Evidence for regulatory function of nucleus-encoded factors on mRNA stabilization and translation in the chloroplast

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A salient feature of organelle gene expression is the requirement for nucleus-encoded factors that act posttranscriptionally in a gene-specific manner. A central issue is to understand whether these factors are merely constitutive or have a regulatory function. In the unicellular alga *Chlamydomonas reinhardtii*, expression of the chloroplast *petA* gene-encoding cytochrome *f*, a major subunit of the cytochrome b_6f complex, depends on two specific nucleus-encoded factors: MCA1, required for stable accumulation of the *petA* transcript, and TCA1, required for its translation. We cloned the *TCA1* gene, encoding a pioneer protein, and transformed appropriate mutant strains with tagged versions of *MCA1* and *TCA1*. In transformed strains expressing decreasing amounts of MCA1 or TCA1, the concentration of these factors proved limiting for *petA* mRNA accumulation and cytochrome *f* translation, respectively. This observation suggests that in exponentially growing cells, the abundance of MCA1 sets the pool of *petA* transcripts, some of which are TCA1-selected for an assembly-dependent translation of cytochrome *f*. We show that MCA1 is a short-lived protein. Its abundance varies rapidly with physiological conditions that deeply affect expression of the *petA* gene *in vivo*, for instance in aging cultures or upon changes in nitrogen availability. We observed similar but more limited changes in the abundance of TCA1. We conclude that in conditions where *de novo* biogenesis of cytochrome b_6f complexes is not required, a rapid drop in MCA1 exhausts the pool of *petA* transcripts, and the progressive loss of TCA1 further prevents translation of cytochrome *f*.

Chlamydomonas reinhardtii | chloroplast gene expression | cytochrome *f* biogenesis | nuclear control | trans-acting factors

Energy transduction in mitochondria or chloroplasts is performed by oligomeric proteins that comprise subunits of dual genetic origin. Due to extensive gene transfer from the organelle to the nucleus, only a subset of subunits is still organelle-encoded, whereas others are expressed in the nucleo-cytosol before being imported into organelles. Assembly of photosynthetic or respiratory protein complexes thus relies on a tight cooperation between different genomes. A cross-talk between these genetic compartments is certainly critical to cope with the drastic unbalance in their gene copy number: The nucleus contains one or few copies of each gene, whereas a plant cell may contain up to 10,000 copies of organelle genomes.

Studies of chloroplast proteins in the green alga *Chlamydomonas reinhardtii* or of mitochondrial proteins in the yeast *Saccharomyces cerevisiae* have disclosed unique traits in their biogenesis: Expression of organelle genes is regulated posttranscriptionally (1–7), mainly at translational or posttranslational steps (7, 8). Genetic analyses of nuclear mutants, defective for organelle gene expression, led to the identification of two major classes of nucleus-encoded transacting factors that target specifically one (or a few) organelle gene(s): Those required for the proper maturation and stabilization of specific organellar transcripts (2, 3) and those that behave as specific translational activators (1, 2, 4).

Numerous studies have focused on RNA/protein interactions governing the action of these nucleus-encoded factors that act most often on the 5′ UTR of their organelle mRNA targets. However, despite our increasing molecular knowledge, little is known about the physiological significance of these transacting factors. They could merely reflect the loss of genetic autonomy of the endosymbiont that now requires their constitutive expression as it requires that of nucleus-encoded ribosomal subunits. Alternatively, they may serve regulatory functions that remain to be determined. Whether variations in their abundance accompanies physiological changes that affect chloroplast gene expression, such as changes in nutrient availability (9), circadian rhythms (10, 11), or gametic differentiation (12), has not been addressed up to now.

Here, we took advantage of the genetic and molecular identification of two nucleus-encoded factors required for expression of the chloroplast *petA* gene encoding cytochrome (cyt.) *f* to investigate their physiological function in *C. reinhardtii*. MCA1, required for *petA* transcript stabilization (13, 14) has recently been cloned (C.L., S. Purton, N. J. Gumpel, J.G.-B., F.-A.W., and Y.C., unpublished work; AF330231), while we report here the molecular characterization of TCA1, required for cyt. *f* translation (15). Using tagged versions of *MCA1* and *TCA1*, we conducted a systematic study of the relationship between abundance of these specific transacting factors *in vivo* and expression of the chloroplast target (*petA*). We conclude that these nucleus-encoded factors are genuine regulators of the expression of the chloroplast *petA* gene.

Results

TCA1 Encodes a Pioneer Protein of 1,103 Residues. MCA1 has been cloned (C.L., S. Purton, N. J. Gumpel, J.G.-B., F.-A.W., and Y.C., unpublished work; AF330231). To complete the molecular characterization of nucleus-encoded factors required for *petA* expression, we cloned *TCA1*, as described in supporting information (SI) *Materials*. Experimental evidence demonstrating the

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The authors declare no conflict of interest.

