

## Molecular Identification and Function of *cis*- and *trans*-Acting Determinants for *petA* Transcript Stability in *Chlamydomonas reinhardtii* Chloroplasts<sup>∇†</sup>

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**In organelles, the posttranscriptional steps of gene expression are tightly controlled by nucleus-encoded factors, most often acting in a gene-specific manner. Despite the molecular identification of a growing number of factors, their mode of action remains largely unknown. In the green alga *Chlamydomonas reinhardtii*, expression of the chloroplast *petA* gene, which codes for cytochrome *f*, depends on two specific nucleus-encoded factors. MCA1 controls the accumulation of the transcript, while TCA1 is required for its translation. We report here the cloning of MCA1, the first pentatricopeptide repeat protein functionally identified in this organism. By chloroplast transformation with modified *petA* genes, we investigated the function of MCA1 *in vivo*. We demonstrate that MCA1 acts on the very first 21 nucleotides of the *petA* 5' untranslated region to protect the whole transcript from 5'→3' degradation but does not process the 5' end of the *petA* mRNA. MCA1 and TCA1 recognize adjacent targets and probably interact together for efficient expression of *petA* mRNA. MCA1, although not strictly required for translation, shows features of a translational enhancer, presumably by assisting the binding of TCA1 to its own target. Conversely, TCA1 participates to the full stabilization of the transcript through its interaction with MCA1.**

Organelle genomes have retained a limited set of genes from their prokaryotic ancestor, the expression of which is tightly controlled by the nucleus. Within organelles, mRNAs may undergo *cis* or *trans* splicing, editing, endo- and exonucleolytic cleavage, and 5'- and 3'-end processing. Their stabilization, translation, and degradation are highly regulated (reviewed in references 3, 5, 23, 26, and 63). Each of these posttranscriptional steps depends on nucleus-encoded factors. Strikingly, most are gene specific, one factor being required for the expression of one or a few organelle mRNAs. Altogether, several hundreds of nucleus-encoded factors may be required for the proper expression of the organelle genome (3, 65).

The green alga *Chlamydomonas reinhardtii* and the yeast *Saccharomyces cerevisiae* have been instrumental in the identification of these factors. Genetic analyses of nuclear mutants, defective for the expression of single organelle genes, have emphasized the existence of two major classes of *trans*-acting factors. Some are required for the proper maturation and stabilization of specific organellar transcripts (reviewed in references 3, 44, and 48), while others are required for their translation (reviewed in references 3, 23, and 78). Among the numerous nucleus-encoded factors involved in the stabilization of specific chloroplast transcripts identified in *Chlamydomonas* (14, 16, 28, 33, 35, 36, 39, 45, 66), only three have been cloned up to now—NAC2, MCD1, and MBB1—that, respectively,

control *psbD*, *petD*, and *psbB* transcript stability (6, 46, 71). They act on the 5' untranslated regions (5'UTRs) of their target transcripts and protect them from 5'→3' ribonucleolytic degradation (14, 50, 71), but the molecular basis for their specific RNA recognition is still poorly understood. MBB1 and NAC2 promote the cleavage of a precursor form to generate the stable, mature form of their target mRNA (49, 50, 72). A 5'-end cleavage is also involved in the formation of *petD* mRNA (58, 70). Understanding whether these 5' processing events are mandatory for the production of stable and translatable mRNAs in the chloroplast is critical for our knowledge of the basic rules of chloroplast gene expression. Furthermore, involvement of MBB1, NAC2, and MCD1 in the translation of their target mRNA has been hypothesized (15, 49, 72) but has proved difficult to test since template mRNAs fail to accumulate in the absence of these specific stabilization factors.

Here, in an attempt to address these issues, we studied the expression of the chloroplast *petA* gene, encoding cytochrome *f*, a major subunit of the cytochrome *b<sub>6</sub>f* complex. Previously, we characterized nuclear mutant strains of *C. reinhardtii* that accumulated the *petA* mRNA but did not synthesize cytochrome *f* (76). We subsequently cloned the *TCA1* gene coding for a *petA*-specific translational activator (53). In the present study we focus on another set of nuclear mutants that are defective for the accumulation of the *petA* mRNA. We present the molecular characterization of MCA1, the nucleus-encoded factor responsible for *petA* mRNA stabilization. *MCA1* turned out to code for the first pentatricopeptide repeat (PPR) protein functionally characterized in *Chlamydomonas*. Using chimeric versions of the *petA* gene, we mapped the nucleotide target of MCA1 to the very 5' end of the *petA* mRNA and demonstrate that the most upstream 21-nucleotide motif is

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TABLE 1. Oligonucleotides used in this study

| Oligonucleotide     | Sequence (5'→3') <sup>a</sup>   | Restriction site |
|---------------------|---|------------------|
| petA <sub>cod</sub> | CGGAGATCTTCCATCGATGAACATGCTTTATTGCTAAAAAAGA   |                  |
| petA <sub>rev</sub> | ACAGCTTGTGGTACTTCGATTTCAACTGCTTTTTGC  |                  |
| pG <sub>cod</sub>   | ATTTGGGGGGGGGGGGGGGGGGAAATAGTAATTATGAATGTAAATTACTIONTATGCTTACT                        |                  |
| pG <sub>rev</sub>   | ATTTCCCCCCCCCCCCCCCCCAAAATTTTATTTTTTTTCTTCTCAACATATATTATATCT                          |                  |
| DA <sub>cod</sub>   | ATTTTTAGCATGTAAACATTAGAAATACAGCAAGTAATTATGAATGTAAATTACTIONTATGCTTACT                  |                  |
| DA <sub>rev</sub>   | TGCTGTATTTCTAATGTTTACATGCTAAAAATTTTTAATATATTATATATCTTTTTTTTAGCAAA<br>TAAAGCATAGTTCATG |                  |
| del <sub>cod</sub>  | CGCCTGCAGCTAGAATAAACTTGTGAGGCTGCTTA   | PstI             |
| del <sub>rev</sub>  | GCGCTGCAGAAATTTATTTTTTTTCTTCTCAACATA  | PstI             |
| petD <sub>cod</sub> | CGCCTAGGACTCACTAAAATTCATTTGCCCGAAGGGACGTCCA   | HindIII          |
| petD <sub>rev</sub> | CGTTAGGCCAAGCAGGTTACCGTAAAGTG   |                  |
| AD <sub>cod</sub>   | GATATAATATATGTTGAGAAGAAAAAATAAAATTTAAATAGTAATTGGAGTAAAAAGAAAA<br>ATAT                 |                  |
| AD <sub>rev</sub>   | TATTTTTTTTCTTCTCAACATATATTATATCTTTTTTTTAGCATTATATATTTTTGTGCGGGATTTA                   |                  |
| RevpetA             | TGCTGCGGTAAAGTAGTAAATACTTGGTT   |                  |
| RevpsaB             | CAGGTGCAAAGCAAACCTTGCATAC   |                  |

<sup>a</sup> Restriction sites are indicated by underscoring.

sufficient to confer MCA1-dependent stability to an unrelated mRNA. Finally, we provide evidence for an enhancer function of MCA1 in translation.

#### MATERIALS AND METHODS

**Strains and growth conditions.** Wild-type, mutant, and transformed strains of *C. reinhardtii* were grown in Tris-acetate-phosphate (TAP) medium (pH 7.2) (29) under continuous low light (5 to 10  $\mu\text{E m}^{-2} \text{s}^{-1}$ ).

*mca1* mutant strains were isolated either by UV mutagenesis (CF-792, CF-152, CF-6, and CF-813 hereafter referred to as the *mca1-2*, *mca1-3*, *mca1-4*, and *mca1-5* strains) (24) or insertional mutagenesis (strain *mca1-1*, previously called MΦ11 in reference 28). Cell-wall-less *mca1* strains were obtained by crossing the *mca1-1* and *mca1-2* strains with mutant strain *cw15*. The nuclear mutant *med1-1* (14, 46), *tca1-1*, *tca1-2*, and *tca1-8* (53, 76) strains, as well as the  $\Delta\text{petA}$  strain, a chloroplast deletion strain (37), were also used in the present study.

**Plasmid constructions.** (i) **Construction of modified *petA* and *petD* genes.** Plasmid pF5mut2 was created by cloning the 841-bp AatII-BstEII fragment from plasmid pWF (37) into vector pBKS, digested by SacI and XhoI, both fragments first blunt ended by treatment with Klenow and T4 DNA polymerase.

To delete nucleotides 22 to 63 from the *petA* 5'UTR, a PCR product was amplified with primers del<sub>cod</sub> and del<sub>rev</sub> from template plasmid pF5mut2 (see Table 1 for the sequence of the primers used in the present study), digested with PstI (a unique restriction site introduced in primers [Table 1]), and religated on itself to yield plasmid pF5mut2-del. The 364-bp BglII-HindIII fragment excised from this plasmid was cloned into plasmid pWF digested with the same enzymes to create plasmid pWF-del.

