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The Initiation Codon Determines the Efficiency but Not the Site of Translation Initiation in *Chlamydomonas* Chloroplasts

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To study translation initiation in *Chlamydomonas* chloroplasts, we mutated the initiation codon AUG to AUU, ACG, ACC, ACU, and UUC in the chloroplast *petA* gene, which encodes cytochrome *f* of the cytochrome b_6/f complex. Cytochrome *f* accumulated to detectable levels in all mutant strains except the one with a UUC codon, but only the mutant with an AUU codon grew well at 24°C under conditions that require photosynthesis. Because no cytochrome *f* was detectable in the UUC mutant and because each mutant that accumulated cytochrome *f* did so at a different level, we concluded that any residual translation probably initiates at the mutant codon. As a further demonstration that alternative initiation sites are not used in vivo, we introduced in-frame UAA stop codons immediately downstream or upstream or in place of the initiation codon. Stop codons at or downstream of the initiation codon prevented accumulation of cytochrome *f*, whereas the one immediately upstream of the initiation codon had no effect on the accumulation of cytochrome *f*. These results suggest that an AUG codon is not required to specify the site of translation initiation in chloroplasts but that the efficiency of translation initiation depends on the identity of the initiation codon.

INTRODUCTION

Protein synthesis is an important target for the regulation of gene expression in chloroplasts of a variety of plant species. Although regulation of protein synthesis occurs at the level of both translation initiation and elongation, translation initiation seems to be the predominant target for regulation (Fromm et al., 1985; Klein and Mullet, 1986; Berry et al., 1988, 1990; Klein et al., 1988; Danon and Mayfield, 1991; reviewed in Gillham et al., 1994; Staub and Maliga, 1994).

The chloroplast translational machinery has many prokaryotic characteristics, including the sedimentation coefficients of ribosomes and their sensitivities to translational inhibitors. Genes encoding the chloroplast counterparts of many components of the *Escherichia coli* translational apparatus have been found in chloroplast and nuclear DNAs (Kostrzewa and Zetsche, 1993; Subramanian, 1993; Lin et al., 1994). Furthermore, it has been shown that *Euglena* chloroplast translation initiation factors can substitute to some extent for their *E. coli* counterparts in vitro (Gold and Spremulli, 1985; Kraus and Spremulli, 1986).

Despite these similarities, significant differences exist in translation initiation between chloroplasts and prokaryotes. First, at least five chloroplast ribosomal proteins have been identified that do not have counterparts in *E. coli* (Subramanian, 1993). Second, in *E. coli*, correct initiation is facilitated by the interaction of the 3′ end of the 16S rRNA with the Shine–Dalgarno sequence in the 5′ untranslated region (UTR; Gold et al., 1981). However, only 50% of the 196 chloroplast genes examined contain putative Shine–Dalgarno sequences in their 5′ UTRs within 12 nucleotides of the initiation codon (Bonham-Smith and Bourque, 1989). In *Chlamydomonas*, a mutation in one such sequence in the chloroplast *petD* gene (*petD* encodes subunit IV of the cytochrome b_6/f complex) had no effect on translation initiation in vivo (Sakamoto et al., 1994), suggesting that this sequence does not function as the ribosome binding site for *petD* translation. Instead, sequence elements farther upstream in the 5′ UTR are essential for translation initiation (Sakamoto et al., 1994). Genetic studies have demonstrated that the translation of several chloroplast mRNAs requires gene-specific factors encoded by the nuclear genome (Kuchka et al., 1988; Rochaix et al., 1989; Drapier et al., 1992; Girard-Bascou et al., 1992). For *psbA* and *psbC* (genes encoding subunits of photosystem II), it has been shown that the nuclear-encoded factor interacts with the 5′ UTR of the transcript (Rochaix et al., 1989; Mayfield et al., 1994; Zerges and Rochaix, 1994).

We have previously used a molecular genetic approach to address the mechanism of initiation codon recognition in chloroplasts. We found that mutating the AUG initiation codon of the *Chlamydomonas petD* gene to either AUU or AUC causes the translation initiation rate to decrease to ~10 to 20% of the wild-type level, resulting in reduced photosynthetic growth at room temperature and an inability to grow photosynthetically

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at 35°C. These studies demonstrated that an AUG initiation codon is not an absolute requirement for translation initiation. However, it was not shown unambiguously that translation initiated at the mutant codons in these strains (Chen et al., 1993). To increase our understanding of the mechanism of initiation codon recognition in chloroplasts, we created five mutations in the initiation codon of the *petA* gene, which encodes cytochrome *f* of the cytochrome b_6/f complex. Analysis of these mutants suggests that the initiation codon strongly influences the rate of translation initiation but that the start site of translation does not specifically require an AUG codon.

RESULTS

Generation of *petA* Initiation Codon Mutants Using Chloroplast Transformation

Five *Chlamydomonas* strains carrying mutations at the initiation codon position of the *petA* gene were created by particle bombardment. A map of the *petA-D* region of the chloroplast genome is shown at the top of Figure 1. The *petD* gene and the *trnR* gene, which encodes $tRNA^{Arg}$, are located downstream of the *petA* gene. Plasmids were constructed that carry changes of AUG to AUU, ACG, ACC, ACU, or UUC at the initiation codon position of the *petA* gene. To facilitate identification of chloroplast transformants carrying these mutations, we introduced an A-to-T change at position −4 to create an SspI site. We also constructed a plasmid carrying this mutation alone to ensure that the phenotypes observed for the initiation codon mutant strains were not due to the −4 mutation. Introduction of these mutations into the chloroplast genome of wild-type *Chlamydomonas* strain P17 was accomplished by particle bombardment with two plasmids; one contained the mutant *petA* gene, and the other carried a selectable marker for spectinomycin resistance encoded by the 16S rDNA. After bombardment, the cells were first selected for spectinomycin resistance and then screened for the presence of the SspI site, which is tightly linked to the initiation codon mutations (see Figure 1).