Abbreviations: cyt., cytochrome; PPR, pentatricopeptide repeat.

Data deposition: The sequence of the *TCA1* cDNA has been deposited in the GenBank database (accession no. EF503563).

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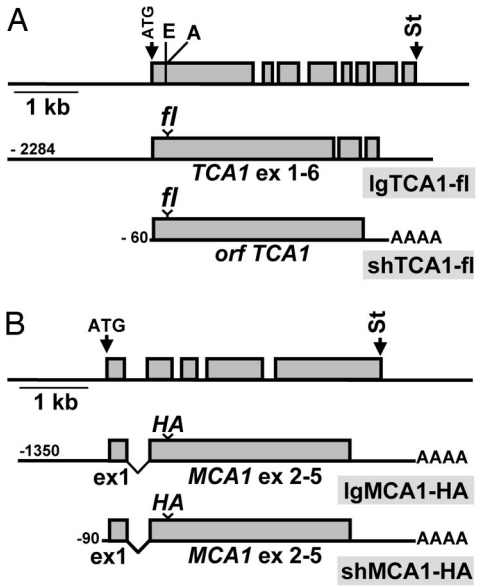

Fig. 1. Tagged versions of TCA1 (*A*) or MCA1 (*B*). In both *A* and *B*, the first line displays a schematic representation of the structure of the gene with initiation and stop codons. Exons are shown as gray boxes. The two following lines represent the constructs used for complementation of *tca1* (*A*) or *mca1* (*B*) mutant strains. The position of the tags with respect to the coding sequence is shown. Length of sequences upstream of the initiation codon (A is taken as +1) is indicated, as well as introns when retained in the construct. PolyA tail indicates constructs derived from a cDNA clone. (*A*) E and A refer to restriction sites EcoRI and AccI, between which the flag tag was inserted in frame.

actual cloning of *TCA1* came from the complementation of *tca1* mutants with genomic fragments or EST clones harboring *TCA1*, the characterization of the deletion in a tagged *tca1* mutant strain (tcal-8; *SI Materials* and SI Figs. 8 and 9), and the identification of mutations within the predicted *TCA1* gene from strains *tca1*-1 and -3 (SI Fig. 10). This gene codes for a protein of 1,103 residues. We found no consensus motifs or patterns that could shed light on the molecular function of the protein. BLAST searches against current databases failed to find orthologs of TCA1 except in *Volvox carterii*, a green alga closely related to *C. reinhardtii* (SI Fig. 11).

Subcellular Localization. Although MCA1 contains a putative N-terminal chloroplast targeting sequence, programs Predotar or ChloroP failed to find one in TCA1. To confirm the chloroplast localization of these factors, chloroplast fractions were prepared from cell wall-deficient strains expressing tagged versions of these proteins. We introduced a triple HA tag in the coding sequence of *MCA1* and a triple flag tag within that of *TCA1* (Fig. 1), at positions presumably neutral (see SI Fig. 12 for details). Tagged proteins, hereafter referred to as MCA1-HA and TCA1-fl, were still functional, because they were capable, upon transformation, to restore phototrophic growth of *mca1* or *tca1* mutants, respectively.

As shown on Fig. 2, both MCA1-HA and TCA1-fl were found in chloroplast fractions. These fractions were devoid of cytosolic proteins but contaminated by mitochondria, because the single large chloroplast of *Chlamydomonas* is tightly associated with several small mitochondria. To rule out a possible targeting of MCA1 and TCA1 to mitochondria, we isolated mitochondrial fractions that were slightly contaminated by chloroplasts, as indicated by a faint reaction against the nucleus-encoded stromal protein GRPE, whose presence was commensurate with traces of MCA1 and TCA1. The cytosolic marker UDP-glucose pyrophosphorylase (UGPase) was found in these fractions, presumably because this enzyme also localizes within Golgi and microsomes (16) that copurified with mitochondria (17). Because MCA1 and TCA1 are heavily detected in chloroplast enriched fractions but barely detectable in mitochondria-enriched fractions, we conclude that the two nucleus-encoded factors are targeted to chloroplast.

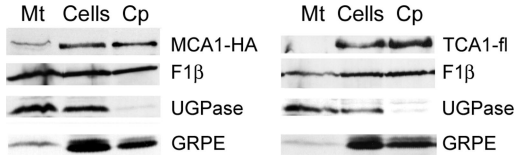

Fig. 2. MCA1 and TCA1 are targeted to the chloroplast. Whole cell (Cells), chloroplast (Cp), and mitochondrial (Mt) fractions, purified from cell wall-deficient strains expressing MCA1-HA and TCA1-fl respectively. UDP-glucose pyrophosphorylase (UGPase), subunit β of mitochondrial ATP synthase (F1β), and GRPE provide controls for fraction purity.