*pG-petA* construct was created by a two-step PCR procedure (31). Two pairs of primers (petA<sub>cod</sub>/pG<sub>rev</sub> and pG<sub>cod</sub>/petA<sub>rev</sub>) allowed amplification from template plasmid pWF of two partially overlapping fragments that were mixed and used as templates in a third PCR with the external primers petA<sub>cod</sub> and petA<sub>rev</sub>. The final amplicon, carrying the poly(G) tract, was digested with BglII and HindIII, two restriction sites on both sides of the mutation, and cloned into plasmid pWF digested with the same enzymes to create plasmid pWF-pG.

Plasmid pWF-DA carrying the modified *petA* gene, *D-petA* (described in Fig. 6A), was similarly created by using petA<sub>cod</sub>/DA<sub>rev</sub> and DA<sub>cod</sub>/petA<sub>rev</sub> primer pairs.

In the three plasmids pWF-del, pWF-pG, and pWF-DA, the SwaI site present in *petA* 5'UTR was destroyed, creating a restriction fragment length polymorphism (RFLP) marker used to assess the homoplasmy of transformed strains.

Plasmid pWQ-AD carrying a modified *petD* gene (*A-petD*, see Fig. 6A) was similarly constructed from template plasmid pWQ, encompassing the *petD* gene (37), using the two-step PCR strategy with the primer pairs petD<sub>cod</sub>-AD<sub>rev</sub> and AD<sub>cod</sub>-petD<sub>rev</sub>. The 780-bp amplicon resulting from the third PCR was digested with HindIII and AvrII (a restriction site introduced within the petD<sub>cod</sub> primer) and cloned into vector pWQ digested by the same enzymes to create plasmid pWQ-AD. In addition to the mutation introduced in the *petD* 5'UTR, this plasmid contained a 381-bp deletion downstream of AvrII. A control plasmid

pWQ- $\Delta\text{AvrII}$ , bearing the same deletion but no modification in the *petD* gene, was constructed by cloning the PCR product amplified with the primers petD<sub>cod</sub> and petD<sub>rev</sub> from plasmid pWQ into the vector pWQ, both being digested with HindIII and AvrII.

Plasmids pWF-pG, pWF-DA, and pWQ-AD were sequenced to confirm the presence of the appropriate mutations. The 5'*psaA-aadA* cassette (77), conferring resistance to spectinomycin, was then introduced into the unique HincII site of plasmids pWF-del, pWF-pG, and pWF-DA or into the unique AvrII site from plasmids pWQ-AD and pWQ- $\Delta\text{AvrII}$ , in reverse orientation with respect to *petA* or *petD* genes, to yield plasmids *paAKWF-del*, *paAKWF-pG*, and *paAKWF-DA* or plasmids *paAKWQ-AD* and *paAKWQ- $\Delta\text{AvrII}$* , respectively.

(ii) **Construction of a chimeric *psbB* gene.** Plasmid *pcA-CP47*, containing the chimeric gene 5'*petA-psbB*, was constructed by cloning a 695-bp DNA fragment containing the *petA* promoter and 5'UTR regions, obtained by digestion of plasmid p5F (11) with NcoI and EcoRV, into vector p38.Nco (72), digested with NcoI and StuI. The recycling *aadA* cassette (21) was then introduced into the unique restriction site AvrII of plasmid *pcA-CP47* filled in with Klenow, in opposite orientation with respect to the *psbB* gene, to yield plasmid *pK'cA-CP47*.

**Transformation experiments.** Chloroplast transformations were performed by tungsten particle bombardment (7) as described earlier (37). Transformants were selected either on TAP medium supplemented with spectinomycin (100  $\mu\text{g ml}^{-1}$ ) under low light (5 to 6  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) or on minimal medium under moderate light (100  $\mu\text{E m}^{-2} \text{s}^{-1}$ ). They were then subcloned on selective medium until they reached homoplasmy. At least three independent transformants were analyzed for each transformation.

Proper insertion of transforming DNA and homoplasmy were checked by RFLP analysis of PCR products encompassing the *petA* 5'UTR, amplified with primers petA<sub>cod</sub> and petA<sub>rev</sub>, since all chimeric *petA* genes used in that study lacked the SwaI site present in the regular 5'UTR or led to amplicons of different size depending on whether the template contained wild-type or chimeric *petA* genes.

Nuclear transformation was performed by electroporation, as described previously (64), using the following parameters: 25  $\mu\text{F}/720 \text{ V cm}^{-1}$ . Transformants were selected for phototrophy on minimal medium (29) under high light (200  $\mu\text{E m}^{-2} \text{s}^{-1}$ ).

**RNA isolation and analysis.** RNA extraction and Northern blot analysis were performed as described previously (17) with probes derived from coding sequences (18).

For primer extension analysis, 15  $\mu\text{g}$  of total RNA, mixed with 1.5 pmol of the <sup>33</sup>P-labeled 5'-phosphorylated oligonucleotides RevpetA and RevpsaB, were denatured at 75°C for 4 min and rapidly cooled down in ethanol-dry ice. Reverse transcription was performed at 54°C for 30 min with avian myeloblastosis virus reverse transcriptase (Finzyme). After LiCl precipitation, half the reaction was run on 5% sequencing gel alongside a sequencing reaction of pWF.

Discrimination between transcription start sites and processing sites of precursor RNAs was done by RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE), using a Generacer kit (Invitrogen) according to the supplier's protocol, except that we omitted the removal of free 5'-phosphates by calf

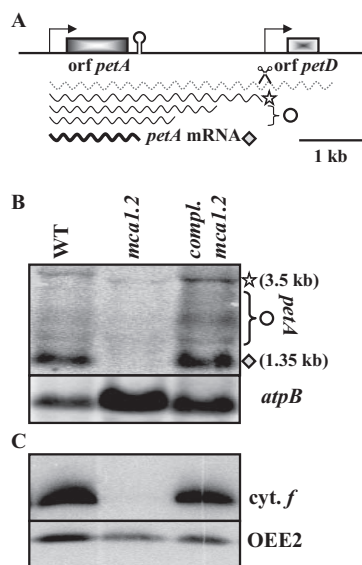


FIG. 1. *mca1* mutant strains lack stable accumulation of *petA* mRNA. (A) Schematic map of the *petA-petD* transcription unit. At the top is shown the relevant chloroplast genomic region, with the transcripts shown below. Bent arrows indicate transcription start sites. The dashed wavy line depicts the transient *petA-petD* cotranscript. This cotranscript is not observed in the wild type (WT, panel B) because of its rapid processing (58, 70). Symbols (diamond, star, and circle) refer to the transcripts visible in panel B. (B and C) Accumulation of *petA* mRNA (B) and cytochrome *f* (C) in wild-type and *mca1-2* strains and in an *mca1-2 cw15* strain complemented by transformation with a *MCA1* cDNA clone. Accumulations of *atpB* mRNA and of the OEE2 subunit from Photosystem II provide the respective loading controls. Sizes of the best-characterized *petA* transcripts are indicated on the right side of panel B.

intestine alkaline phosphatase. RLM-RACE was performed on 5  $\mu$ g of total RNA from wild-type *Chlamydomonas*, pretreated or not with tobacco acid pyrophosphatase. Reverse transcription was done with oligonucleotide RevpetA, and the subsequent PCR amplification was performed using the oligonucleotides RevpetA and Generacer 5' as primers. PCR products were analyzed on agarose gels and scanned for ethidium bromide fluorescence on a Typhoon Trio+ (GE Healthcare Life Sciences).

**Protein isolation and analysis.** Protein isolation, separation, and immunoblot analysis were carried out on exponentially growing cells ( $2 \times 10^6$  cells  $\text{ml}^{-1}$ ) as described previously (37). Cell extracts were loaded on an equal chlorophyll basis. Cytochrome *f* accumulation, normalized to that of the oxygen evolving enhancer 2 (OEE2) subunit from Photosystem II as an internal standard, was quantified from PhosphorImager scans of immunoblots revealed with  $^{125}\text{I}$ -labeled protein A, using ImageQuant software (Molecular Dynamics, Sunnyvale, CA), as described previously (11).

## RESULTS

**Stabilization of *petA* mRNA is impaired in *mca1* mutants.** In *Chlamydomonas*, *petA* is the first cistron of a dicistronic unit (Fig. 1A) that also includes the *petD* gene, encoding subunit IV from the cytochrome *b<sub>6</sub>f* complex (70). The *petA* transcription pattern, schematized in Fig. 1A, can be observed in Fig. 1B. Large transcripts corresponding to processing intermediates of the *petA-petD* transcription unit (55) are clearly visible, together with the shorter mature *petA* mRNA in the wild-type lane.