As shown in Figure 2A, a 563-bp fragment spanning the initiator region of the *petA* gene could be amplified by polymerase chain reaction (PCR), using primers petA4 and petA5. The PCR product from the wild-type recipient strain does not contain an SspI site, whereas PCR products from mutant transformants can be digested with SspI. Transformants identified in this way were at first heteroplasmic: the amplified fragments were only partially digested by SspI, suggesting that they contained both wild-type and mutant copies of the *petA* gene. Two rounds of single-colony isolation were performed, and DNA was prepared and analyzed by PCR. Figure 2B shows the SspI-digested PCR products from the recipient strain and the transformants; the PCR products from the transformants were completely digested by SspI, demonstrating that they are homoplasmic. These strains were named A-AUG, A-AUU, A-ACG, A-ACC, A-ACU, and A-UUC.

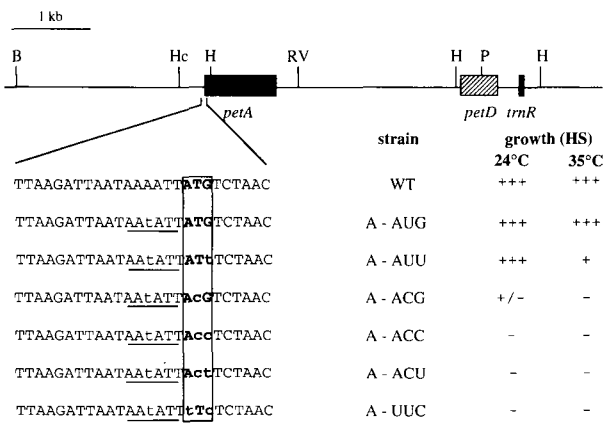

Figure 1. The *petA-D* Region of the *Chlamydomonas* Chloroplast Genome and Initiation Codon Mutant Strains Created in This Study.

The locations of the coding regions for *petA*, *petD*, and *trnR* are indicated by boxes. The nucleotides surrounding the initiation codon of the *petA* gene are shown, with the mutated nucleotides in lowercase letters. The underlined nucleotides form an SspI restriction site. The initiation codon position is surrounded by the box. The names of these strains and their growth phenotypes on minimal medium are listed on the right. +++, wild-type growth; +, reduced growth; +/−, extremely slow growth; −, no growth. Restriction sites are B, BamHI; H, HindIII; Hc, HincII; P, PstI; and RV, EcoRV. WT, wild type.

Growth Phenotypes of *petA* Initiation Codon Mutants

The growth phenotypes of various *petA* initiation codon mutants were examined on YA and HS plates, as shown in Figure 3 and summarized in Figure 1. YA plates contain acetate so that photosynthesis is not required for growth, whereas HS plates lack acetate. A strain carrying an AUG-to-AUU change at the initiation codon of the *petD* gene was included for comparison; this strain (D-AUU) displays a temperature-sensitive photosynthetic growth phenotype on HS medium (Chen et al., 1993). A-AUG was indistinguishable from the wild type under all growth conditions, demonstrating that the A-to-T mutation at position −4 has no effect on cell growth. A-ACC, A-ACU, and A-UUC did not grow photosynthetically at either 24°C or 35°C but did grow heterotrophically on YA medium. A-UUC was somewhat bleached when grown on YA medium; such light sensitivity is common among a variety of nonphotosynthetic mutants. At 24°C, A-ACG grew very slowly under conditions in which photosynthesis was required (the growth is not visible in Figure 3) and failed to grow on HS plates at 35°C. At 24°C on HS plates, A-AUU appeared to grow as well as the wild type, but its growth rate was reduced relative to the wild type at 35°C. An identical mutation at the *petD* initiation codon caused reduced photosynthetic growth at 24°C and rapid bleaching at 35°C. Under photosynthetic conditions at

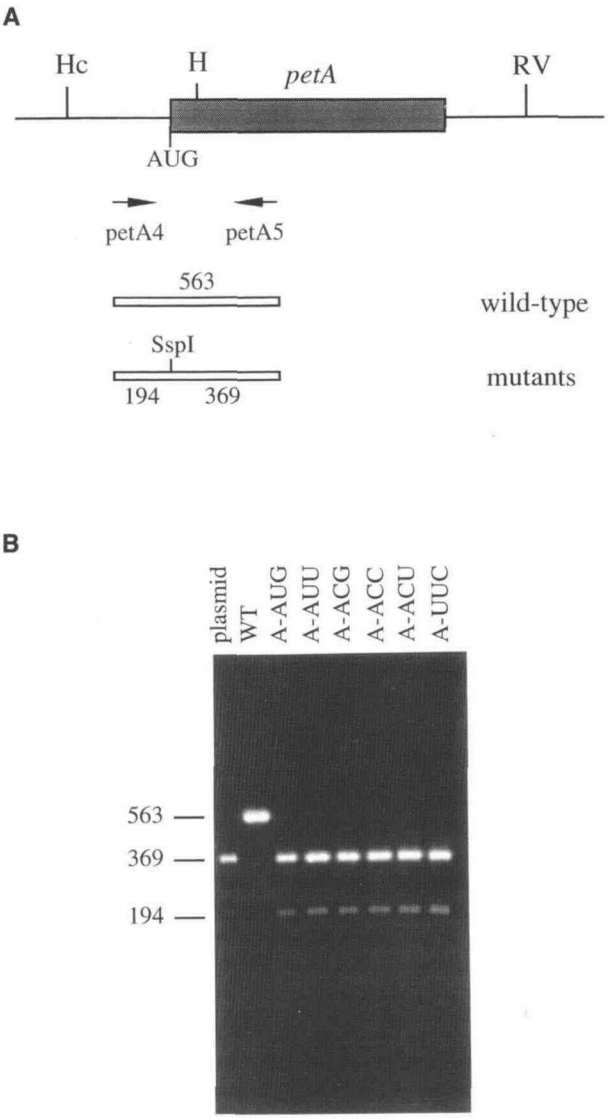

Figure 2. Identification of *petA* Initiation Codon Mutants.

(A) The strategy used to distinguish transformants carrying appropriate mutations from wild-type, untransformed cells. The primers petA4 and petA5 are represented by arrows and flank the initiation codon. As expected, PCR-amplified DNA fragments from transformants but not the recipient strain contained an SspI site. H, HindIII; Hc, HincII; RV, EcoRV.

(B) SspI-digested petA4/petA5 PCR products from a mutant plasmid (plasmid BRV3.8-AUG), the wild-type recipient strain (WT), and transformant strains (A-AUG to A-UUC).