MCA1-HA Is Limiting for the Accumulation of *petA* Transcripts. The *petA* transcript does not accumulate in *mca1* mutants (13) in the absence of the MCA1 protein that protects its 5′ UTR from degradation (C.L., S. Purton, N. J. Gumpel, J.G.-B., F.-A.W., and Y.C., unpublished work). Using a tagged version of the protein, we determined how variations in MCA1 accumulation affect the expression of the *petA* gene in independent transformants. Complementation of *mca1* mutants occurs by random insertion of transforming DNA, resulting in various levels of expression of MCA1, depending on integration sites and, possibly, on the number of integrated copies of the tagged gene. To get a broader range of MCA1 expression, we used two constructs: a short construct (shMCA1-HA), whose expression depends on the strength of the promoter trapped upon insertion, and a longer one (lgMCA1-HA) including the promoter and 5′UTR of *MCA1* (Fig. 1*B*).

Fig. 3*A* shows the results obtained for a set of six clones transformed with construct lgMCA1-HA. As expected, contrasted accumulations of MCA1-HA were observed. The abundance of *petA* mRNA increased with increasing amounts of MCA1-HA. Using a larger set of 13 transformants that included clones transformed with construct shMCA1-HA and that covered a range of 6-fold variations in MCA1-HA, we plotted the *petA* mRNA dependence over MCA1-HA. The shape of the curve (Fig. 3*B*) suggests that MCA1 functions *in vivo* as a multimer. The curve could not be fully extended to the high-values region, probably because our constructs do not allow wild-type levels of expression of MCA1-HA. The strain that displayed the highest expression of MCA1-HA accumulated only 60% of the wild-type level of *petA* mRNA, but, nevertheless reached wild-type levels of cyt. *f*. All other transformants had lower expression of cyt. *f* (Fig. 3*C*). Typically, 20% of *petA* mRNA allowed >40% of the wild-type expression of cyt. *f* (SI Fig. 13), pointing to additional controls on the rate of production of cyt. *f* besides the mere concentration in *petA* mRNA.

Sensitivity of Cytochrome *f* to Changes in TCA1 Abundance. We assessed similarly the contribution of TCA1 in cyt. *f* translation. To maximize variations in TCA1-fl expression, we complemented *tca1* mutants with two constructs coding for a tagged version of TCA1 (Fig. 1*A*): the tagged cDNA clone (shTCA1-fl) and a larger construct containing upstream regulatory sequences and the last two introns of *TCA1* (lgTCA1-fl).

The set of clones transformed with construct shTCA1-fl accumulated from 10% to 80% of the wild-type cyt. *f* level (Fig. 4 *A* and squares in *B*), whereas all clones transformed with the lgTCAl-fl construct expressed high cyt. *f* levels (triangles in Fig. 4*B*). Data were gathered on a single plot. In exponentially growing cells, cyt.

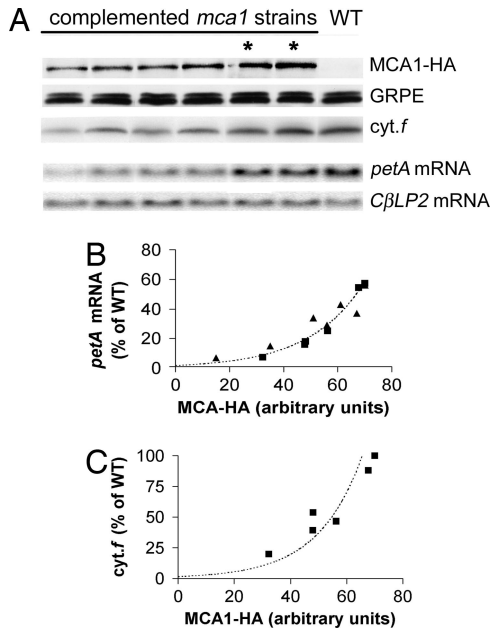

Fig. 3. MCA1 is rate-limiting for *petA* mRNA accumulation. (*A*) Accumulation of MCA1-HA, cyt. *f*, and GRPE (loading control) detected with specific antibodies in independent clones complemented with construct lgMCA1-HA. Accumulation of *petA* and *CβLP2* (loading control) mRNAs in the same strains is shown below. Asterisks indicate the clones used for experiments in Figs. 5–7 (strains *M-HA*). (*B*) Relationship between MCA1-HA abundance (arbitrary units) and *petA* mRNA accumulation (reported to that in the wild type). The graph combines data obtained from clones transformed with construct shMCA1-HA (triangles) and from clones presented in *A* (squares). (*C*) Relationship between MCA1-HA (arbitrary units) and cyt. *f* accumulation (percent of the wild-type level) in strains from *A*.