Previously, we isolated several mutants of *C. reinhardtii* that were incapable of phototrophic growth because they lacked

stable accumulation of the *petA* mRNA (24, 28). As can be seen in Fig. 1B, where we overloaded the mutant lane with respect to the control, the *petA* mature mRNA is fully missing in the mutant *mca1-2* strain, while trace amounts of larger *petA*-containing transcripts are still slightly visible. Accumulation of the *petD* mRNA is not affected (data not shown).

Genetic analysis demonstrated that all mutations are nuclear, recessive and allelic (see Table S1 in the supplemental material). Two strains were chosen for further study: M $\Phi$ 11, isolated after insertional mutagenesis using a modified *ARG7* cassette (28), now referred to as the *mca1-1* strain, and CF792, generated by UV mutagenesis (24), referred to here as the *mca1-2* strain. Since the *mca1-1* strain still showed active transcription of *petA* (28), its failure to accumulate the transcript was attributed to increased nucleolytic degradation. The gene mutated in those strains was thus called *MCA1* (for maturation/stabilization of cytochrome *b<sub>6</sub>f* complex *petA* mRNA).

***MCA1* encodes a PPR protein.** As we reported previously, phototrophic growth of the *mca1-1* mutant is restored by complementation with a cosmid containing a 31-kb fragment of wild-type genomic DNA (28), which is also able to complement the *mca1-2* strain (data not shown). An 18-kb *AvrII-NdeI* subfragment, complementing both *mca1-1* and *mca1-2* mutations, was further restricted to a *HindIII* 7.7-kb region, which upon transformation, restored the photosynthetic capability of the *mca1-2* strain but not of the *mca1-1* strain (data not shown). This suggested that, in addition to *MCA1*, at least one other gene required for phototrophic growth had been disrupted during insertion of the modified *ARG7* cassette into the *mca1-1* strain. This other gene, *TDA1*, controlling translation of the  $\alpha$  subunit of the ATP synthase complex, is characterized in a separate study (C. Loisel et al., unpublished data). To avoid any uncontrolled effect of this second mutation on cytochrome *f* expression, we chose to continue our experimental investigations primarily with the *mca1-2* strain. However, as illustrated below, we consistently obtained similar results when we compared the *mca1-1* and *mca1-2* strains.

The sequence of the 7.7-kb insert and its surrounding regions was deposited in the EMBL database under accession no. AF330231 and corresponds to nucleotides 335254 to 347320 of contig 75 from the *C. reinhardtii* draft genome (v3.0) (<http://genome.jgi-psf.org/Chlre3/Chlre3.home.html>). The sequence of the CL01e04 cDNA clone from this region, obtained from Kazusa DNA Research Institute (<http://www.kazusa.or.jp/en/plant/>), supported gene model estExt\_fgenes2\_kg.C\_750005. The polyadenylation consensus signal typical of *C. reinhardtii*, TGTAAG (67), is found 15 bp upstream of the poly(A) tail. Sequence comparison between the cDNA and the genomic scaffold showed that *MCA1* gene is composed of five exons (Fig. 2A). The cDNA lacks the 5'UTR and the first two codons of *MCA1*. However, it was able to complement the *mca1-2* mutation, as demonstrated by the restoration of *petA* mRNA and cytochrome *f* in transformed strains (Fig. 1B and C). This confirmed that we actually cloned *MCA1*. Furthermore, sequence analysis of *MCA1* in the *mca1-2* and *mca1-3* strains revealed that they each harbored single point mutations within the *MCA1* coding sequence, changing, respectively, codons UGG(W<sub>270</sub>) and GAG(E<sub>368</sub>) to an amber codon (Fig. 2A).

*MCA1* codes for a 1,068-residue protein (Fig. 2B), predicted to be targeted to the chloroplast by the programs Wolf PSORT



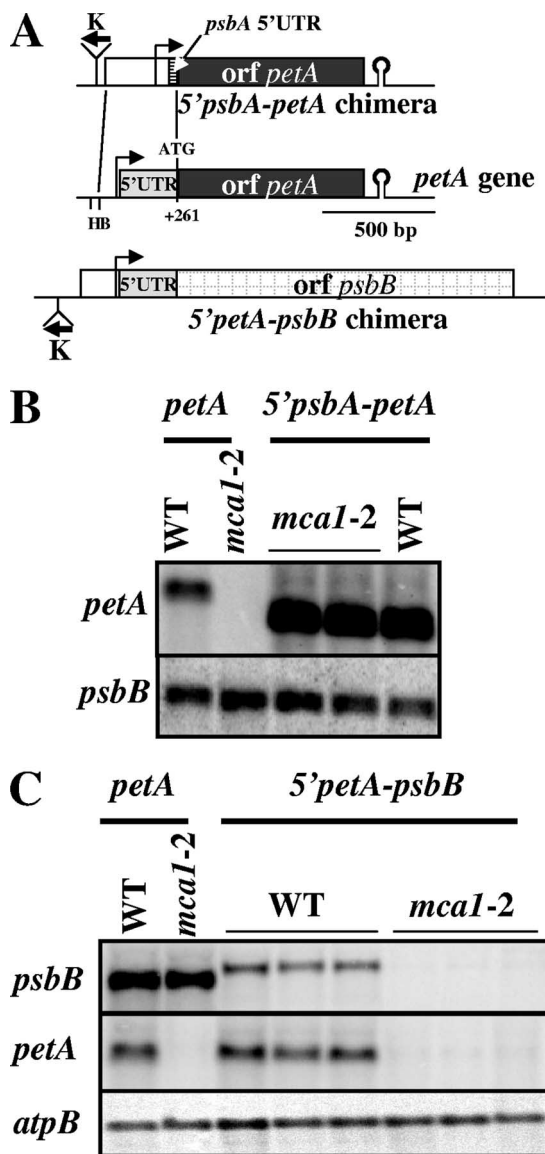


FIG. 3. The *petA* 5'UTR is the target of the MCA1 factor. (A) Schematic map of the chimera used for these experiments. The chloroplast genomic region encompassing the *petA* gene is shown in the middle line. *5'psbA-petA* and *5'petA-psbB* chimera, whose expression is analyzed in panels B and C, are, respectively, shown above and below the *petA* gene. Bent arrows indicate transcription start sites, while "K" stands for the *aadA* cassette, inserted in the opposite orientation with respect to *petA* to allow the selection of transformed strains. (B) A chimeric *petA* mRNA, expressed from the unrelated *psbA* 5'UTR, accumulates in the absence of MCA1. The accumulation of *petA* mRNA (either regular or the smaller chimeric *5'psbA-petA* mRNA) in wild-type and *mca1-2* nuclear backgrounds is shown. Two independent transformants expressing the chimeric *5'psbA-petA* gene in the *mca1* nuclear context are shown. The accumulation of *psbB* transcripts in the same strains provides a loading control. (C) Chimeric transcripts, driven by the *petA* 5'UTR, fail to accumulate in an *mca1* mutant strain. The accumulation of *psbB* mRNAs (either regular or the slightly larger chimeric *5'petA-psbB* mRNA) in wild-type and *mca1-2* nuclear backgrounds is shown. Three independent transformants expressing the chimeric *5'petA-psbB* gene are shown for each nuclear background. Accumulation of the *atpB* transcript in the same strains provides a loading control.

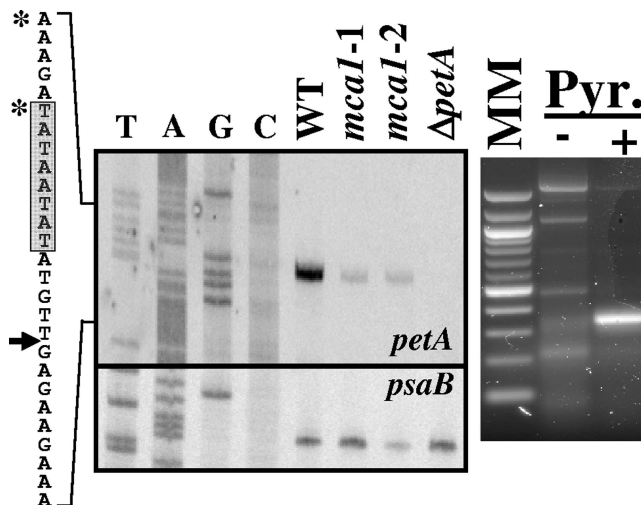


FIG. 4. MCA1 controls the abundance, but not the localization of the 5' end of *petA* transcript. The left panel shows mapping of the *petA* 5' ends by extension of primer RevpetA in wild-type, *mca1-1*, and *mca1-2* strains and in the control strain  $\Delta petA$ . Mapping of the *psaB* 5' end by extension of the primer RevpsaB in the same reaction mixture provides a loading ladder for comparison. Arrow points to the 5' end of the *petA* transcript on the sequence shown on the left, while the promoter consensus sequence is highlighted by a gray box. Asterisks stress two 5' termini described by Matsuda and coworkers (41). We did not find these 5' ends in our experiments and concluded that they probably originated from nonspecific extensions. The right panel shows the 5' end of the *petA* transcript analyzed by RLM-RACE after (lane +) and without (lane -) taP treatment of mRNAs. First lane, 100-bp DNA ladder.

ground (43) (Fig. 3B, last lane). Since the *mca1* transformed strains are capable of photoautotrophic growth and have a wild-type phenotype, we concluded that *petA* is the sole gene involved in photosynthesis whose expression is altered in the absence of MCA1.

