atpB* mRNA accumulates normally (Figure 3D).

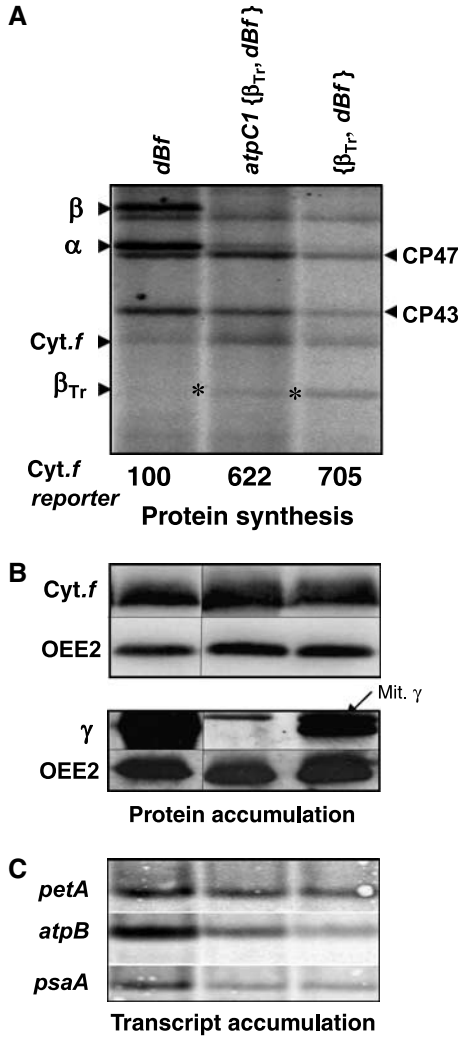

Figure 4 The expression of 5′*atpB*-driven genes is no longer dependent on the presence of subunit γ when full-length subunit β is lacking. (**A**) Translation of the main chloroplast-encoded CF1 subunits and reporter cytochrome *f* in strains *dBf*, {β_{Tr}, *dBf*} and *atpC1* {β_{Tr}, *dBf*}. *Points to truncated subunit β. Rates of synthesis of the cytochrome *f* reporter, relative to the reference strain *dBf* and normalised to that of the apo-CP43 subunit from PSII are shown. (**B**) Accumulation of subunits α, β and γ, of cytochrome *f* and OEE2 (loading control). In this figure and others, the vertical black line indicates lanes from the same gel that have been cropped because they were not originally adjacent on the gel. (**C**) Accumulation of transcripts for *petA*, *atpB* and *psaA* (loading control).

Using the two steps procedure described in the legend of Table I we then substituted the resident *petA* gene of strains β_{Tr} and *atpC1* {β_{Tr}} with the 5′*atpB-petA* chimera. In the resulting transformants {β_{Tr}, *dBf*} and *atpC1* {β_{Tr}, *dBf*}, the 5′*atpB*-driven-cytochrome *f* reporter was more translated than in the control strain *dBf* (Figure 4A). This contrasts with the decreased expression observed in strain *atpC*1 {*dBf*} (Figure 2B) that accumulates full length subunit β unable to assembled in CF1. In agreement with pulse-labelling data, the accumulation of the cytochrome *f* reporter was increased by 50% in strains {β_{Tr}, *dBf*} and *atpC1* {β_{Tr}, *dBf*} relative to *dBf* (Figure 4B), as opposed to its 95% decrease in strain *atpC*1 {*dBf*} (Figure 2C). Thus, subunit γ is not required to stimulate 5′*atpB*-driven translation, ruling out the trans-activation hypothesis. Our data point to a negative feedback mechanism: the production of unassembled β subunits represses translation initiation of *atpB* mRNA.

Oligomeric forms of subunits α and β, but not 'free' subunit β, repress translation of atpB mRNA

That the unassembled β subunit represses its own translation in the absence of the γ subunit but not in the absence of the α subunit (see Figure 1A) suggests that it does not adopt the same unassembled conformation in the two situations. A major difference between γ- or α-deficient mutant strains is that α/β oligomers can assemble in the former strain only. We thus deleted the *atpA* gene from the chloroplast genome of the *atpC1* strain in order to prevent formation of α/β oligomers in absence of the γ subunit.