In **(A)** and **(B)**, numbers indicate the size of the amplified DNA fragments in base pairs.

either 24°C or 35°C, A-AUU grew more robustly than did D-AUU because of higher accumulation of the cytochrome b_6/f complex, as discussed later.

Cytochrome *f* Accumulation in the *petA* Mutant Strains

The photosynthetic growth phenotypes of the *petA* initiation codon mutants suggested that A-AUU and A-ACG were still able to produce the cytochrome *f* apoprotein, whereas A-ACC, A-ACU, and A-UUC produced insufficient apoprotein to support photosynthetic growth. To determine the level of cytochrome *f*, we performed immunoblotting experiments using cells grown in HSA (acetate-containing) medium. As shown in Figure 4A, cytochrome *f* accumulation was similar in the wild type and A-AUG, demonstrating that the A-to-T mutation at position −4 did not affect cytochrome *f* accumulation. A-AUU accumulated ~20% of the wild-type level of cytochrome *f* when grown in HSA. The 80% reduction in cytochrome *f* accumulation caused by the AUU mutation was similar to our previous observations for D-AUU, which accumulated ~10 to 20% of the wild-type level of subunit IV and was weakly photosynthetic at 24°C. A-ACG accumulated 2 to 5% of the wild-type level of cytochrome *f*, which probably accounts for the severely reduced photosynthetic growth of this strain. Although nonphotosynthetic, A-ACC and A-ACU still accumulated trace amounts of cytochrome *f*, ~1% of the wild-type level and <1% of the wild-type level, respectively. The cytochrome *f* apoprotein was undetectable in A-UUC. Proteins from a cytochrome *f* deletion strain (ΔpetA; Kuras and Wollman, 1994) were included in the immunoblot to show that the band detected by the antibodies in the wild-type and initiation codon mutants was indeed cytochrome *f*.

It has been shown previously that the accumulation of cytochrome b_6/f complex subunits is coordinately regulated; when cytochrome *f* or subunit IV is limiting, accumulation of other subunits is also reduced (Lemaire et al., 1986; Chen et al., 1993). Because the accumulation of the cytochrome b_6/f complex polypeptides was similar in A-AUU and D-AUU when cells were grown in HSA medium, as shown in Figure 4B, we were surprised that photosynthetic growth of A-AUU was more robust than that of D-AUU (see Figure 3). We therefore measured the accumulation of cytochrome *f* and subunit IV in the wild type, D-AUU, and A-AUU when grown in HS medium. Under these conditions, accumulation of both cytochrome *f* and subunit IV was increased in A-AUU compared with D-AUU (Figure 4B). Quantification of ^{125}I-protein A signals using a PhosphorImager revealed that cytochrome *f* accumulation in A-AUU was approximately four times higher than that in D-AUU and corresponded to 40% of the wild-type level. The increased accumulation of the cytochrome b_6/f complex in A-AUU probably accounts for its enhanced photosynthetic growth phenotype. Cytochrome *f* accumulation was significantly higher when A-AUU cells were grown in HS medium (two- to threefold) and modestly increased in wild-type cells but showed little if any increase in D-AUU cells. We speculate that *petA* expression is higher in minimal medium than in acetate-containing medium for all strains but that the accumulation of cytochrome *f* depends on the abundance of the limiting subunit(s) in the

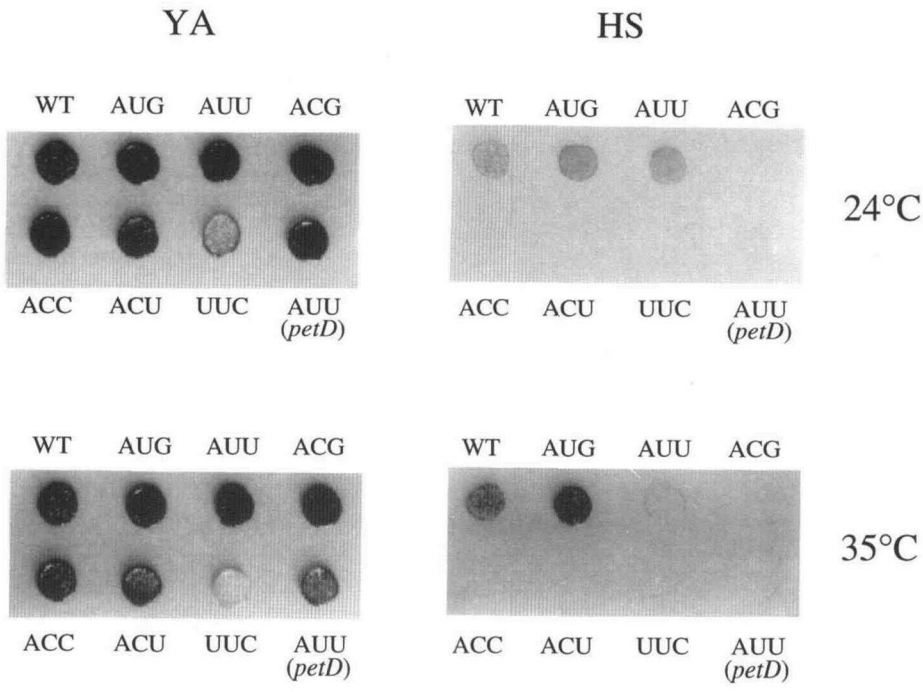

Figure 3. Growth of Wild-Type, A-AUG, A-AUU, A-ACG, A-ACC, A-ACU, A-UUC, and D-AUU Cells on YA or HS Media at Room Temperature and at 35°C.

D-AUU contains AUU instead of AUG at the initiation codon position of the *petD* gene (Chen et al., 1993).

cytochrome b_6/f complex. In A-AUU, cytochrome *f* presumably limits the accumulation of the cytochrome b_6/f complex; this is probably true as well for wild-type cells. However, in D-AUU, subunit IV presumably limits the accumulation of the cytochrome b_6/f complex, and unassembled cytochrome *f* is degraded.