That the target for MCA1 was fully comprised within the *petA* 5'UTR was confirmed by using another chimera, *5'petA-psbB*, made of the *petA* 5'UTR fused to the *psbB* coding sequence and 3'UTR (Fig. 3A). This chimera, associated with a spectinomycin resistance cassette to allow selection of transformed strains (25), was introduced by transformation in place of the endogenous *psbB* gene in the chloroplast genome of wild-type and *mca1-2* strains. The chimeric transcript turned out to be fully MCA1 sensitive since it accumulated in a wild-type genetic context but was absent in *mca1* mutants (Fig. 3C).

**The *petA* mRNA displays a single 5' end, which is MCA1 independent.** In chloroplasts, nucleus-encoded factors often stabilize a processed form of their target transcript (50, 72). This prompted us to map the *petA* mRNA 5' end(s) by primer extension in wild-type and *mca1* strains with the expectation that the 5' end of the sole precursor transcript would be observed in *mca1* mutant strains, whereas both mature and precursor ends would be detected in the wild type. Figure 4 (left panel) shows that a single 5' end was detected in the wild type. The same single 5' terminus was also detected to a much lower extent, in *mca1-1* or *mca1-2* mutant strains. This terminus, located 13 bp downstream of the first nucleotide of the TAT AATAT consensus sequence for chloroplast promoters in

*Chlamydomonas* (−10 box), probably corresponds to the actual start site for *petA* transcription. When carrying out RLM-RACE on *Chlamydomonas* RNA treated with tobacco acid pyrophosphatase (taP), we observed an amplification product of the expected size (Fig. 4, right panel). In contrast, we did not observe this amplicon when the experiment was performed on RNA not treated with taP, but rather several faint unspecific amplification products of various sizes. The *petA* mRNA is thus almost exclusively found with a triphosphorylated 5' end, which is not a substrate for ligation with an RNA oligonucleotide. The *petA* transcript therefore is an unprocessed transcription product, the abundance of which is controlled by MCA1 (53), through a mechanism that does not involve the maturation of its 5' end.

**MCA1 protects the *petA* mRNA from 5'→3' RNases.** Stabilization factors previously studied in *Chlamydomonas* chloroplasts protect their target transcript from a 5'→3' degradation process (14, 49, 72). This conclusion was reached by using poly(G) tracts that form very stable secondary structures impeding the progression of 5'→3' exoribonucleases. We inserted 19 consecutive G residues in the *petA* 5'UTR at position +22 (+1 being the 5' end of the mRNA), which also corresponds to position −239 with respect to the A residue from the initiation codon. This construct was introduced in place of the endogenous *petA* gene in the chloroplast genome of wild-type and *mca1* strains. In a wild-type nuclear context, the *pG-petA* mRNA accumulated twice as much as the endogenous *petA* transcript (Fig. 5A). Thus, the accumulation of *petA* mRNA is limited by 5'→3' degradation processes even in the presence of MCA1. This conclusion is consistent with MCA1 being in limiting amounts in the chloroplast, as we previously demonstrated (18, 53). In *mca1* mutant strains that do not accumulate the *petA* transcript, the *pG-petA* mRNA accumulated to a level close to that of the regular transcript in the wild type (Fig. 5A). If the poly(G) tract impedes the progression of 5'→3' RNases, then the 5' end of this transcript should map at the 5' border of the poly(G) tract in *mca1* mutant strains. We mapped the 5' ends of *pG-petA* transcripts by primer extension (Fig. 5B). Prominent bands in the −239/244 and −252/258 regions map at the beginning and the end of the poly(G) tract in both wild-type and *mca1* nuclear backgrounds. The group of shorter bands originates from arrested extension at the beginning of the strongly structured poly(G) tract, as often observed in similar experiments (49, 72). The group of longer bands corresponds to the expected 5' end of a transcript protected from 5'→3' RNase degradation by the poly(G) tract. Accumulation of these bands in the wild-type nuclear context is in agreement with the overaccumulation of the *pG-petA* mRNA. In wild-type strains, but not in *mca1* strains expressing the *pG-petA* chimera, a larger band at position −280 maps to the genuine 5' end of the *petA* transcript, just downstream of the −10 box. Its intensity is lower than that of the 5' end of the regular *petA* mRNA in the wild type, since most extensions are arrested at the level of the poly(G) tract.

Thus, in the absence of MCA1, the *petA* mRNA is degraded by a 5'→3' ribonucleolytic activity which is blocked by the poly(G) tract. In the wild type, MCA1 protects only a fraction of neosynthesized *petA* mRNAs from this ribonucleolytic activity.

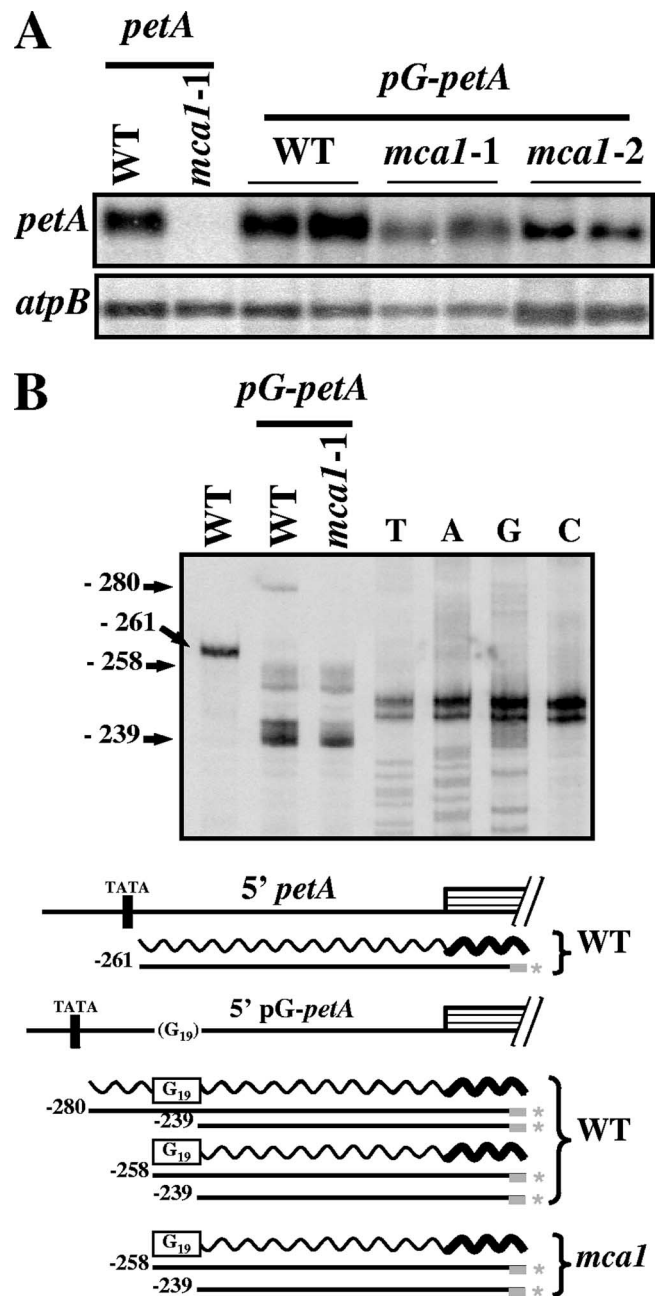


FIG. 5. Insertion of a poly(G) tract at the beginning of the 5'UTR stabilizes the *petA* mRNA. (A) Accumulation of transcripts expressed from the *pG-petA* chimera in two independent transformed clones derived from wild-type, *mca1-1*, or *mca1-2* recipient strains. The accumulation of the endogenous *petA* transcript in wild-type and *mca1-1* strains is also shown. *atpB* mRNA provides a loading control. (B) 5' ends of *petA* transcripts in the wild type and in a transformed strain expressing the *pG-petA* chimera, determined by primer extension. The labeled primer RevpetA was also used to generate a sequencing ladder from template plasmid pWF-pG. The length (numbered from the initiation codon) of extension products is indicated on the left. Below are schematically shown the structure of the 5' region of the *petA* gene, as well as the transcripts (wavy lines) and extension products (straight lines) found in each strain. The position of the −10 box is indicated. The thick gray bar depicts the oligonucleotide primers, labeled at the 5' end (\*).