Expression of subunit β in the resulting strains, *atpC1* {Δ*atpA*}, is shown on Figure 5A and B. Whereas synthesis of subunit β was drastically reduced in strain *atpC1*, the double mutant, *atpC1* {Δ*atpA*}, showed high rate of translation of subunit β, similar to that in the Δ*atpA* deletion strain. Accordingly, the accumulation of subunit β in strain *atpC1* {Δ*atpA*} was similar to that in Δ*atpA*, much higher than in the *atpC1* mutant. We conclude that subunit β *per se*, is unable to down-regulate its own synthesis: the negative feedback due to defective CF1 assembly requires the combined expression of α and β subunits, i.e. is rather born by α/β hetero-oligomers.

Oligomerisation of subunits α and β in a γ-deficient strain

This prompted us to examine CF1 assembly intermediates in strains Δ*atpA*, Δ*atpB*, *atpC1* and wild type. Soluble fractions, containing subunits not yet assembled into a membrane-bound ATP synthase, were analysed by CN–PAGE followed by denaturating Urea–SDS–PAGE in the second dimension and revealed after immunoblotting using a mixture of α and β specific antibodies (Figure 6).

In the wild type, subunits α and β were mainly observed in a complex of 500–600 kDa, absent in either strains Δ*atpA* or Δ*atpB*, that probably corresponds to the CF1 moiety of the ATP synthase. However, free β subunits—but not free α subunits—were detected in significant amount in the region around 60 kDa. In addition, subunit α, together with subunit β, was found in the region around 250 kDa (indicated by a black bar), and to a lesser extent in the 120–140 kDa region. However, 120–140 kDa was the major position of subunit α when expressed in absence of subunit β, as in strain Δ*atpB*, even if trace amounts of larger α-containing oligomers could

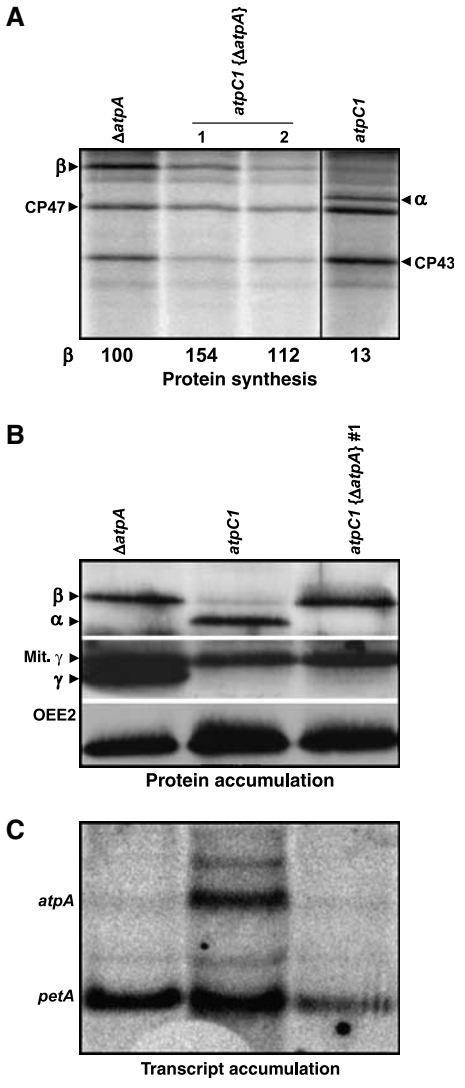

Figure 5 In absence of subunit γ, the presence of subunit α is required for subunit β to repress translation of *atpB* mRNA. Levels of translation (**A**) protein (**B**) and transcript (**C**) accumulation for the main CF1 subunits in strains Δ*atpA*, *atpC1* and *atpC1* {Δ*atpA*} (two independent transformed clones). Levels of subunit β synthesis, relative to that of apoCP43, appear similar in the various strains, but strain *atpC1*. OEE2 (B) and *petA* transcript (C) provide loading controls.

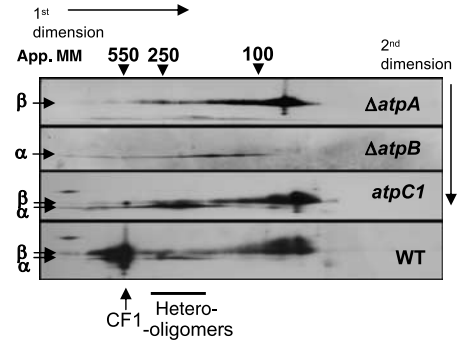

Figure 6 Oligomeric forms of α and β accumulate in the absence of subunit γ. CF1 assembly intermediates from soluble extracts of wild-type, Δ*atpA*, Δ*atpB* and *atpC1* cells were separated on colourless native gels in the first dimension and denaturating 12–18% Urea-gels in the second dimension. Apparent molecular mass in the first dimension of molecular standards is shown on the top of the figure. After electrotransfer, the presence of subunits α and β was revealed using a mixture of antibodies specific for each subunit. The strength of the signal is therefore not an indication on the relative accumulation of the two subunits. The bottom black bar stresses the appropriate region for α/β heteromers in wild-type and *atpC1* strains.