f is a stable protein (7, 18). Therefore, its accumulation level is a faithful measure of its rate of translation. TCA1-fl proved limiting for cyt. *f* synthesis with a roughly linear dependence for clones expressing medium or low levels of TCA1-fl. When reaching 60% of the wild-type level of cyt. *f*, the concentration of TCA1-fl became less critical for cyt. *f* expression as illustrated by the asymptotic

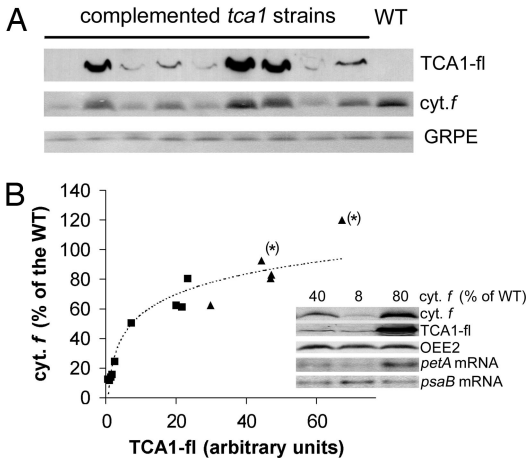

Fig. 4. TCA1 is rate-limiting for cyt. *f* accumulation. (*A*) Accumulation of TCA1-fl, cyt. *f* and GRPE (loading control) in independent clones transformed with construct shTCA1-fl. (*B*) Correlation between TCA1-fl abundance and cyt. *f* accumulation. Data are from clones from *A* (squares) and from clones transformed with lgTCA1-fl (triangles). Clones indicated by an asterisk were used for experiments on Figs. 5–7 (strains *T-fl*). (*Inset*) The recovery of *petA* mRNA in three representative complemented strains.

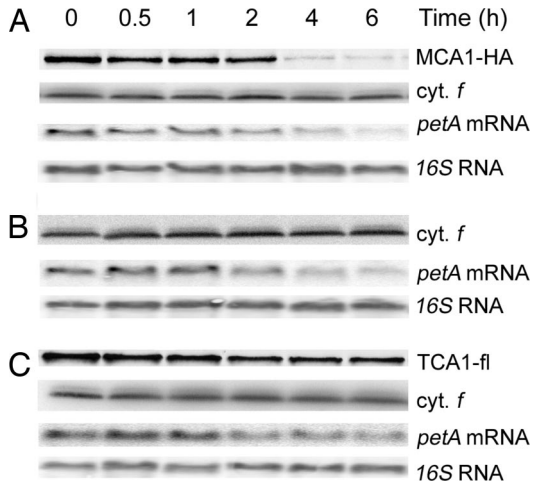

Fig. 5. Stability of MCA1-HA and TCA1-fl. Accumulation of MCA1-HA (*A*), TCA1-HA (*C*), cyt. *f* in *M-HA* (*A*), wild-type (*B*), or *T-fl* (*C*) strains treated with cycloheximide for the indicated times. Accumulation of *petA* mRNA and 16S rRNA (loading control) in the same strains is also shown.

evolution toward wild-type level. In the various transformants, the accumulation of *petA* mRNA was partly restored (Fig. 4*B Inset*), starting with only 20% of the wild-type level in the *tca1* mutant used as a recipient strain. We note however that the content in *petA* mRNA did not reach the wild-type level, ranging from 20% to 60%.

Half-Life of TCA1-fl and MCA1-HA. Thus, decreased accumulation of MCA1 or TCA1 actually decreases the expression of the *petA* gene. These two factors could serve a regulatory function *in vivo*, especially if they are short-lived enough to undergo rapid changes in abundance. We therefore followed the decay of MCA1-HA and TCA1-fl, together with that of cyt. *f* and *petA* mRNA, in transformants that were incubated with cycloheximide, an inhibitor of cytosolic translation. Wild-type cells were similarly treated as a control. As shown on Fig. 5*A*, the half-life of MCA1-HA was of ≈2 h after cycloheximide addition, and the protein became barely detectable after 4 h. Cyt. *f* showed no significant changes, because preexisting cyt. b_6f complexes are stable over this time period (18). In contrast, the *petA* transcript decreased in parallel with the loss in MCA1-HA, whereas 16S rRNA remained unaffected. These drops were not due to a destabilization of MCA1 by its HA-tag insertion because *petA* mRNA decays with exactly the same time course in wild type (Fig. 5*B*). Decay of MCA1-HA was not indirectly caused by a drop in *petA* mRNA because MCA1-HA accumulates normally in Δ*petA* strains (SI Fig. 14*A*). In contrast to MCA1-HA, TCA1-fl proved quite stable, because 80% of the protein was still present 8 h after cycloheximide addition (Fig. 5*C*).