**The MCA1 target is located within the first 30 nucleotides of the 5'UTR of *petA* mRNA.** The MCA1-dependent protection of the *petA* mRNA suggests that MCA1 interacts, directly or indirectly, with the very 5' end of the *petA* 5'UTR to prevent access of 5'→3' RNases. In order to better define the target of MCA1 within the *petA* 5' UTR, we constructed a reciprocal pair of chimeric genes, each linked to a spectinomycin resistance cassette for selection of transformants.

In the first one, hereafter referred to as *A-petD* (Fig. 6A), the -10 box and the first 31 transcribed nucleotides from *petD* were replaced by the -10 box and 30 first transcribed nucleotides from the *petA* gene. In the second construct, *D-petA* (Fig. 6A), we substituted the -10 box up to the 26th transcribed nucleotide of *petA* by the -10 box and first 29 transcribed nucleotides from *petD*. This construct includes stabilization element I, which is crucial for *petD* mRNA stability (57) that comprises the nine most upstream nucleotides of the *petD* transcript (30) and could contribute to the target of MCD1, the *petD* specific stabilization factor (14).

By chloroplast transformation, we substituted the endogenous *petA* or *petD* genes with their chimeric versions *D-petA* or *A-petD* in the wild type and in the *mca1* and *mcd1* strains lacking, respectively, the MCA1 and MCD1 stabilizing functions. When introduced in the wild type, the *A-petD* transcript accumulated significantly, although to a 10-fold-decreased level than the regular *petD* mRNA (Fig. 6B). Unlike the *petD* transcript, its *A-petD* chimeric version accumulated to similar levels in wild-type and *mcd1* strains but failed to accumulate when expressed in *mca1* genetic background (Fig. 6B). Thus, the *A-petD* transcript behaved as the original *petA* transcript: it is stabilized by MCA1 but insensitive to MCD1. Therefore, an essential target sequence for MCA1 is comprised within the most upstream 30 nucleotides in the 5'UTR of *petA*.

In the reciprocal experiments, the *D-petA* transcript was stabilized in a wild-type nuclear background, although to a lower level than the regular *petA* mRNA (20 to 35%, Fig. 6C). At variance with this, *D-petA* mRNA accumulated to similar levels in *mca1* and wild-type strains but failed to accumulate in *mcd1* mutants (Fig. 6C). Thus, the *D-petA* transcript behaved as the original *petD* transcript, being stabilized by MCD1 but insensitive to MCA1. The target for MCD1 is comprised within the 30 most upstream nucleotides of the *petD* 5'UTR.

This domain-swapping assay demonstrated that the specificities of MCA1 and MCD1 reside within the most upstream regions of the 5'UTRs of their respective target mRNAs.

**TCA1 does not promote translation through the MCA1 target.** Translation of the *petA* mRNA is strictly dependent on the presence of TCA1, the *petA* specific translational activator that acts on its 5'UTR (53, 76). We wondered whether MCA1 and TCA1 shared the same target on the *petA* 5'UTR. We assessed for translation the transformed strains harboring the MCA1-sensitive *A-petD* construct. These strains were incapable of photoautotrophic growth because they completely lack accumulation of subunit IV. This defective expression cannot be accounted for by the 10-fold-decreased content in chimeric *petD* mRNA since strains with 10% of the wild-type level of *petD* mRNA accumulate more than 50% of subunit IV (20), whereas 5 to 10% of subunit IV is sufficient to sustain photoautotrophic growth (8, 56). Higgs et al. have proposed that "MCD1 itself appears to be a translation factor, in addition to

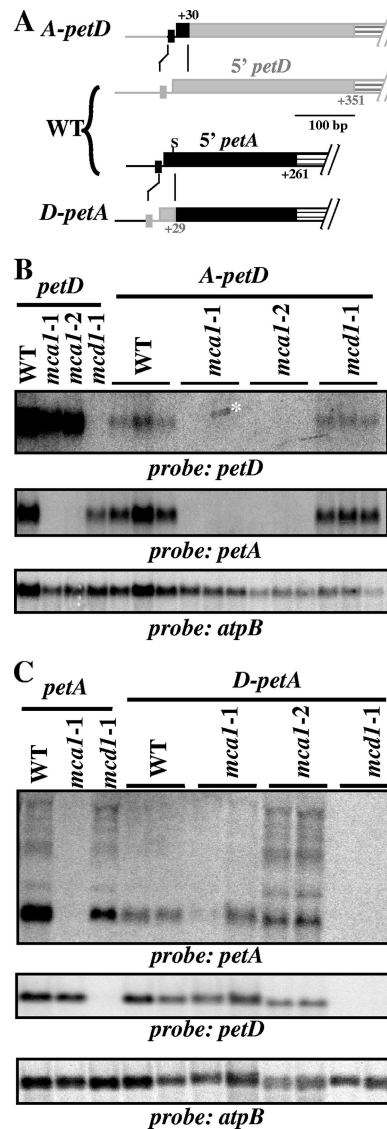


FIG. 6. The stability of chimeric transcripts depends on their very 5' end. (A) Schematic representation of the chimeric genes used for these experiments. The 5' regions of the *petA* and *petD* genes are shown in the middle. Filled boxes represent the respective 5'UTR (in black for *petA* and gray for *petD*), while dashed boxes symbolize the beginning of the coding sequences. Rectangles immediately upstream of the 5'UTRs depict the -10 boxes of the two genes. In the 5'UTR of the *petA* gene, "S" indicates a SwaI restriction site, lost in the *D-petA* chimera, used for RFLP analysis of transformants. The top and bottom lines show, respectively, the structure of the *A-petD* and *D-petA* chimeras, with the origin of the sequence elements indicated by the color code. (B) The 30 first nucleotides of the *petA* 5'UTR confer an MCA1-dependent stability to the *A-petD* transcript. The *A-petD* chimera was introduced in the chloroplast of wild-type, *mca1-1*, *mca1-2*, and *mcd1-1* strains. The accumulation of *petA*, *petD* (and *atpB* as a loading control) transcripts was monitored in three independent transformed strains for each genetic background, as well as in recipient strains. The asterisk points to unspecific stain overlapping two lanes. (C) *D-petA* transcripts are stabilized through interactions with MCD1. The *D-petA* chimera was introduced in the chloroplasts of wild-type, *mca1-1*, *mca1-2*, and *mcd1-1* strains. The accumulation of *petA* and *petD* (and *atpB* as a loading control) mRNAs was monitored in two independent transformants, as well as in recipient strains.

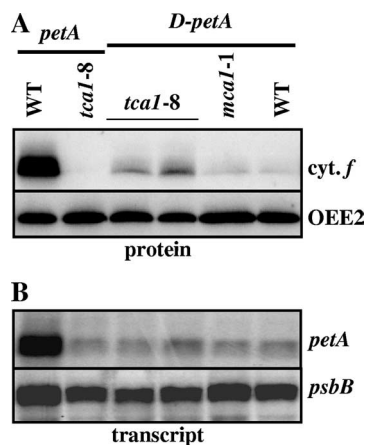


FIG. 7. The *D-petA* chimera is still translated in the absence of TCA1. (A and B) Accumulation of cytochrome *f* (A) and *petA* transcript (B) expressed from the *D-petA* chimera introduced by transformation in the chloroplast genomes of *tca1-8* (two independent transformants), *mca1-1*, and wild-type recipient strains. The accumulation of cytochrome *f* and of the regular *petA* mRNA in wild-type and *tca1-8* strains is also shown. OEE2 and *psbB* mRNA provide the respective loading controls.

promoting RNA stability” (30). Most probably, absence of the crucial stability element I within the *A-petD* mRNA prevents MCD1 to bind the chimeric transcript and to activate its translation.

Thus, stabilization of the *A-petD* chimeric transcript by MCA1 allowed its accumulation but not its translation. We conclude that part, if not all, of the target for TCA1 is located downstream the first 30 nucleotides from the *petA* 5'UTR.