also be detected. In strain Δ*atpA*, most of the β subunits were found as free polypeptides, below 100 kDa, with traces in larger complexes (up to 250 kDa) that may correspond to β homo-oligomers. In strain *atpC1*, subunit β was mostly fully unassembled, below 100 kDa, but a significant fraction was also found in the region around 250 kDa suggesting the presence of oligomers that were dramatically enriched in subunit α. Thus, in absence of subunit γ, an increased fraction of subunit α relocates in oligomeric complexes of same app. MM as those containing subunit β.

As a further confirmation, subunit β was co-immunoprecipitated with an antibody raised against subunit α from a soluble cellular extract of strain *atpC1* (data not shown), indicating that both subunits were indeed associated as hetero-oligomers. This mixed population of α- and β-containing oligomers reflects the accumulation of α/β assembly intermediates and provides a molecular basis for the α-sensitive CES behaviour of the β subunit in a γ-deficient context.

Unassembled subunit β trans-activates translation of the α subunit

To study the role of the *atpA* 5′UTR in the CES behaviour of the α subunit, we used the *dAf* strain that expresses a protein complex **d** (ATP synthase) *atp**A***-driven cytochrome *f*, since it bears instead of the endogenous *petA* gene a 5′*atpA-petA* chimera. This reporter gene is translated at a level similar to—or slightly higher than—regular cytochrome *f* (Choquet *et al*, 1998). To compare the expression of the *atpA* 5′UTR-driven cytochrome *f* in the presence or absence of subunit β, we introduced by transformation the truncated *atpB* allele into the *dAf* strain. Indeed, the β_{Tr} strain shows the same decrease in translation of subunit α (Figure 3B) as strain Δ*atpB* (Figure 1A): truncated subunit β did not sustain translation of subunit α. Transformants {β_{Tr}, *dAf*}, recovered on TAP-spectinomycin plates, were analysed for cytochrome *f* expression.

The levels of synthesis and accumulation of the cytochrome *f* reporter were about three times lower when subunit β could not accumulate (compare lanes *dAf* and {β_{Tr}, *dAf*} on Figure 7A and B). Since the chimeric *petA* mRNA accumulated to high level in both strains (Figure 7D), we conclude that 5′*atpA*-driven translation is decreased in cells lacking subunit β, the assembly partner of α. The *atpA* 5′UTR is therefore sufficient to confer a β-dependent CES behaviour to a reporter gene, pointing to a specific down-regulation of synthesis operating at the level of translation initiation.

To address the mechanism of this down-regulation and determine whether subunit β stimulates the expression of subunit α or whether the unassembled subunit α exerts a negative feed-back on translation of 5′*atpA*-driven mRNAs, we studied the expression of the 5′*atpA*-driven cytochrome *f* in the combined absence of the assembly partner subunit β and of the CES subunit α, following the same rationale as we described above for subunit β. By multiple successive chlor-