Abundance of MCA1-HA and TCA1-fl Varies Depending on Physiological Conditions. We then addressed possible changes in abundance of MCA1 and TCA1 in different physiological conditions. Because such variations could result from transcriptional regulations, we used strains transformed with the largest *TCA1* or *MCA1* constructs that most likely preserved transcriptional regulatory sequences. In addition, we selected transformants, hereafter referred to as *M-HA* and *T-fl* (see legends of Figs. 3*A* and 4*B*), that showed an expression of the *petA* gene as close as possible to that observed in the wild type. All experiments were performed on two independent transformants that displayed identical behaviors.

Cyt. *f* is actively translated in exponentially growing cells that undergo *de novo* biogenesis of thylakoid membranes. It should

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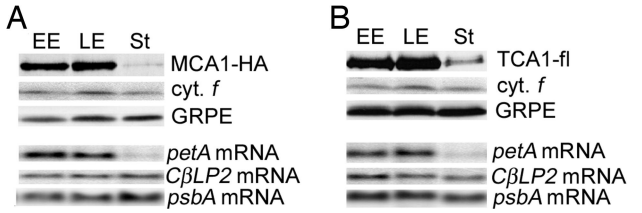

Fig. 6. Accumulation of MCA1-HA and TCA1-fl decreases during aging of cultures. Wild-type, *M-HA* (*A*), or *T-fl* (*B*) strains were inoculated at a density of 0.5×10^6 cells per ml and grown up to 5 days. Abundance of MCA1-HA (*A*), TCA1-fl (*B*), and cyt. *f* proteins and of *petA* and *psbA* transcripts were monitored at early exponential (EE) ($1–2 \times 10^6$ cells per ml), late exponential (LE) ($4–5 \times 10^6$ cells per ml) and stationary (St) ($6–7 \times 10^6$ cells per ml) phases of growth. GRPE and *CβLP2* provide the respective loading controls.

be poorly expressed in nondividing cells, i.e., in stationary phase, when *de novo* biogenesis is not required. We therefore studied the accumulation of TCA1-fl and MCA1-HA together with the expression of *petA* in cultures of wild-type, *M-HA* or *T-fl* strains grown up to 5 days. Cells were counted daily, and aliquots were harvested for RNA and protein extraction at early exponential (EE), late exponential (LE), and stationary (St) phase of growth. Cyt. *f* remained stable, even after cells reached the stationary phase in wild-type (data not shown) and complemented strains (Fig. 6). By contrast, *petA* transcripts decreased dramatically, up to 10-fold, when cells entered the stationary phase. This drop was not due to a general decay in chloroplast transcripts because *psbA* mRNA remained unaffected (Fig. 6). The loss of *petA* transcripts correlated with loss in MCA1-HA (Fig. 6*A*), consistent with its role in mRNA stabilization. TCA1-fl also showed much reduced abundance in stationary cultures, which was not due to the decrease in *petA* mRNA abundance because TCA1-fl accumulation is not altered in Δ*petA* strains (SI Fig. 14*B*). Thus, nucleus-encoded factors required for *petA* gene expression were turned off in nondividing cells. MCA1-HA was rapidly restored after dilution of the culture with fresh medium (it was significantly detected after 30 min), whereas recovery of TCA1-fl was slower (SI Fig. 15).

Previous studies have shown that cyt. *f* expression in *Chlamydomonas* is tightly controlled by nitrogen availability: The abundance of *petA* mRNA decreases with a concomitant degradation of cyt. b_6f complex upon nitrogen starvation (9). Wild-type, *M-HA*, and *T-fl* cells were cultured in nitrogen-deficient medium for up to 5 days and then transferred to nitrogen-repleted medium for several hours. We note that the kinetics of losses in cyt. *f* were slightly different in the three strains. They proved highly sensitive to the physiological conditions of the cell cultures and to the genetic background of the strains. Maximal loss in cyt. *f* was obtained after 96 h of nitrogen starvation for the *M-HA* strain and the wild type and after 120 h for the *T-fl* strain (Fig. 7). The original content in cyt. *f* was fully restored 24 h after N-repletion. As shown on Fig. 7*A*, changes in the level of *petA* mRNA strictly paralleled changes in the level of MCA1-HA. They were both dramatically reduced after 48 h of N-starvation but largely restored within the first hour of N-repletion. We note that MCA1-HA and *petA* mRNA subsequently decayed during restoration of the full cyt. *f* complement. The same behavior was observed for *petA* mRNA in the wild type (Fig. 7*B*). Whereas TCA1-fl also dropped dramatically upon 120 h of N-starvation in conditions where cyt. *f* was severely decreased, it required up to 6 h of N-repletion to regain its original level, i.e., a longer time than for the restoration of MCA1-HA and *petA* mRNA levels. Cyt. *f* restoration displayed an even longer lag, illustrating the dependency of *petA* translation upon TCA1 accumulation and the time required for the chloroplast to accumulate its original level of cyt. *f* once its translation capability has been restored.