**TCA1 hampers translation of the *D-petA* mRNA.** We next examined whether these target elements for TCA1 were preserved in the *D-petA* construct. To study its translation, we relied on the fact that, in exponentially growing cells, cytochrome *f* is a stable protein whose accumulation faithfully reflects the rate of translation, a property particularly useful at low expression levels (9, 11, 37). The *D-petA* mRNA turned out to be translatable since cytochrome *f* could be detected in transformed cells with a wild-type nuclear background (Fig. 7A). As expected from the absence of the MCA1 target in the *D-petA* construct, translation of cytochrome *f* proved quite independent of the presence of MCA1, similar low levels of accumulation being observed in a wild-type or *mca1* nuclear background (Fig. 7A).

We then tested whether translation of the *D-petA* mRNA still required TCA1 by introducing the *D-petA* chimera in the chloroplast genome of a *tca1* mutant strain. In this *tca1* nuclear context, the *D-petA* mRNA accumulated to the same extent as in the wild-type and *mca1* nuclear contexts (Fig. 7B). Surprisingly, cytochrome *f* was better expressed in the absence of TCA1 than in a wild-type nuclear background (Fig. 7A). Thus, the first 29 nucleotides from the *petD* 5'UTR, which confer MCD1-dependent stability to the *D-petA* chimera, allow its translation in the absence of TCA1. Moreover, that translation is enhanced in the absence of TCA1 suggests an interaction between TCA1 and the chimeric transcript that hampers MCD1-mediated translation.

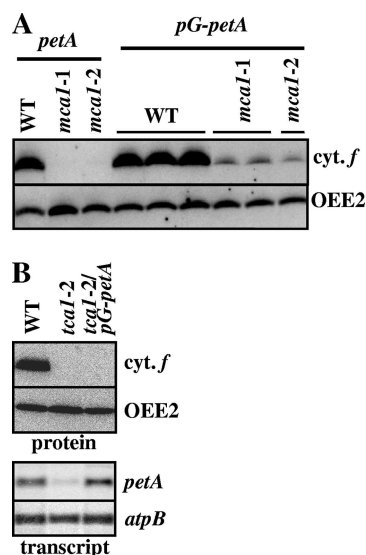


FIG. 8. Cytochrome *f* can be translated from the *pG-petA* transcript in the absence of MCA1 but not in the absence of TCA1. (A) Accumulation of cytochrome *f* (and OEE2 as a loading control) detected by a specific antibody in independent transformants expressing the *pG-petA* chimera derived from wild-type, *mca1-1*, or *mca1-2* recipient strains. The accumulation of cytochrome *f* in the recipient strains is also shown for comparison. (B) Expression of the *pG-petA* chimera, introduced by transformation in the chloroplast genome of the *tca1-2* recipient strain. The accumulations of *petA* mRNA and cytochrome *f* are shown in the wild-type and *tca1-2* recipient strains and in two independent transformants. *psbB* mRNA and the OEE2 protein provide the respective loading controls.

**MCA1, or its mRNA target, enhances *petA* translation.** That MCA1 cannot promote translation of the *A-petD* chimeric transcript by no means excludes its contribution to the translation of a regular *petA* mRNA. The *pG-petA* mRNA that still accumulates in the absence of MCA1 gave us the opportunity to address this point by comparing the abundance of cytochrome *f* translated from this transcript in *tca1*, *mca1*, and wild-type nuclear backgrounds (Fig. 8).

In a wild-type nuclear background cytochrome *f* was slightly more expressed from the overaccumulated *pG-petA* transcript than from the regular *petA* mRNA (Fig. 8A): hence, the poly(G) tract inserted at position +22 did not impair translation. Despite the high accumulation of the *pG-petA* transcript in a *tca1* nuclear context, cytochrome *f* was nevertheless not translated from the *pG-petA* mRNA in the absence of TCA1 (Fig. 8B): translation of the *pG-petA* mRNA, like that of the endogenous *petA* transcript, still requires TCA1.

In the absence of MCA1, cytochrome *f* was still expressed from the *pG-petA* transcript, but much less efficiently than in a wild-type nuclear context (Fig. 8A). The threefold drop in *pG-petA* mRNA accumulation in *mca1* mutant strains could not account for the ~10-fold decrease in cytochrome *f* expression. Thus, MCA1, even if not strictly required for translation of the *petA* mRNA, could behave as a translational enhancer. Alternatively, translational enhancement may involve nucleotides 1 to 21 that are not protected by the poly(G) tract in the absence of MCA1 (Fig. 5B).

We also noted that in *tca1* mutant strains, the accumulation of the *pG-petA* mRNA remained unchanged, at variance with

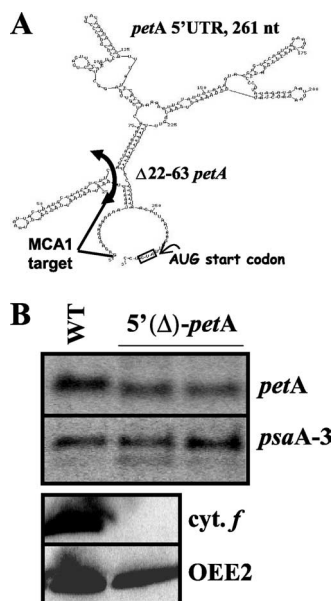


FIG. 9. Nucleotides 22 to 63 from the *petA* 5'UTR are required for the translation but not for the accumulation of the mRNA. (A) In silico prediction of the possible secondary structure of the *petA* 5'UTR, using the program RNAfold. Note that the proposed structure lacks experimental support. (B) Transcript accumulation from the regular *petA* gene in the wild type and from 5'( $\Delta$ )*petA* in transformed strains. *psaA* provides a loading control. Below is shown the accumulation of cytochrome *f* (and OEE2 as a loading control) in the same strains.

the regular *petA* transcript, whose level is reduced fivefold (76) (Fig. 8B). In the absence of TCA1, the *petA* mRNA is thus degraded by a 5'→3' ribonucleolytic activity, from which it is normally protected by MCA1. The lower content in *petA* transcripts in *tca1* mutants thus suggests a weakened interaction between the *petA* transcript and MCA1 in the absence of TCA1.

**MCA1 and TCA1 could act on neighboring targets within the *petA* 5'UTR.** The experiments described above (Fig. 7 and 8) suggest that TCA1 interacts with sequences downstream of the MCA1 binding domain and that the presence of MCA1 and/or its nucleotide targets enhances TCA1-driven translation, while TCA1 improves the MCA1-dependent protection of *petA* transcripts from 5'→3' degradation. To check whether the target of TCA1 includes sequences just downstream of those recognized by MCA1, we constructed a modified *petA* gene that still contained the target of MCA1 (the first 21 transcribed nucleotides from *petA* mRNA) but lacked nucleotides 22 to 63, predicted by RNA folding programs (Mfold) (80) to form a stem-loop structure (Fig. 9A). We then substituted the endogenous *petA* gene in wild-type and *mca1* strains with this 5'( $\Delta$ )*petA* gene. The 5'( $\Delta$ )*petA* RNA accumulated to ca. 75% of the level observed for the endogenous *petA* transcript, when expressed in a wild-type nuclear background (Fig. 9B). In contrast, it did not accumulate in *mca1* mutant strains (data not shown), indicating that it still contains the major target of MCA1, which could thus be restricted to positions 1 to 21.

Cytochrome *f* was undetectable in transformed cells, either with the wild-type or the *mca1* nuclear genome (Fig. 9B): the

5'( $\Delta$ )*petA* mRNA was not translatable despite its significant accumulation. Nucleotides 22 to 63 are critical for translation, probably as part of the TCA1 target. Thus, the targets for MCA1 and TCA1 comprise neighboring, if not overlapping, sequence elements in the *petA* 5'UTR.

## DISCUSSION

**MCA1 codes for a PPR protein.** Here, we report the characterization of MCA1, a nucleus-encoded factor specifically required for stabilization of the chloroplast *petA* mRNA in *Chlamydomonas*, since *mca1* mutant strains still transcribe but do not accumulate the *petA* mRNA (28). MCA1 turned out to encode the first PPR protein functionally characterized in *C. reinhardtii*.

PPR proteins are presumably involved in specific interactions with organelle RNAs. They form a large protein family, present in all eukaryotes but greatly expanded in land plants (68). More than 450 PPR proteins were identified in a systematic search on the *Arabidopsis* genome, most of them predicted as targeted to organelles (38). This is also true for the PPR protein MCA1, since we previously reported that it was enriched in chloroplast fractions (53).

Only a few PPR proteins have been extensively studied up to now, but they most often act on one (or a few) specific target mRNA(s) and participate in any of the various posttranscriptional steps of organelle gene expression (reviewed in reference 1): *cis* and *trans* splicing (61), RNA editing (51), RNA processing (42, 47), RNA stabilization (the present study), and RNA translation (22, 40, 60). Other PPR proteins targeted to plant mitochondria are fertility restorers: they prevent expression of mRNAs encoding cytotoxic peptides (13, 75). How these proteins mediate such widely different functions within organelles is still poorly understood. In vascular plants, additional conserved motifs present at the C terminus of some PPR proteins may carry catalytic activity (38, 59). However, PPR proteins from nonplant eukaryotes, including green algae, lack these domains and most probably just recognize and/or bind RNAs. PPR proteins are thus believed to perform their function by recruiting catalytic factors (38).