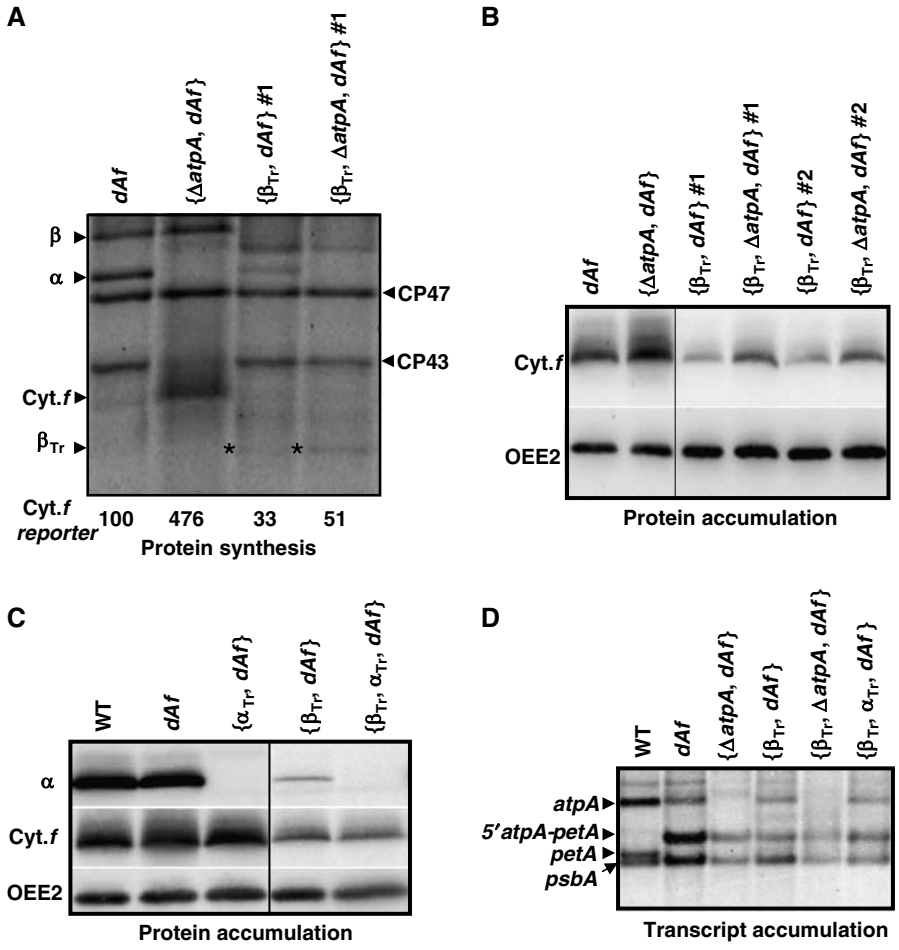

Figure 7 Initiation of translation of subunit α is not autoregulated but transactivated by subunit β. (**A**) Chloroplast translation products in *dAf*, {Δ*atpA*, *dAf*}, {β_{Tr}, *dAf*} and {β_{Tr}, Δ*atpA*, *dAf*} strains. The rate of translation of cytochrome *f* in the various strains, normalised to that of apoCP47 and relative to the reference strain *dAf* is indicated below for this representative experiment. (**B**, **C**) Accumulation of cytochrome *f* and OEE2 as a loading control in strains *dAf*, {Δ*atpA*, *dAf*}, {β_{Tr}, *dAf*}, {β_{Tr}, Δ*atpA*, *dAf*} (B) or in wild type and strains expressing the 5′*atpA-petA* chimeric gene, either alone (*dAf*) or together with a truncated subunit α {α_{Tr}, *dAf*}, a truncated subunit β {β_{Tr}, *dAf*} or both {β_{Tr}, α_{Tr}, *dAf*}. (C) On panel B, two independent transformants are shown for strains {β_{Tr}, *dAf*} and {β_{Tr}, Δ*atpA*, *dAf*}. (**D**) Accumulation of mRNAs for *atp*A, *petA* and *psbA* (loading control) in the wild type and in strains *dAf*, {Δ*atpA*, *dAf*}, {β_{Tr}, *dAf*} {β_{Tr}, Δ*atpA*, *dAf*} and {β_{Tr}, α_{Tr}, *dAf*}. Note the reduced accumulation of *atpA* mRNA in presence of the 5′*atpA-petA* chimeric gene.

oplast transformations, we created strains {β_{Tr}, Δ*atpA*} and {β_{Tr}, Δ*atpA*, *dAf*} (Table I), taking advantage of the inherent instability of the recycling cassette that allows spontaneous elimination of the *aadA* marker upon culture in the absence of antibiotics. Pulse labelling of the {Δ*atpA*, *dAf*} strain showed highly increased translation of the chimeric gene in the absence of subunit α (Figure 7A). In marked contrast, translation of the chimeric gene in transformant {β_{Tr}, Δ*atpA*, *dAf*} lacking both subunits α and β was lower than in the control strain *dAf* (Figure 7A). These opposite changes in rates of synthesis were reflected at the level of cytochrome *f* accumulation, being 1.5 times higher in strain {Δ*atpA*, *dAf*} than in strain *dAf*, but four times lower in strain {β_{Tr}, Δ*atpA*, *dAf*} (Figure 7B). Here again, there was no corresponding changes in the accumulation of the chimeric *petA* transcripts (Figure 7D), which indicates actual changes in the translation rate.