The increased accumulation of cytochrome *f* under photosynthetic growth conditions could result from increased RNA levels, an increased translation rate of *petA* mRNA, or increased stability of cytochrome *f*. We were unable to detect any difference in the levels of *petA* mRNA in HSA- and HS-grown wild-type, D-AUU, and A-AUU cells (data not shown). Wild-type, D-AUU, and A-AUU cells grown in HSA or HS medium and pulse labeled for 5 min with $^{35}S\text{-}Na_2SO_4$ did not show increased synthesis of cytochrome *f* in HS medium relative to other thylakoid membrane proteins, suggesting that the increase in cytochrome *f* accumulation did not result from increased translation (data not shown). However, because the synthesis of the other thylakoid membrane proteins may also have been affected by the growth conditions, the pulse labeling results are not fully conclusive.

Cytochrome *f* Synthesis Rates in the Initiator Mutant Strains

To determine whether decreased cytochrome *f* accumulation in the initiation codon mutants is due to decreased synthesis, cells were pulse labeled with $^{35}S\text{-}Na_2SO_4$ for 5 min. Labeled cytochrome *f* was immunoprecipitated from crude cell extracts using antibodies raised against a glutathione *S*-transferase–cytochrome *f* fusion protein (see Methods). We electrophoresed one-fifth of the extracts used for immunoprecipitation reactions in 12% SDS–polyacrylamide gels to determine the amount of labeled proteins in each sample, as shown in Figure 5A. By normalizing the amount of immunoprecipitated cytochrome *f* to either the most prominent labeled band or the sum of all other labeled proteins, we were able to determine the relative rates of synthesis of cytochrome *f* in each of the *petA* initiation codon mutants. The results in Figure 5B show that A-AUU synthesizes cytochrome *f* at ~25% of the wild-type level, whereas A-ACG, A-ACC, and A-ACU synthesize cytochrome *f* at ~2.5, 2, and 0.8% of the wild-type level, respectively (see numbers at bottom). To ensure that the antibodies were in excess for immunoprecipitation reactions, we included a reaction in which twice the amount of extract from the wild-type cells was incubated with the same amount of antibodies used for the other reactions. Figure 5B shows that, as expected, twice as much labeled cytochrome *f* was immunoprecipitated, as determined by PhosphorImager quantification.

The rates of cytochrome *f* synthesis are correlated with the levels of cytochrome *f* accumulation in *petA* initiation codon mutants, suggesting that decreased accumulation of cytochrome *f* in these mutant strains is caused by the decreased translation initiation rates of the mutant *petA* messages. It is remarkable that strains such as A-ACC or A-ACU, which carry two-nucleotide mutations at the initiation codon position, still synthesize detectable amounts of the cytochrome *f* protein.

petA mRNA Accumulation in the *petA* Mutant Strains

To determine whether the decrease in cytochrome *f* synthesis in the *petA* initiation codon mutants is correlated with decreased *petA* mRNA abundance, we performed RNA filter hybridizations. Figure 6 shows that *petA* mRNA abundance in A-AUG, A-AUU, A-ACG, A-ACC, A-ACU, and A-UUC was similar to that in the wild-type strain when normalized to *atpB* mRNA; this was confirmed by PhosphorImager quantification. Therefore, decreased synthesis of cytochrome *f* does not result in decreased levels of *petA* mRNA. This contrasts with our finding that in D-AUU or D-AUC, the reduced translation rates of the

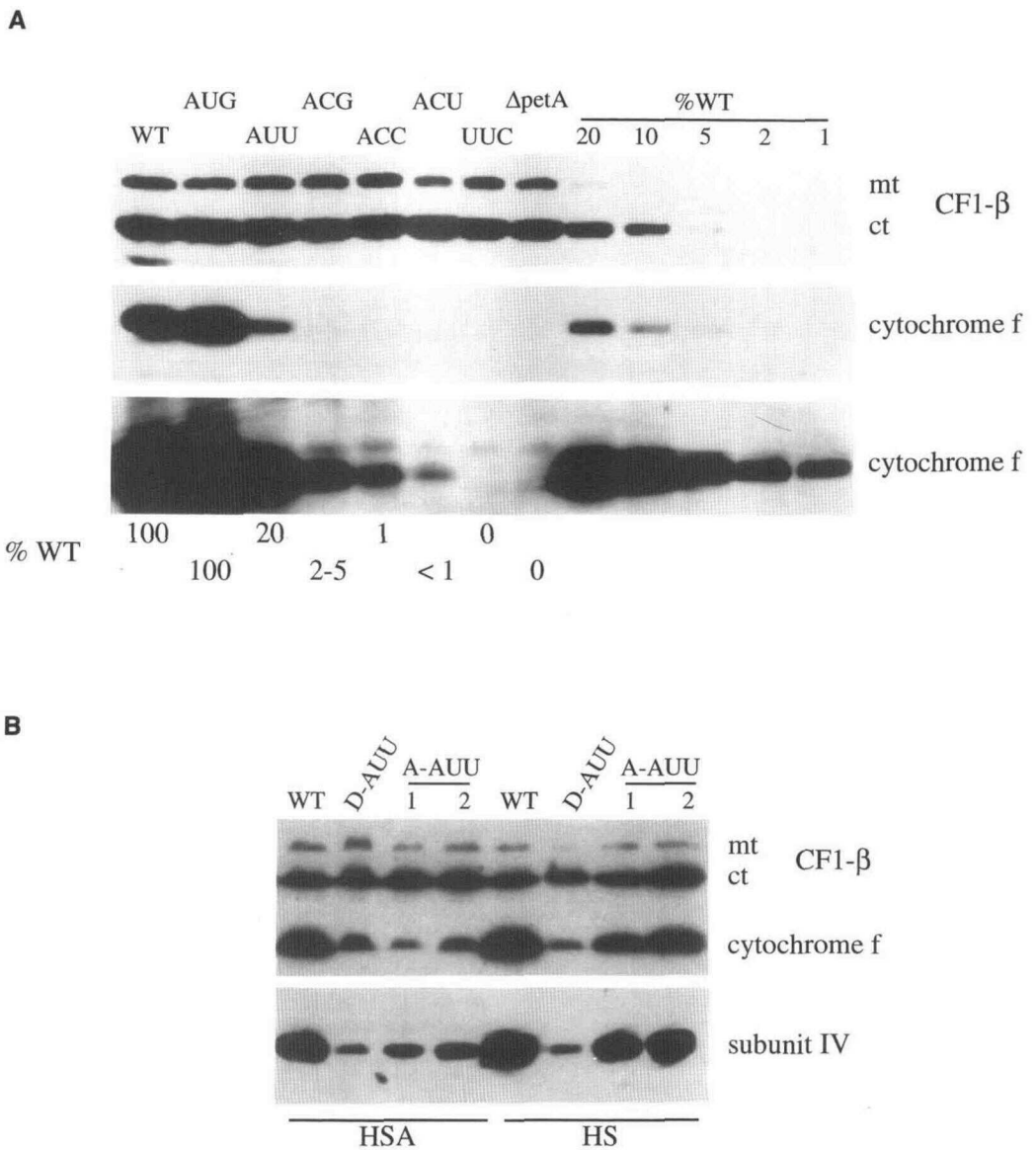

Figure 4. Cytochrome *f* Accumulation in Wild-Type and Mutant Strains.