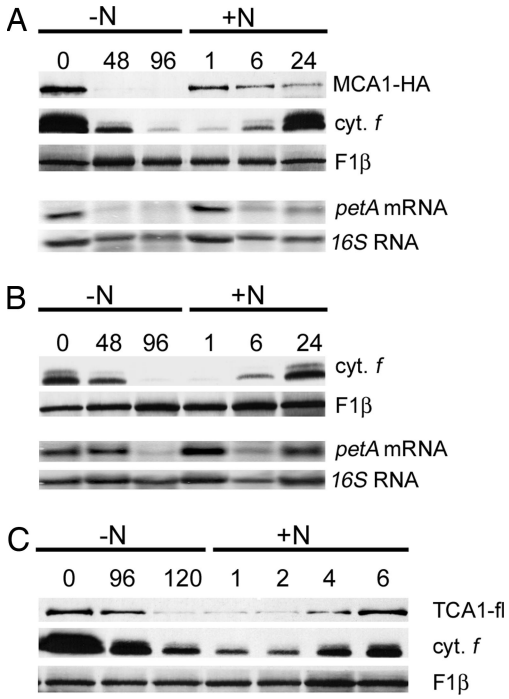

Fig. 7. MCA1-HA and TCA1-fl are reversibly down-regulated during nitrogen starvation. Accumulation of MCA1-HA (*A*), TCA1-fl (*B*), cyt. *f*, and F1β (loading control) in *M-HA* (*A*), wild-type (*B*), and *T-fl* (*C*) cells subjected to nitrogen starvation (−N) followed by repletion (+N) for the indicated times (in hours). Protein samples were loaded on an equal cell number basis. Accumulation of *petA* and 16S (as a loading control) transcripts in the same cells is also shown.

This loss of the two nucleus-encoded factors involved in cyt. *f* expression was selective, because NAC2, another nucleus-encoded factor required for *psbD* mRNA stabilization remained largely unaffected in the same conditions (data not shown).

Together, these experiments demonstrate that physiological changes in the abundance of MCA1 and TCA1 actually control the expression of the *petA* gene *in vivo*.

Discussion

A growing number of nucleus-encoded factors controlling post-transcriptional steps of chloroplast gene expression have been cloned during the past decade (2, 4–6). Remarkably, in most cases, these factors either stand as pioneer proteins (19, 20) or belong to protein families identified only by sequence motifs involved in protein/protein [tetratricopeptide repeat (TPR) motifs] (21, 22) or protein/RNA [pentatricopeptide repeat (PPR) motifs] interactions (23–26). The nucleus-encoded factors examined in the present study combine these traits because MCA1, required for *petA* mRNA stabilization, is a PPR protein (C.L., S. Purton, N. J. Gumpel, J.G.-B., F.-A.W., and Y.C., unpublished work) whereas TCA1, the specific translation factor (15), whose sequence identification is presented here, is a pioneer protein. BLAST search identified a single *TCA1* ortholog in another Chlamydomonadale: *V. carterii*. Another *petA*-specific translational activator, identified in maize (23), is a PPR protein and shows no similarity with TCA1. Nuclear control on chloroplast gene expression, although widespread in the plant kingdom, may rely on diverse proteins in the different lineages. In some cases, however, we cannot exclude that homologous nucleus-encoded regulatory factors may no longer be recognized due to their rapid evolution, as suggested by the marked divergence between TCA1 sequences in *C. reinhardtii* and *V. carterii*.

Decreased Levels in MCA1 and TCA1 Deeply Affect *petA* Expression. Our transformation strategy allowed us to decrease expression of MCA1 or TCA1 over an order of magnitude, even if a comparison with the wild-type level is lacking. The tagged versions of the nucleus-encoded factors used in this study are unlikely to modify their original properties: The half-life of *petA* mRNA upon inhibition of their synthesis or the kinetics of changes in *petA* gene expression in various physiological conditions were well preserved in transformants *M-HA* and *T-fl*, when compared with the wild type, even though there were small variations between the three strains in the kinetics of cyt. *f* and *petA* mRNA losses upon N-starvation. Because we explored a wide range of TCA1 and MCA1 concentrations below those in the wild type, as deduced from the levels of *petA* mRNA and/or cyt. *f* in the transformants, we could determine the major features of the dependence of *petA* gene expression on the concentration of these factors. For the lowest levels of *petA* mRNA accumulation (<20% of the wild-type level), we observed a parabolic dependence of the transcript level over the concentration in MCA1 that suggests a cooperative action. Another PPR protein required for transcript processing/stabilization in the chloroplast of *Arabidopsis*, HCF152, likely functions as a homodimer (26). Several nucleus-encoded factors stabilizing chloroplast mRNAs in *Chlamydomonas* belong to high molecular weight complexes (21, 22), but their copy number within these complexes remains unknown. In the range of 20% to 60% accumulation of the wild-type content in *petA* mRNA, the increases in transcript and MCA1-HA abundance were roughly proportional. Because MCA1-HA and *petA* mRNA showed identical variations upon physiological changes (see below), we conclude that, in most conditions, MCA1 is limiting for *petA* mRNA stabilization. This behavior supports a long lasting interaction of MCA1 with its mRNA target rather than a catalytic role in the conversion of *petA* transcripts from an unstable to a stable form.