Thus, in the absence of subunit β, elimination of subunit α does not restore high translation of *atpA* mRNA, ruling out the autoregulation hypothesis and pointing to a specific role of subunit β in the trans-activation of translation of the *atpA* mRNA.

We note, however, that cytochrome *f* was synthesised and accumulated a little more in mutants defective in both *atpA* and *atpB* expression {β_{Tr}, Δ*atpA*, *dAf*} than in mutants defective for *atpB* expression only {β_{Tr}, *dAf*} (Figure 7B). We attribute this difference to better access of the chimeric transcript to nuclear factors required for *atpA* expression when the resident *atpA* gene is deleted. As an example, *atpA* mRNA is less accumulated in strains *dAf* and {β_{TR}, *dAf*} than in wild type (Figure 7D), suggesting a competition between 5′*atpA* UTRs for interaction with RNA stabilising factors. The similar accumulation of the reporter cytochrome *f* (Figure 7C) in strain {β_{Tr}, *dAf*} and in a double mutant strain expressing a truncated version of both α and β subunits {β_{Tr}, α_{Tr}, *dAf*} (described on Supplementary Figure S1), in which specific nuclear factors would not be reallocated to the *dAf* transcript, supports this reallocation hypothesis.

Subunit α aggregates when expressed to a high level in absence of subunit β

The unusual CES control of subunit α through a transactivation of its translation by subunit β prompted us to investigate

Table II Distribution of subunits α and β (in % of total amount) between the fractions in the three strains studied

Fraction	WT		*atpC1* {Δ*atpA*}	{β_{Tr}, *aA*α}
	α	β	β	α
Buffer A	42	34	83	18
Mb[a]	50	61	15	11
Buffer B	0	2	0	5
Pellet	8	1	<0.5	66

[a]Mb, thylakoid fraction at the interface between buffers A and B.

the behaviour of unassembled subunits α and β. To that end, we used strain *atpC1* {Δ*atpA*} that accumulates high levels of subunit β in the absence of subunit α (Figure 5B) and constructed a strain, *aA*α, expressing a 5′*ps**aA***-driven subunit **α** (see Supplementary Figure S2 for the construction and the characterisation of this strain). In strain {β_{Tr}, *aA*α} where subunit α is translated under the control of this unrelated 5′UTR (*psaA* encodes a PSI subunit, the expression of which is independent of ATP synthase biogenesis), it escapes CES regulation and still shows high rates of translation when subunit β does not accumulate (see Supplementary Figure S2).

Cells from strains *atpC1* {Δ*atpA*} and {β_{Tr}, *aA*α} (and from the wild type as a control) were broken by French press and cell lysates were overlaid on a high density sucrose cushion (for details see Material and methods). After ultracentrifugation, soluble proteins were recovered in the upper layer, membranes were recovered at the interface layer and protein aggregates sedimented through the sucrose cushion, as a pellet fraction. The distribution of subunits α and β, as quantified from immunoblots, is reported in Table II. In the wild type, subunits α and β are mostly in the membrane fraction, but also in the upper layer, probably because a fraction of CF1 was stripped off when cells were broken. We found a small fraction of subunit α in the pellet. In strain *atpC*1 {Δ*atpA*}, more than 80% of subunit β was in the upper layer, indicating that unassembled subunit β remains soluble. In marked contrast, more than 60% of subunit α from strain {β_{Tr}, *aA*α} was found in the pellet. Thus, unassembled subunit α forms high density aggregates, able to cross the sucrose cushion. However, subunit α did not form large inclusion bodies similar to those observed in (Ketchner *et al*, 1995), since it was not recovered in the pellet after low speed centrifugation (data not shown).

Discussion

How bacteria, mitochondria and chloroplasts produce the major subunits of the ATP synthase in the uneven stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$ required for their functional assembly within (C)F1 has remained unresolved over the years. This unique assembly requirement occurs in widely different genetic contexts. In prokaryotes these subunits are co-transcribed from the *unc* operon (McCarthy, 1988), whereas they are all expressed independently from nuclear genes when assembled in the mitochondria or from a combination of nuclear and organelle genes when assembled in the chloroplast. The higher expression of α and β versus γ/δ/ε in prokaryotes was suggested to originate from a combination of differences in transcript stability and translation rates (McCarthy and Bokelmann, 1988; McCarthy *et al*, 1988, 1991). In mitochondria, two dedicated molecular chaperones Atp11p/Atp12p bind newly imported α and β subunits and control the formation of αβ heteromers before their assembly in F1 (Ackerman, 2002). Whether specific molecular chaperones also contribute to the formation of αβ heteromers in the chloroplast as they do in mitochondria is not known. Chloroplast α and β subunits, however, are not imported in but translated within the organelle. Previous screens for chloroplast ATP synthase mutants in Chlamydomonas or Arabidopsis did not identify nuclear loci for chaperones. Although a survey of the current version of the Chlamydomonas genome identifies putative orthologs for *atp11* and *atp12*, these are most likely required for the biogenesis of the mitochondrial ATP synthase whose α and β subunits are nucleus-encoded, as they are in yeast.