(A) Cells were grown in HSA liquid medium, and whole-cell proteins were separated in an SDS–polyacrylamide gel, transferred to nitrocellulose, and reacted with specific antibodies. The accumulation of the mitochondrial (mt) and chloroplast (ct) forms of CF1-β served as loading controls. A dilution series of the wild-type (WT) extract was used to estimate cytochrome *f* accumulation in the mutants. At bottom, a long exposure of the cytochrome *f* immunoblot is shown to demonstrate cytochrome *f* accumulation in the weakly expressing mutants. The relative levels of cytochrome *f* in various strains are indicated by the numbers below the gels.

(B) Cells were grown in HSA or HS liquid medium as indicated. Two independent isolates of the A-AUU transformants are shown. Cytochrome *f* and subunit IV accumulation were quantified in this experiment using ^{125}I-protein A (see text). Abbreviations are as given in **(A)**.

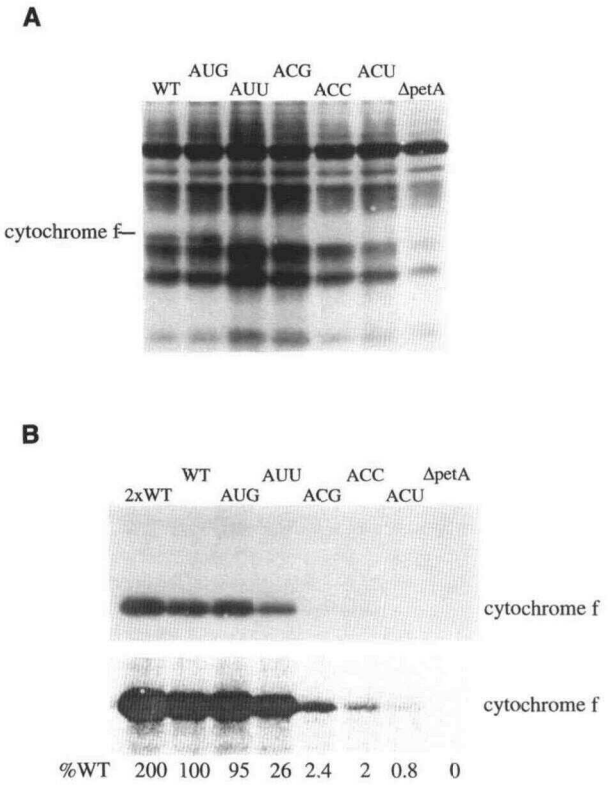

Figure 5. Pulse Labeling of Chloroplast Polypeptides in Wild-Type and Various *petA* Mutant Strains.

(A) Total cell extracts were prepared from cells that had been pulse labeled with ^{35}S-Na_2SO_4 for 5 min, and proteins were electrophoresed in a 12% SDS–polyacrylamide gel. The gel was then electroblotted onto a nitrocellulose membrane, which was exposed to x-ray film for 3 days. The labeled cytochrome *f* protein is indicated.

(B) Cytochrome *f* was immunoprecipitated from the extracts shown in **(A).** Twice as much extract from the wild-type strain was included in one immunoprecipitation reaction (lane labeled 2xWT) to ensure that the cytochrome *f* antibodies were in excess. The amount of labeled cytochrome *f* was quantified using the PhosphorImager. The labeling of cytochrome *f*, shown by the numbers below the gels, was normalized to the wild type using either the most prominent band or all labeled proteins in the lane, as shown in **(A).** Identical results were obtained from both calculations.

mutant *petD* mRNAs destabilize them; as a result, they accumulate only to 50% of the wild-type *petD* mRNA level (Chen et al., 1993).

Site of Translation Initiation in *petA* Initiator Mutants

The aforementioned experiments show that most *petA* initiation codon mutant strains still synthesize and accumulate cytochrome *f*. We considered the possibility that upstream or downstream codons might serve partially or entirely as alternative initiation codons. Because cytochrome *f* is processed after amino acid 31 following insertion into the thylakoid membrane, N-terminal sequencing is uninformative for determining the translation start site (Willey et al., 1984; Johnson et al., 1991; Matsumoto et al., 1991; Smith and Kohorn, 1994). However, the observation that no cytochrome *f* accumulates in A-UUC argues strongly against the use of alternative initiation codons. Similarly, the fact that mutants such as A-ACC and A-ACU accumulate low but different levels of cytchrome *f* argues that the mutated codon, rather than an alternative codon common to both strains, is utilized for translation initiation. To obtain independent evidence that an alternative codon is not used for translation initiation, we introduced UAA stop codons into three locations at or near the AUG initiation codon, as shown in Figure 7A. If cytochrome *f* accumulation were unaffected by a UAA codon positioned immediately upstream of the AUG codon (strain A-UAA-AUG), we could conclude that upstream codons do not contribute significantly to translation initiation. Using similar reasoning, we introduced a UAA codon immediately downstream of AUG (strain A-AUG/UAA) to determine whether any codon downstream of the second codon can serve as the initiation codon. To exclude the possibility that the second codon serves as the initiation codon, we introduced a UAA mutation at the initiation codon position (strain A-UAA). SspI sites tightly linked to these mutations were created by introducing additional AA-to-TT (−5 and −4 positions for A-UAA/AUG) or A-to-T (−4 position for A-AUG/UAA and A-UAA) changes. These strains were created by chloroplast transformation, as described for the initiation codon mutant strains.