At <60% of the wild-type cyt. *f* level, the accumulation of TCA1-fl was strictly limiting for cyt. *f* expression: Increase in cyt. *f* was roughly proportional to that of TCA1-fl. However, it showed saturation features at higher expression levels, consistent with our finding that cyt. *f* translation is limited by its rate of assembly within cyt. b_6f complexes (27). In the yeast *S. cerevisiae* also, Pet111p is limiting for *cox2* mRNA translation, but its overexpression increased accumulation of COX2p (28), presumably because the rate of COX2p synthesis is not limited by its assembly in cyt.-oxidase (29).

In *mca1*- and *tca1*-complemented cells, 60% of the wild-type level of *petA* mRNA allowed wild-type expression of cyt. *f*. Approximately 30% of the transcripts still allowed up to 60% of wild-type cyt. *f* expression. Thus, in the wild type, MCA1 stabilizes *petA* mRNA in excess of what is needed for cyt. *f* translation. A somewhat larger excess was reported previously upon treatment with rifampicin (8), which probably preserved transiently the pool of nucleus-encoded factors.

The Level of MCA1 and TCA1 Varies with Physiological Conditions. A regulatory role of nucleus-encoded factors has been suggested for the light regulation of *psbA* gene expression in *Chlamydomonas*. Translation of the *psbA* mRNA is activated by a multimeric protein complex whose binding to the 5′ UTR depends on the redox and energy state of the chloroplast (reviewed in ref. 30). Regulation of *psbA* expression would rely on modulations of the activity of this complex rather than of its abundance. It is still unclear, however, whether this complex is a genuine and specific activator of *psbA* expression or whether it plays a more general role in chloroplast translation. In *Arabidopsis*, expression of ATAB2, a putative translational activator of some reaction center subunits, is induced by light and controlled by photoreceptors (31). In the yeast *S. cerevisiae*, expression of PET494p, a nucleus-encoded specific translational activator of the *COXIII* mRNA, is reduced 5-fold under anaerobiosis and shows even higher variations, depending on the carbon source (32). These observations argue for a regulatory role of these proteins through changes in their abundance. Unfortunately, variations in the expression of their organelle-encoded targets were not investigated in these studies.

Here, we observed spectacular changes in the accumulation of MCA1 and TCA1 in physiological conditions where the cell demand for *de novo* synthesis of cyt. *f* changes. In aging cultures, where cyt. *f* expression is no longer required as cells stop growing, a drop in both factors is accompanied by a loss in *petA* mRNA. Similarly, losses and gains in MCA1 mimic changes in *petA* mRNA upon N-starvation followed by N-repletion. Thus, changes in the abundance of MCA1 rapidly regulate *petA* mRNA accumulation. When cyt. b_6f complexes are actively degraded in the absence of nitrogen and resynthesized after repletion (12), the translational activator for cyt. *f*, TCA1, shows similar changes. Despite the fast recovery of MCA1 and *petA* mRNA (less than 1 h), which is rapid compared with a doubling time of >8 h, the slow reconstitution of the pool of TCA1 upon N-repletion explains the lag in cyt. *f* restoration, supporting its regulatory function.

Together, these results demonstrate that MCA1 and TCA1 superimpose layers of regulation on the physiology-dependent expression of the *petA* gene.

Major Traits for the Nuclear Regulation of Cytochrome *f* Expression. Previous studies have established that chloroplast transcription is not limiting for transcript accumulation (8, 33). Here, we show that the amount of MCA1 determines the fraction of transcribed *petA* mRNAs that are stabilized. Part of these stable *petA* mRNAs are selected for translation depending on the amount of TCA1 available in the cells.

MCA1 being short-lived, its abundance varies widely upon environmental and metabolic changes. When *de novo* biogenesis of the cyt. b_6f complex is not required, as shown here for aging cultures or changes in nitrogen availability, it appears as a major regulator of cyt. *f* expression by preventing accumulation of *petA* mRNA.