Here, using Chlamydomonas as an experimental system, we show how chloroplasts use the nuclear-encoded subunit γ to regulate the production of α and β subunits. A CES process comprising intertwining transactivation and negative feedback loops ensures the production of αβ oligomers in the proper stoichiometry required for their interaction with subunit γ. High rates of translation of subunit α require the presence of its assembly partner subunit β, whose translation is repressed when α/β oligomers accumulate, unless they are converted into a γ-containing protein complex (Figure 8). Whether this regulation scheme drawn from the present study with Chlamydomonas also applies to higher plant chloroplasts requires specific studies of mutant strains showing fully prevented expression of the β or γ subunits. In the two reports where synthesis of either subunit γ from Arabidopsis or subunit β from maize were compromised (McCormac and Barkan, 1999; Bosco *et al*, 2004), they were still synthesized to some extent, which may explain why the feedback regulations we report here were not observed.

A transactivation rather than a negative feedback loop controls translation of subunit α

Our results show that the decreased translation of subunit α in absence of subunit β is born by the *atpA* 5′UTR, as described for the other CES proteins in the chloroplast of *C. reinhardtii* (Choquet *et al*, 1998, 2003; Wostrikoff *et al*, 2004; Minai *et al*, 2006). Like other CES proteins, subunit α appears to be stable when synthesized at high rates in the absence of its assembly partners because of impaired CES control. However, in marked contrast to the other instances of CES regulation described so far, the regulation of α synthesis does not rely on a negative feedback of the unassembled subunit on its own translation. We demonstrate that α translation is transactivated by subunit β: a 5′*atpA*-driven reporter gene remains poorly expressed when the accumulation of β subunits is prevented, whether the α subunit accumulates or not. We attribute the selection of a transactivation rather than a negative feedback mechanism, to the propensity of unassembled subunit α to self-aggregate. Aggregation shields the highly hydrophobic sequences surrounding the nucleotide binding site from water. These being ultimately buried at the subunit interface in the $\alpha_3\beta_3$ hexamer. Subunit α aggregates when over-expressed in *E. coli*, while over-expressed subunit β remains soluble (Du and Gromet-Elhanan, 1999). Both in Chlamydomonas chloroplasts (Ketchner *et al*, 1995) and yeast mitochondria (Lefebvre-Legendre *et al*, 2005), subunit α accumulates in inclusion bodies in some assembly-defective strains. Here we con-

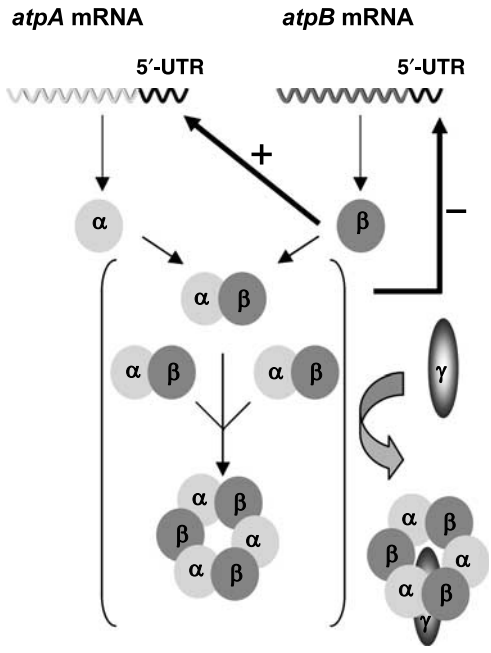

Figure 8 A model for intertwining CES regulations controlling CF1 biogenesis. Subunit β transactivates initiation of translation of subunit α by a presently unknown mechanism. Heteromers of subunits α and β assemble in a γ-binding competent form. When accumulating in excess of the stoichiometry required for functional assembly with subunit γ, a negative feed-back loop prevents further translation of subunit β, which in turn deactivates translation of subunit α.

firmed that the unassembled α subunit self-aggregates when escaping the CES regulation because expressed from an unrelated 5′-UTR. Most probably, subunit α, once aggregated, would not be available for autoregulation, causing an increased and fruitless expression, sustaining further aggregation, a process that was indeed observed in the FUD16 mutant of Chlamydomonas (Ketchner *et al*, 1995).