**MCA1 specifically recognizes the very 5' end of the *petA* mRNA.** Understanding the basis and specificity of PPR/RNA interactions is thus crucial to elucidate the function of this wide and yet largely unknown protein family. This requires the precise identification of PPR protein targets in vivo. In higher plant chloroplasts, interactions between the PPR protein HCF152 and its target RNA—the transcript of the polycistronic unit *psbB-psbT-psbH-petB-petD*—have been demonstrated in vitro by UV cross-linking (42, 47). More recently, targets of PPR proteins in the chloroplast of maize were identified in vivo by microarray analysis of RNA immunoprecipitated with antibodies against the PPR proteins CRP1 or PPR4, respectively required for translation of *petA* and *psaC* mRNAs or for *trans* splicing of *rps12* mRNA (60, 62).

In *Chlamydomonas*, short nucleotide sequences required for the stable accumulation of several transcripts have been identified by site-directed mutagenesis of their 5'UTRs (2, 30, 49), such as the stability element I (nucleotides 1 to 9) that is required for stable accumulation of the *petD* mRNA (30). Here, we show that the first 29 nucleotides from the *petD*

mRNA are sufficient to confer an MCD1-dependent stability to a chimeric transcript. Probably, this major target sequence of MCD1 can further be restricted to stability element I because substitutions of nucleotides 10 to 24 had no effect on *petD* gene expression (30).

We also identified the sequence elements sufficient to confer *in vivo* MCA1-dependent stability to a chimeric transcript. We first demonstrated that the target of MCA1 was located within the *petA* 5' UTR, a feature shared by most stabilization factors studied up to now in *Chlamydomonas* (14, 50, 72). Our 5' end swapping experiments then pointed to the first 30 nucleotides from the *petA* transcript as the main target of MCA1, which can be further restricted to nucleotides 1 to 21 (it may even be shorter), as it is conserved in the 5'( $\Delta$ )*petA* construct that bears a deletion from nucleotides 22 to 63. This short nucleotide stretch confers MCA1-dependent stability to the *A-petD* reporter transcript, demonstrating that it is recognized by MCA1. Furthermore, we found no evidence for other critical sequence elements that would allow an independent binding of MCA1 to the *petA* mRNA: the presence of MCA1 in *mcd1* strains expressing the *D-petA* chimera did not prevent the degradation of the chimeric mRNA, despite it contained the whole *petA* sequence, only deprived from the very first 26 first nucleotides (Fig. 6B). Conversely, this *D-petA* transcript proved insensitive to the absence of MCA1: it accumulates to identical levels in wild-type and *mca1* genetic contexts (Fig. 6C). These experiments, however, do not exclude a marginal participation of other *petA* sequences to the interaction with MCA1, which would explain the limited accumulation of the *A-petD* transcript in a wild-type nuclear context.

PPR motifs are predicted to fold into pairs of antiparallel  $\alpha$  helices (helices A and B, see Fig. 2C) (68), and tandem PPR repeats would form a superhelical binding surface. Three polar or charged amino acids exposed on the external side of helix A would make specific contact with target nucleotides (68). Although it has been proposed that each PPR repeat could bind three nucleotides (60), the number of PPR repeats in MCA1 (14), close to the maximal size of its mRNA target (21 nucleotides), rather supports an alternative model where each repeat would bind a single nucleotide, as established from the crystal structure of the analogous PUF repeats (19, 73, 74). We have here an unique opportunity for a better understanding of the structural interactions between PPR motifs and their nucleotide sequence target since both interacting partners (MCA1 and the *petA* 5'UTR) can exquisitely be modified by site-directed mutagenesis.

To address the specificity of the MCA1/*petA* 5'UTR interaction, we BLAST searched for other occurrences of the major MCA1 target in chloroplast genomes. This sequence was found only once in the chloroplast genome of *Scenedesmus oblicus*, a closely related unicellular green alga, 205 nucleotides upstream of the initiation codon of the *petA* gene (see Fig. S2 in the supplemental material). It was also found 214 nucleotides upstream of the *petA* translation start codon from another *Chlamydomonas* species, *C. raudensis* (27), 37 nucleotides downstream of a putative  $-10$  box (see Fig. S2 in the supplemental material). Once available, the chloroplast genome sequence of *Volvox* should also prove informative in that respect. In contrast, this target was not found elsewhere in the chloroplast genome of *C. reinhardtii*, even when the query sequence

was restricted to the first 14 nucleotides of the *petA* transcript. We only found one occurrence of the related sequence GAG AAGATAAAA in the transcribed intergenic region between *psaC* and *petL* (see Fig. S2 in the supplemental material), but it does not correspond to the 5' end of a transcript. Moreover, the accumulation of *psaC* and *petL* transcripts is unaltered in *mca1* mutant strains (C. Loiselay, unpublished results).

**MCA1 protects the *petA* transcript from 5'→3' degradation.** MCA1 protects its target RNA from 5'→3' ribonucleolytic degradation since insertion of a poly(G) tract rescues the accumulation of the downstream sequence of the *petA* transcript in the absence of MCA1. A single 5' end of *petA* mRNA was found in both wild-type and *mca1* strains that corresponds to the transcription initiation site. In that respect, MCA1 differs from MBB1, NAC2, or MCD1, whose mRNA stabilizing function is associated with a 5' end cleavage that converts an unstable mRNA precursor molecule to a stable and mature form (49, 50, 72). Our previous study of the MCA1 dependency of *petA* mRNA abundance also suggested a stable interaction between the MCA1 factor and its target mRNA, rather than a catalytic role of this factor in the conversion of *petA* transcripts from an unstable to a stable form (53). In agreement with this, MCA1 has no predicted catalytic domain. Non-PPR sequences within MCA1 are unlikely to carry enzymatic activity since, apart from the N and C termini, they are poorly conserved in size and sequence in the closely related species *V. carteri*. MCA1 thus probably recognizes and binds the very 5' end of the *petA* 5'UTR, thereby preventing the access of RNases to the *petA* transcript.

**In *Chlamydomonas*, the *petA* expression system comprises one stabilization factor and one translational activator.** Several chloroplast genes require specific factors for both mRNA stabilization and translation. This was documented for the expression of *psbC* (33, 54, 79), *psaB* (12, 69), and *psbD* (34–36, 61) genes, although the precise number of specific factors of each type remains unknown. In the case of the chloroplast gene *petA*, we identified only two distinct nuclear loci, encoding MCA1 and TCA1, respectively, that control mRNA accumulation and translation (24, 28, 53, 76). The number of mutant alleles identified thus far for each locus—13 and 9, respectively—argues that these two factors are the sole specific partners in *petA* gene expression. Since cytochrome *f*, the product of the *petA* gene, is highly conserved among organisms performing oxygenic photosynthesis, we looked for orthologs of MCA1 and TCA1 in other photosynthetic eukaryotes. Like TCA1 (53), MCA1 has an ortholog in *V. carteri* (66% identity), but none was found in higher plants or diatoms. This may reflect the rapid evolution of nucleus-encoded factors in green algae, as we suggested for TCA1 (53). Alternatively, an MCA1 homolog may not be required in vascular plants because of a different gene organization. In *C. reinhardtii*, *petA* is the first gene of the *petA-petD* dicistronic unit, and MCA1 binds to the very 5' end of the transcribed mRNA. In contrast, *petA* is the last cistron of a  $\sim 3.5$ -kb tetracistronic transcript (comprising *psaI*, *ycf4*, *cemA*, and *petA*) in land plants (4), which may not require *petA*-specific factors for its stable accumulation. Interestingly, expression of *petA* in maize does involve another PPR protein, CRP1, that is required for the translation of the *petA* and *psaC* mRNAs (4, 22, 60). Like MCA1, CRP1 comprises 14 PPR repeats, but these are organized in a continuous block.