TCA1 responds less rapidly to physiological changes. Its regulatory role should rather be found in its probable participation in the CES behavior of cyt. *f*. Control by epistasy of synthesis (CES) is a translational regulation dependent on protein assembly that governs the stoichiometric accumulation of the various subunits of a same protein complex (7). Unassembled cyt. *f* down-regulates its translation (18, 27), most probably by trapping TCA1 by way of its C-terminal regulatory region (34). In *tca1* transformants displaying high expression of cyt. *f* (> 50% of the wild-type level), large changes in TCA1 levels have limited effects on cyt. *f* expression, because unassembled cyt. *f* would bind any excess TCA1.

Thus, the short-lived MCA1, through rapid changes in its expression, is most likely responsible for an arrest of cyt. *f* expression when *de novo* biogenesis of cyt. b_6f is not required, whereas TCA1 participates in the fine tuning of the stoichiometric production of cyt. *f* during cyt. b_6f assembly.

Experimental Procedures

Media, Culture Conditions, and Strains. Wild-type (derived from 137C), mutant and complemented strains of *C. reinhardtii* were grown in Tris-acetate-phosphate (TAP) medium, pH 7.2 (35), under continuous light (5–10 $\mu E \cdot m^{-2} \cdot s^{-1}$; E = 1 mol of photons) on a rotatory shaker (120 rpm). Strains *tca1*-1, *tca1*-8 (SI Fig. 8), and *mca1*-2 were used as recipient strains for nuclear transformation experiments, as described in SI Table 1. When required, cell wall deficient or arginine auxotroph derivatives were obtained from crosses with appropriate mutant strains and screening of progeny for the desired phenotypes.

Nitrogen starvation was performed by culturing cells (inocu-

lated at 0.25×10^6 cells per ml) in N10 medium (35), whereas repletion was done by resuspending cells harvested by centrifugation in Tris-acetate-phosphate (TAP) medium.

Constructs and Nucleic Acids Manipulations. Standard nucleic acid manipulations were performed according to ref. 36. DNA constructs, detailed in the *SI Materials*, were sequenced before transformation in *C. reinhardtii*. Primers used in this study are listed in SI Table 2. RNA extractions and RNA blot analyses were carried out as described in ref. 37. RNA accumulations were normalized to that of the *CβLP*2 transcript, which is poorly sensitive to physiological conditions (38) or 16S rRNA.

Transformation Experiment. Nuclear transformation was performed by electroporation, as described in ref. 39, by using the following parameters: 10μF/1,800 V·cm^{-1} for cell wall-deficient strains and 25μF/2,500–3,000 V·cm^{-1} for strains with cell wall. Transformants were selected for phototrophy on minimum medium (35) under high light (200 μE·m^{-2}·s^{-1}).

Chloroplast and Mitochondria Preparation. Chloroplast and mitochondria were isolated from 2 liters of synchronized cultures (12 h light, 12 h dark: six cycles). Cells were harvested, resuspended in 30 ml of NEB1 buffer (0.3 M sorbitol/40 mM Hepes-KOH, pH 7.2/5 mM $MgCl_2$) supplemented with protease inhibitors (Roche, Indianapolis, IN) and broken in a nebulizer (18 psi). Cell lysates were centrifuged at $2,000 \times g$ for 10 min. Mitochondria were purified from the supernatant as described in ref. 17. Pellet, resuspended in 40% Percoll-NEB1, was overlaid on a continuous 80–40% Percoll-NEB1 gradient. After 30 min of centrifugation at $3,000 \times g$, chloroplasts were collected, diluted in NEB1, spun down at $1,100 \times g$, and resuspended in 0.1M DTT/Na_2CO_3 for gel analysis.

Protein Preparation, Separation, and Analysis. Protein isolation, separation and immunoblot analysis were carried out on exponentially growing cells (2×10^6 cells per ml^{-1}), as described in ref. 18. Cell extracts were loaded on an equal chlorophyll basis, unless otherwise specified. Cyt. *f* accumulation (after normalization to that of the OEE2 protein from PSII) was quantified according to ref. 34. TCA1-f1 and MCA1-HA were detected by ECL using monoclonal antibodies [anti-flag M2 (Sigma, St. Louis, MO) and anti HA.11 (Covance, Princeton, NJ)] and HRP-conjugated antibody against mouse IgG (Promega, Madison, WI). Their accumulation (normalized to that of GRPE) was quantified from scanned films by using the program ImageQuant (Molecular Dynamics, Sunnyvale, CA). To test the correlation between signal intensity and protein abundance, signal intensity was plotted against protein concentration for serial dilutions of protein extracts. A linear correlation was observed in all experiments (SI Fig. 16). Quantification of *petA* mRNA, cyt. *f*, and MCA1-HA or TCA1-f1 were repeated three times on a subset of four *mca1*-complemented clones and three *tca1*-complemented clones: Variations remained <15% of the obtained value.

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