There are several ways in which subunit β may transactivate translation of subunit α. Our working hypothesis that subunit β traps a translational repressor for subunit α is currently under investigation.

A negative feedback from αβ oligomers controls translation of the β subunit

We show here that synthesis of subunit β depends on the availability of subunit γ. This regulation operates at the level of translation initiation since it is controlled by the *atpB* 5′UTR. It cannot result from a trans-activation mechanism, because the 5′*atpB*-driven cytochrome *f* is still highly expressed in the absence of both the γ and β subunits, pointing to a negative feedback mechanism. However a mere autoregulation of translation of subunit β can be excluded as well, since both subunit β and the 5′*atpB*-driven reporter are highly expressed in the combined absence of subunits α and γ. Since the down regulation of 5′*atpB*-driven translation in the absence of subunit γ is lost when either subunits α or β are missing, molecular species comprising these two subunits must be required for the negative feedback to occur. Indeed, α/β oligomers accumulate in the absence of subunit γ in Chlamydomonas chloroplasts (this study) as well as in yeast mitochondria (Paul *et al*, 1994).

The titration of α/β assembly intermediates by the amount of subunit γ that reaches the chloroplast compartment after its import from the cytosol thus offers an efficient strategy to assemble the $\alpha_3\beta_3\gamma$ core of the CF1 moiety of the ATP synthase (Figure 8). These assembly intermediates would release a negative feedback on translation of subunit β- that in turn activates translation of subunit α- only when CF1 complexes are formed. This regulatory feedback loop accounts for contrasting observations on the relative rates of translation of subunits α and β, close to a 1:1 stoichiometry in this study, but in a 3:1 ratio in our previous studies (Drapier *et al*, 1992). In the latter experiments, cycloheximide that prevents cytosolic translation was added 10 min before pulse labelling. During this time period the stromal pool of nucleus-encoded subunit γ, available for assembly of new CF1, was depleted, reducing drastically translation of subunit β in the wild type (see Supplementary Figure S1) but not in strains defective for the expression of subunit α where α/β oligomers could not be formed. In contrast, the rate of translation of subunit α is less sensitive to a drop in γ since the pool of unassembled β subunits—acting as transactivator for α translation—is rather large in the wild type of Chlamydomonas (see Figure 6).

Together, these two feedback regulatory loops also explain the high level of translation of the 5′*atpA*-driven reporter in absence of subunit α that results from the high expression of the trans-activating free subunit β.

Multiple levels of CES control in ATP synthase biogenesis

The CES contribution to the biogenesis of the chloroplast ATP synthase may be wider than the translational coupling between subunits α, β and γ described in this article. The role of subunits ε and δ remains to be investigated. A CES regulation also participates in CF0 biogenesis: suIV from CF0 is poorly synthesised in mutants lacking expression of suIII, the assembly partner which assembles in ten-fold excess to the other subunits within CF0 (Lemaire and Wollman, 1989b). Similarly, in mitochondria of yeast, mutants lacking expression of Atp9p, equivalent to CF0-suIII, show reduced synthesis of the mitochondrial-encoded subunits Atp8p (homologous to CF0-suIV) and Atp6p (Jean-Francois *et al*, 1986). Thus, the biogenesis of the ATP synthase relies on a variety of assembly-dependent regulations of synthesis of those subunits that are translated in the organelles.

Materials and methods

Strains and growth conditions

Wild type, derived from 137C, mutant and transformed *C. reinhardtii* strains were grown in TAP medium (Harris, 1989) under continuous low light ($5–10\,\mu E\,m^{-2}\,s^{-1}$). Also used in this study are strains *atpC1* (Smart and Selman, 1991), *dAf* (formerly AFFF in Choquet *et al* (1998)) and Fud50, hereafter referred to as Δ*atpB* (Woessner *et al*, 1984).