The accumulation of cytochrome *f* in stop codon mutant strains was determined by immunoblotting. Figure 7B shows that no cytochrome *f* was detected in A-AUG/UAA, suggesting that codons downstream of the second codon do not serve as initiation codons in vivo. Furthermore, no cytochrome *f* was detected in A-UAA, suggesting that the second codon does not serve as the initiation codon in vivo. In A-UAA/AUG, cytochrome *f* accumulated to the wild-type level, suggesting that initiation upstream of the AUG codon does not contribute significantly to cytochrome *f* accumulation. These results, taken together with the data shown in Figure 4, suggest that the use of an alternative *petA* initiation codon is minimal or nonexistent in vivo.

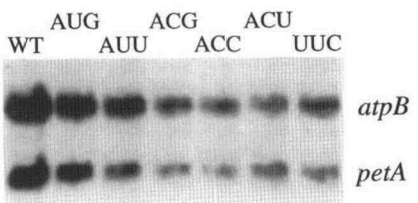

Figure 6. *petA* mRNA Accumulation in Wild-Type and Various *petA* Initiation Codon Mutant Strains.

RNA was separated in a 1.2% agarose–formaldehyde gel, transferred to a nylon membrane, and hybridized with probes for *petA* and *atpB*. The latter probe was used to normalize the amount of RNA loaded in each lane. WT, wild type.

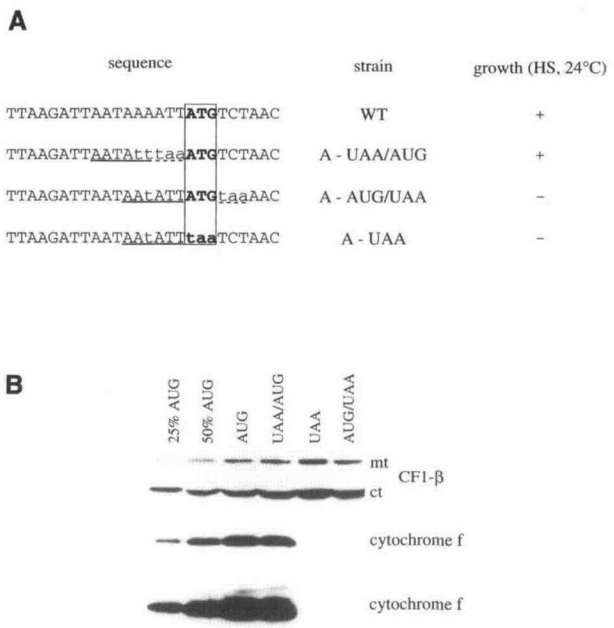

Figure 7. Creation of *Chlamydomonas* Strains with Termination Codons near the *petA* Initiation Codon.

(A) The sequence around the initiation codon position of the *petA* gene, including nucleotides changed to create an SspI site tightly linked to the introduced termination mutations, is shown for each transforming plasmid. Mutated nucleotides are shown in lowercase letters, underlined nucleotides form an SspI restiction site, and dashed lines indicate the newly introduced stop codons. The initiation codon position is enclosed by a box. The photosynthetic growth phenotype of each mutant in HS medium at 24°C is shown at right. +, wild-type (WT) growth; −, no growth.

(B) Cytochrome *f* accumulation in the wild type and various stop codon–containing strains. The 25% AUG and 50% AUG indicate dilutions of the A-AUG extract; protein loading can be estimated by the amount of mitochondrial (mt) and chloroplast (ct) CF1-β. An overexposure of the cytochrome *f* blot is shown at bottom to illustrate that even low levels of cytochrome *f* cannot be detected in A-UAA and A-AUG/UAA.

DISCUSSION

AUG Is Not Obligatory for Translation Initiation in *Chlamydomonas* Chloroplasts

We have shown previously that strains in which the *Chlamydomonas* chloroplast *petD* initiation codon AUG was changed to either AUU or AUC retained the ability to translate subunit IV at 10 to 20% of the wild-type level (Chen et al., 1993). We have now made both one- and two-nucleotide mutations in the initiation codon of a second chloroplast gene, the *petA* gene, and demonstrated that most of the mutations examined do not abolish translation. This suggests that at least two and perhaps all chloroplast genes can tolerate mutations in their initiation codons. The failure to accumulate cytochrome *f* in A-UUC and the results from the stop codon strains argue that alternative initation codons are not used in vivo. This strongly suggests that in each initiation codon mutant strain that accumulates cytochrome *f*, the mutant codon is used as the initiation codon. Thus, the *petA* initiation codon AUG seems to dictate only the efficiency of translation initiation rather than specifying the site of translation initiation. Although AUG is nearly universally used as an initiation codon in chloroplast genes, *C. reinhardtii* and *C. eugametos psbC* genes (and possibly those of land plants) appear to use GUG as their initiation codon (Rochaix et al., 1989; Turmel et al., 1993).

In experiments to be reported elsewhere, we found that mutations immediately upstream of the initiation codon position in A-AUU had a strong effect on cytochrome *f* accumulation. In particular, changing the initiation region from AUUAUU (where the initiation codon is underlined) to UAAAUU abolished cytochrome *f* accumulation. We believe that this probably results from an impaired interaction between the 16S rRNA and *petA* mRNA in the context of a weak initiation codon rather than preventing the use of an alternative upstream initiation site. As mentioned previously, the latter possibility is inconsistent with the low but codon-specific levels of cytochrome *f* in A-ACG, A-ACC, and A-ACU and the observation that A-UUC does not accumulate any detectable cytochrome *f*.

Our analysis of the *petA* initiation codon mutants allows us to draw certain conclusions regarding the tolerance of non-AUG initiation codons. Two single-nucleotide mutations, AUG to AUU or ACG, decreased translation to ~20% and 2 to 5% of the wild-type level, respectively. The two-nucleotide mutations, AUG to ACC or ACU, caused translation to decrease to 1 to 2% and 0.8% of the wild-type level, respectively. The two-nucleotide mutation UUC, which changed the first and the third nucleotides of the initiation codon, abolished translation initiation. The phenotypes of these mutants suggest that (1) single-nucleotide mutations at the third position are tolerated more easily than those at the second position; (2) single-nucleotide mutations are tolerated more easily than two-nucleotide mutations; and (3) mutations in the first position are more deleterious than those at the second position.