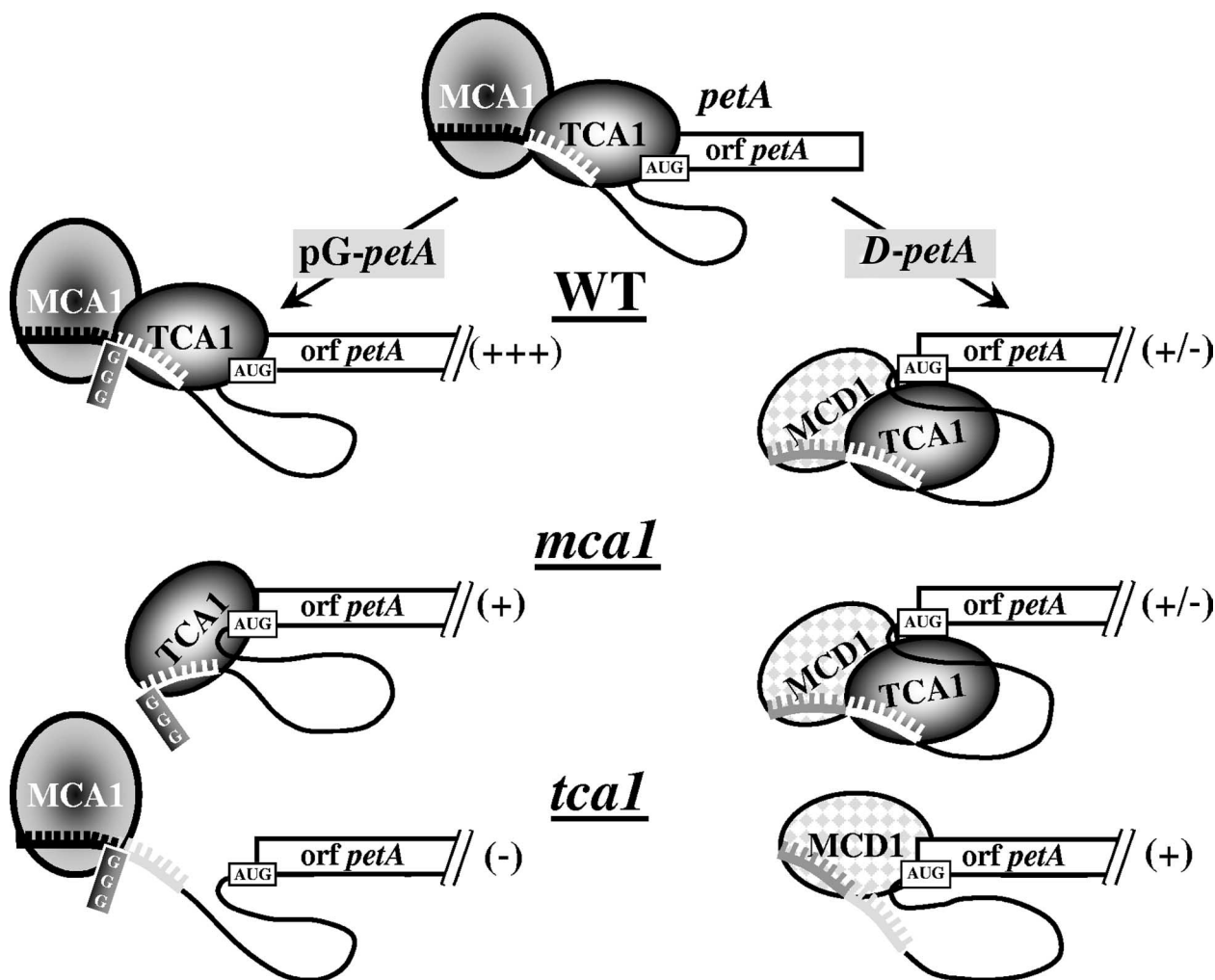


FIG. 10. Working model for the expression of the *petA* genes used in the present study. At the top is shown the expression of the endogenous *petA* gene in the wild type. The MCA1, TCA1, and MCD1 factors are shown with their respective targets, respectively, drawn in black, white, and gray. The interaction between the initiation codon and TCA1 is suggested. The expression of the *pG-petA*, on the left, and *D-petA*, on the right, transcripts are schematically depicted below in (from top to bottom) wild-type, *mca1*, and *tca1* strains. For each situation, we indicated in parentheses the efficiency of the translation: +++, robust; +, reduced but significant (~10% of the wild-type level); +/-, low (<5%); -, no synthesis of cytochrome *f*.

Whether these two proteins shared a common origin but diverged in the different lineages cannot be currently excluded. In *Chlamydomonas*, however, translation of *petA* mRNA relies on a distinct nucleus-encoded factor, TCA1, a pioneer protein devoid of PPRs (53, 76).

**Are some nuclear factors that stabilize chloroplast mRNAs also required for their translation?** For a number of chloroplast genes only mRNA stabilization factors or translation factors have been identified to date. In some cases, it is likely that additional *trans*-acting factors still await identification. However, some chloroplast genes may actually require a single factor that would both stabilize their mRNA and allow its translation. Insertion of poly(G) tracts into *petD*, *psbB*, or *psbD* 5'UTRs restores the accumulation of these transcripts in the absence of their respective stabilization factor, but not their translation (14, 49, 50, 72), suggesting that these stabilization factors could be required for translation as well. Similarly, in the mitochondria of the yeast *S. cerevisiae*, mutations that re-

stored the accumulation of the *COB* RNA coding for cytochrome *b*, in the absence of its specific stabilization factor, CBP1p, did not restore the synthesis of cytochrome *b*, which, again, suggests a role for CBP1p in the translation of its target transcript (32).

Our results strongly support a role for the stabilization factor MCD1 in translation. That element I, the putative nucleotide target of MCD1 (see above), is required for efficient *petD* mRNA translation has been abundantly documented (30, 56, 57). Here, we demonstrate that this MCD1 target allows the translation of a chimeric *petA* mRNA in the absence of its regular translation activator, TCA1. This argues for a role of MCD1 in translation activation, either by itself or by recruiting a translation activator, which would, however, be devoid of sequence specificity since *petA* and *petD* 5'UTRs have no common motif.

Recruitment prevails for the expression of the *psbD* mRNA, whose translation relies on the binding of a 40-kDa protein to

a U-rich region of the *psbD* 5'UTR (52, 62). Once bound, this protein activates translation by destabilizing a secondary structure that sequesters the initiation codon (34). RNA binding of this protein shows poor sequence specificity, and its interaction with the *psbD* 5'UTR depends on the presence of NAC2, the *psbD*-specific stabilization factor (52), with which it interacts (62). Thus, sequence specificity is rather born by NAC2, which, nevertheless, is not able alone to activate translation.

**Interaction of MCA1 and TCA1 in the expression of *petA* mRNAs.** Our work shows that MCA1 does not activate translation by itself since the *A-petD* chimeric transcript is not translated in vivo. Either it cannot efficiently recruit TCA1 or, if it does, downstream sequences from the *petA* 5'UTR are subsequently required for translation activation. In both hypotheses, TCA1 should have its own target(s), not restricted to the first 30 nucleotides of the *petA* 5'UTR. Deletion of nucleotides 22 to 63 prevented translation but not accumulation of the *petA* mRNA, suggesting that they contribute to the target of TCA1, adjacent to that of MCA1. This organization in tandem of the two target sites is further supported by the higher translation of the *D-petA* mRNA in the absence of TCA1. Probably, the target of TCA1 is preserved in the *D-petA* mRNA, and the interaction of TCA1 with the *D-petA* transcript hampers translation driven by MCD1/*petD* elements.

MCA1 is dispensable for translation since the *pG-petA* transcript is still translated in its absence, but not in the absence of TCA1. However, the absence of MCA1 results in poor translation, suggesting that MCA1 acts as an enhancer of translation. Alternatively, the target of TCA1 may extend upstream of position +22 and be degraded in the absence of MCA1 because it remains unprotected by the poly(G) tract. Even if not strictly required for TCA1 recruitment, the interaction of MCA1 with its mRNA target proved sensitive to the presence of TCA1 on its neighboring target (Fig. 10): in the absence of TCA1, much less *petA* transcript accumulates, due to the same poly(G)-sensitive 5'→3' nucleolytic degradation that fully destroys *petA* mRNAs in the absence of MCA1. Thus, the decreased translation efficiency in the absence of MCA1 and the decreased mRNA stability in the absence of TCA1 argue for an interaction between these two *trans*-acting factors for efficient expression of the *petA* gene.

Preliminary biochemical analyses suggest a physical interaction between MCA1 and TCA1 within a high-molecular-weight protein complex, the characterization of which is now under progress (C. Raynaud et al., unpublished data). We note also that a cooperative binding of MCA1 and TCA1 to the *petA* 5'UTR could account, at least in part, for the low accumulation of the *A-petD* transcript, since this latter does not contain the TCA1 target.

Being most likely restricted to three components—MCA1, TCA1, and their chloroplast gene target—the *petA* gene expression system now represents a unique model for an in-depth characterization of the regulation of the expression of a chloroplast gene. Further biochemical analysis of the behavior of TCA1 and MCA1 combined with site-directed mutations of the three partners should provide critical information on the physiological significance of their molecular interactions.

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