Constructs and nucleic acids manipulations

Standard nucleic acids manipulations were performed according to (Sambrook *et al*, 1989). Before transformation in *C. reinhardtii*, constructs were sequenced to assess the presence of appropriate mutations. DNA constructs are detailed in the Supplementary Section. Northern blot analyses were carried out as described in (Drapier *et al*, 2002). Polysomes were prepared and analysed as described in (Minai *et al*, 2006). mRNA distribution was quantified in each lane from a PhosphorImager scan of the ^{33}P labelling and

expressed as the percentage of total transcript. For each strain, polysome association was calculated as the percentage of transcript found in fractions P to 5.

Transformation experiments

Cells were transformed by tungsten particle bombardment (Boynton *et al*, 1988) as described in (Kuras and Wollman, 1994). Transformants were selected on Tap-Spec (100 μg ml^{-1}). Proper insertion of transforming DNA and homoplasmy were assessed as detailed in the Supplementary Data. At least three independent transformants were analysed for each transformation.

Protein analysis

Pulse and pulse-chase experiments, protein electrophoresis and immunoblotting were performed according to (Kuras and Wollman, 1994), with the following modifications: for pulse labelling, cycloheximide (10 μg ml^{-1}) was added simultaneously with ^{14}C acetate. Cell extracts, loaded on equal chlorophyll basis, were analysed by SDS–PAGE in the presence of 8 M urea, on 8% acrylamide gels for pulse-labelling experiments and on 12% or 12–18% acrylamide gels for immunoblot analysis. Anti-cyt. *f*, -OEE2, -subunit α and –subunit β antibodies, used for [^{135}I]protein A revelation were raised in the laboratory against proteins isolated from *C. reinhardtii* and are respectively described in (de Vitry *et al*, 1989; Drapier *et al*, 1992; Kuras and Wollman, 1994). Antibodies used for ECL detection, raised against isolated proteins from *C. reinhardtii*, were from the Pr Berzborn's antibody collection and kindly given by Dr A Sokolenko (University of Munich).

Values for the level of synthesis of cytochrome *f*, α or β subunits (either endogenous or chimeric) were quantified from PhosphorImager (Molecular Dynamics) scans of dried pulse-labelling gels, using program ImageQuant (Sunnyvale, CA). They were corrected for background, by measuring in each lane an empty window of same area below the protein of interest. Synthesis of the unrelated apo-CP47, -CP43, D1 and D2 polypeptides from the PSII complex was similarly quantified. To correct for variations in the uptake and incorporation of radiolabelled acetate in the various strains, the rates of translation of the cytochrome *f* reporter or of CF1 subunits were normalised to the rates of synthesis of these PSII polypeptides. All pulse-labelling experiments were repeated at least twice and performed on three independent transformants. Despite small variations due to the physiological state of cultures or variations in genetic backgrounds, we consistently found similar results when repeating experiments. Quantification of the accumulation of cytochrome *f*, determined from PhosphorImager scans of immunoblots revealed with ^{125}I protein A, was normalised to that of the OEE2 protein from the PSII complex. Colourless-Native Polyacrylamide Gel Electrophoresis (CN–PAGE) was carried out according to (Majeran *et al*, 2005).

Sucrose cushions

200 ml of cells (2×10^6 cells ml^{-1}) were harvested by centrifugation, resuspended in 5 ml of ice-chilled buffer A (5 mM Hepes–NaOH, pH 7.5, 10 mM EDTA, 0.3 M Sucrose) and broken in a French press (6000 PSI). 1.5 ml of cell lysate was overlaid on a 1.5 ml cushion of buffer B (Hepes-NaOH, pH 7.5, 10 mM EDTA, 1.8 M Sucrose) and centrifuged for 2 h at 260 000 *g* in TL55 rotor (Beckman). After centrifugation membranes were recovered at the interface between the two layers, diluted in 2.5 ml buffer A, pelleted by centrifugation to wash off contaminating soluble material and resuspended in 1.5 ml of 0.1 M DTT/ 0.1 M Na_2CO_3. Buffer A and buffer B layers were recovered separately. Walls of the tube were carefully cleaned and the pellet was resuspended in 1.5 ml of 0.1 M DTT/ 0.1 M Na_2CO_3 by brief sonication. After boiling in SDS, equal volumes of each fractions were loaded on the gel and analysed by immunoblotting. Cytochrome *f* was found in the membrane fraction but also for a minor part in the pellet. This provided an estimation of the fraction of unbroken cells that was substracted from the values obtained for subunits α or β.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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