The relatively high tolerance of third position changes in the initiation codon is consistent with the wobble rule for the interactions between codons and anticodons. In mature initiation complexes in prokaryotes, the fMet-tRNA interacts with the initiation codon using its anticodon 3′-UAC-5′. The role of fMet-tRNA in the formation of the 30S initiation complex has long been the subject of debate (Gold et al., 1981). The currently favored model for 30S initiation complex formation in prokaryotes is that the 30S ribosomal subunit binds to either the fMet-tRNA or the mRNA in a random order, suggesting that fMet-tRNA has at most a minor role in initiation codon selection during translation initiation (Gold et al., 1981). Indeed, recent cross-linking data suggest that mRNA–rRNA contacts occur over an extensive region surrounding the initiation codon

to position the ribosome correctly (reviewed in McCarthy and Brimacombe, 1994). However, the relative translation rates in the *petA* initiation codon mutants can be explained roughly by the strength of the potential base-pairing interaction between the fMet-tRNA anticodon and the mutant codons. This suggests that the strength of codon–anticodon interactions may determine the rates of translation initiation. Because the mutant codons are probably used as the initiation codon rather than a nearby codon that might have a stronger base-pairing interaction with the fMet-tRNA (for example, in the A-UUC strain, the immediate upstream codon is AUU), fMet-tRNA does not appear to determine the site of translation initiation. Instead, it appears only to facilitate initiation from a site whose location is determined by other factors.

Translation Initiation in Chloroplasts

Because neither the AUG initiation codon nor the fMet-tRNA appears to determine the start site of translation in chloroplasts, this site must be specified by other factors. The available experimental evidence suggests that for the barley large subunit of ribulose-1,5-bisphosphate carboxylase (*rbcL*) and the *Chlamydomonas psbA* genes, a prokaryotic mechanism is used for translation initiation, based on in vitro binding studies in the former instance or site-directed mutagenesis and in vivo analysis of chloroplast transformants in the latter (Kim and Mullet, 1994; Mayfield et al., 1994). Although a number of *Chlamydomonas* chloroplast mRNAs contain Shine–Dalgarno–like sequence motifs and the 3′ end of the *Chlamydomonas* 16S rRNA contains complementary sequences in the expected location (Dron et al., 1982), many chloroplast mRNAs do not have a Shine–Dalgarno–like sequence at positions −5 to −10. When a candidate Shine–Dalgarno sequence was mutated in the *Chlamydomonas* chloroplast *petD* gene, no effect on translation initiation was observed (Sakamoto et al., 1994).

Two possible mechanisms that translate mRNAs lacking obvious Shine–Dalgarno elements at positions −5 to −10 are the following: (1) the 30S subunit recognizes a Shine–Dalgarno sequence farther upstream and then scans to the start site, as has been proposed for barley *psbA* (Kim and Mullet, 1994); and (2) an unrelated sequence element serves as a protein or rRNA binding site, which might mediate the mRNA–30S ribosomal subunit interaction. Such sequences might be located in the 5′ UTR close to the initiation site. For example, a nine-nucleotide sequence (3′-UGGCGCCGA-5′), which is present in a single-stranded region of the small ribosomal rRNAs termed the 530 loop, has been implicated in regulating translation initiation by interacting with the three nucleotides immediately upstream of the initiation codon (Lagúnez-Otero, 1993). Other sequences likely to be involved in chloroplast translation initiation lie farther upstream, such as those identified for *psbA*, *psbC*, and *petD* mRNAs in *Chlamydomonas* (Rochaix et al., 1989; Mayfield et al., 1994; Sakamoto et al., 1994).

METHODS

Construction of *petA* Transforming Plasmids

pHcRV1.6 contains a 1.6-kb HincII-EcoRV subfragment of BamHI fragment 7 of the *Chlamydomonas reinhardtii* chloroplast genome (Harris, 1989). It contains the entire *petA* gene. Site-directed mutagenesis was performed on pHcRV1.6 according to Kunkel (1985) to produce nine mutations at or near the initiation codon position, as shown in Figures 1 and 7. The following primers were used for mutagenesis: petA6, 5′-GTTTCGTTTTAAGATTAATAATATTA(T/C)(G/T/C)TCTAACCAAGTATTTAC-3′, which was used to create the A-AUG, A-AUU, A-ACG, A-ACC, A-ACU, and A-UUC mutants; petA11, 5′-GTTTCGTTTTAAGATTAATAATATTTAATCTAACCAAGTATTTAC-3′, which was used to create the A-UAA mutant; petA12, 5′-GTTTCGTTTTAAGATTAATATTTAAAT(G/T)TCTAACCAAGTATTTAC-3′, which was used to create the A-UAA/AUG mutant; petA13, 5′-GTTTCGTTTTAAGATTAATAATATTAT(G/T)TAAAACCAAGTATTTACTAC-3′, which was used to create mutant A-AUG/UAA. The petA6, petA11, and petA12 primers correspond to nucleotides −25 to +20 with respect to the first nucleotide of the initiation codon of the *petA* gene. The petA13 primer corresponds to nucleotides −25 to +23 with respect to the initiation codon. Following mutagenesis, larger plasmids carrying these mutations, the pBRV3.8 series, were obtained by replacing the HincII-EcoRV fragment from the wild-type pBRV3.8 plasmid, which contains a 3.8-kb BamHI-EcoRI fragment, with the mutant HincII-EcoRV fragments (Figure 1).

Chlamydomonas Strains, Culture Conditions, and Chloroplast Transformation

The ΔpetA strain, which contains a deletion in the coding region of the *petA* gene, was kindly provided by F.-A. Wollman (Institut de Biologie Physico-Chimique, Paris; Kuras and Wollman, 1994). P17 (Stern et al., 1991) is a wild-type *Chlamydomonas* strain derived from CC373 (ac-u-c-2-21; Shepherd et al., 1979) by bombardment with the *atpB* gene and was used as the recipient strain to create the *petA* mutant strains.

Chlamydomonas strains were grown in YA, HSA, or HS medium (Harris, 1989) under continuous medium-intensity light (5 to 8 W/m^2). YA and HSA media contain acetate and support nonphotosynthetic growth. HS medium lacks acetate, and cells require photosynthesis for growth.

petA initiation codon mutations were introduced into the chloroplast genome by cotransformation (Kindle et al., 1991) with the pCrBH4.8 (spectinomycin-resistant) plasmid. A 2:1 ratio of *petA* DNA (the pBRV3.8 series) to pCrBH4.8 was used, and the *petA* pBRV3.8 plasmids were linearized with BamHI to increase cotransformation efficiency.

Chlamydomonas DNA Minipreps and Polymerase Chain Reaction

Approximately 10^4 cells were scraped from a plate and resuspended in 100 μL of proteinase K buffer (10 mM Tris-HCl, pH 8, 10 mM EDTA, 10 mM NaCl). SDS was added to a final concentration of 2.5%, and proteinase K was added to a final concentration of 200 μg/mL. The samples were incubated at 55°C for 1 hr and extracted with phenol-chloroform, and the DNA was precipitated with ethanol. The DNA was

dissolved in 15 μL of H_2O, and 5 μL was used for polymerase chain reaction (PCR).

The positions of the primers petA4 and petA5, which were used for screening transformants, are shown in Figure 2A. Their sequences are as follows: petA4, 5′-GCTAGAATAAACTTGTTGAGGC-3′, corresponding to nucleotides −197 to −175 with respect to the initiation codon, and petA5, 5′-CTGCCGGAACGCGATCTGG-3′, corresponding to nucleotides +348 to +366.

PCR was performed in a volume of 50 μL using 1.5 mM $MgCl_2$, 40 μM deoxynucleotide triphosphates, 200 ng of each primer, and two units of Promega Taq polymerase for each reaction. Thirty cycles of amplification were performed at an annealing temperature of 55°C and a polymerization temperature of 72°C.

Chlamydomonas Total Protein Preparation and Immunoblotting

Protein preparation and immunoblotting were performed as described previously (Chen et al., 1993). Antibodies raised against spinach CF1-β (β subunit of the chloroplast ATPase) were obtained from R. McCarty (Johns Hopkins University, Baltimore, MD) and used at a 1:100,000 dilution. To raise antibodies against *Chlamydomonas* cytochrome *f*, a HindIII-AccI fragment from the coding region of the *petA* gene was filled in with the Klenow fragment of DNA polymerase I and inserted into the SmaI site of the fusion protein vector pGEX-2T, which generates glutathione *S*-transferase fusion proteins in *Escherichia coli* (Smith and Johnson, 1988). *E. coli* cells were induced with 0.15 mM isopropyl β-D-thiogalactopyranoside for 2 hr, resuspended in SDS sample buffer, boiled for 5 min, and loaded into preparative 10% SDS–polyacrylamide gels. The induced fusion protein band was excised from the gel, electroeluted, and quantified by SDS-PAGE using BSA as a standard. Fusion protein (120 μg) was initially injected into a rabbit, followed by two boosts of 50 μg each. The crude serum was tested by immunoblotting on filters containing total proteins from wild-type and ΔpetA strains. The crude serum reacted with a prominent protein of the expected size in the wild-type strain but not in the ΔpetA strain; two very faint additional signals were present in both strains. Immunoblots were incubated with a 1:5000 dilution of the crude serum. After incubation with the antiserum, the blots were reacted with horseradish peroxidase–linked secondary antibodies, and the signals were visualized with the Enhanced Chemiluminescence (ECL) kit from Amersham. For some experiments, ^{125}I-protein A was used instead of the horseradish peroxidase–linked secondary antibodies. The signals were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RNA Isolation and RNA Filter Hybridizations

Cells were grown to mid-log phase in HSA medium. RNA was isolated and filter hybridizations were performed as described previously (Stern et al., 1991). RNA accumulation was quantified using a PhosphorImager. The *atpB* probe was a PCR product amplified from the plasmid p17 using primers DBS2 and NS1b. DBS2, 5′-GACCGTATACAAGAGCTAC-3′, corresponds to nucleotides −454 to −470 relative to the initiation codon of *atpB*. NS1b, 5′-GACCGTATACAAGAGCTAC-3′, corresponds to nucleotides 1471 to 1453 relative to the initiation codon of *atpB*. The *petA* probe was a HindIII-PvuII fragment located in the coding region.

In Vivo Pulse Labeling and Immunoprecipitations

Chlamydomonas cells were labeled for 5 min with ^{35}S-Na_2SO_4 in the presence of cycloheximide, as described by Sakamoto et al. (1994) After labeling, cells were washed in 1 mL of 50 mM sodium-Tricine containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 mM ε-amino-*n*-caproic acid, 50 μg/mL aprotinin, and 1 mM EDTA. Cells were then resuspended in 100 μL of lysis buffer (50 mM Tris-HCl, pH 8, 2% SDS, 5% β-mercaptoethanol) and boiled for 2 min. The lysate was centrifuged at 12,000*g* in a microcentrifuge for 3 min to remove insoluble material. The supernatant was diluted 20-fold with dilution buffer (1 mM phenylmethylsulfonyl fluoride, 2% Nonidet P-40, 2 mM EDTA, 5 mM ε-amino-*n*-caproic acid, and 1 mM benzamidine in TBS [0.5 M NaCl, 20 mM Tris-HCl, pH 7.5]). Five microliters of preimmune serum was added to the lysate and incubated at 4°C for 1 hr. Fifty microliters of *Staphylococcus aureus* cells washed with the dilution buffer lacking the protease inhibitors was then added, and incubation was continued for 30 min. The cells were collected in a microcentrifuge, and the supernatant was transferred to a new tube. Five microliters of cytochrome *f* antibodies were added to the supernatant, and the mixture was incubated on a rocking platform at 4°C overnight. Fifty microliters of *S. aureus* cells was then added to the mixture, and incubation was continued for 30 min. The cells were then collected in a microcentrifuge. The cell pellet was washed with the following buffers: 1 mL of dilution buffer without protease inhibitors for two washes, followed by 1 mL of 10 mM Tris-HCl, pH 7.5/0.1% Nonidet P-40 for the third wash. The cells were resuspended in 20 μL of 1.5 × SDS sample buffer, boiled for 5 min, and loaded into 12% SDS–polyacrylamide gels. One-fifth of the amount of extract used for immunoprecipitation was electrophoresed to quantify total labeled proteins.

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