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Evidence for Nuclear Control of the Expression of the *atp*A and *atp*B Chloroplast Genes in Chlamydomonas

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We analyzed three nuclear mutants of *Chlamydomonas reinhardtii* altered in the expression of the chloroplast genes *atp*A or *atp*B coding for the α or β subunit of the chloroplast ATP synthase. These mutants revealed the existence of three nuclear products controlling the expression of the two chloroplast genes: the first one acts on the translation of the *atp*A transcript, and the two others act specifically on the stability of either the *atp*B or the *atp*A mRNAs. The nuclear mutation responsible for the decreased stability of the *atp*B mRNA prevented translation of the corresponding polypeptide. In contrast, the mutation responsible for the decreased stability of the *atp*A mRNA had limited effect on the translation of the α subunit, thereby allowing its accumulation and assembly in an active ATP synthase. Although acting originally on the expression of only one of the two main coupling factor 1 subunits, the three mutations caused a change in the translation rate of the other subunit, as viewed in 5-min pulse labeling experiments. This is indicative of a concerted expression of the α and β subunits at an early post-translational step, or during translation, that may be critical for the assembly of the chloroplast ATP synthase.

INTRODUCTION

The major proteins of the photosynthetic apparatus in the chloroplast are multisubunit protein complexes that comprise subunits encoded by both the chloroplast and nuclear genomes. How the synthesis of these various subunits is coordinated to allow proper assembly in a protein complex is still poorly understood. In higher plants, the chloroplast genes encoding subunits of the ATP synthase are organized in two transcriptional units within the same single-copy region of the chloroplast genome: *atp*B (subunit β) plus *atp*E (subunit ε) are in one unit, and *atp*I/*atp*H/*atp*F/*atp*A (subunits IV/III/I/α) are in the other (Westhoff et al., 1981; Cozens et al., 1986; Hennig and Herrmann, 1986). This organization suggests that the coordinated expression of these chloroplast genes may involve transcriptional regulations. This is not the case in the unicellular green alga *Chlamydomonas reinhardtii*, where the genes are dispersed around the entire chloroplast genome on either of its two single-copy regions (Woessner et al., 1987).

Both the intrinsic (coupling factor 0 [CF_0]) and catalytic (coupling factor 1 [CF_1]) sectors of the chloroplast ATP synthase result from the assembly of chloroplast- and nuclear-encoded subunits. The biogenesis of the chloroplast ATP synthase therefore requires some coordination in the expression of the nuclear and chloroplast genes encoding the various subunits of the complex. Several nuclear genes have already been shown to control the expression of photosystem II (PSII) and PSI chloroplast genes in Chlamydomonas: nuclear factors would be required for the stabilization and for the translation of mRNAs of chloroplast-encoded PSII subunits (Jensen et al., 1986; Kuchka et al., 1988; Kuchka et al., 1989; Rochaix et al., 1989; Sieburth et al., 1991). The nuclear control on the mRNA processing of one of the two main PSI subunits has also been extensively studied (Choquet et al., 1988; Goldschmidt-Clermont et al., 1990). In contrast, the targets for the action of nuclear gene products controlling the synthesis of chloroplast-encoded subunits of the ATP synthase have not yet been characterized.

In a previous study, Lemaire and Wollman (1989a, 1989b) have characterized the nine subunits of the chloroplast ATP synthase complex in Chlamydomonas and studied some aspects of the enzyme assembly by comparing the patterns of synthesis and accumulation of its subunits in various mutants defective in photophosphorylation. Among them were two nuclear mutants (F54 and thm24) affected in the synthesis of the α and/or β subunits. The phenotype of these mutants pointed to the existence of a nuclear control on the expression of the chloroplast-encoded subunits of the complex.

In this study, we have analyzed at the transcriptional and post-transcriptional levels three nuclear mutants of Chlamydomonas, F54, thm24, and ncc1, which display specific alterations in the expression of the chloroplast-encoded α and β subunits. We report that distinct nuclear gene products are required for the stabilization of the *atp*A and *atp*B transcripts as well as for the translation of the *atp*A transcript. Moreover, this comparative study shows that the synthesis of the α and β subunits are closely coordinated at the translational step.

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RESULTS

Comparative Analysis of the Contents in α and β Subunits in the Wild-Type and Nuclear Mutant Strains

It has been previously reported that mutants F54 and thm24 were unable to assemble active chloroplast ATP synthase (Bennoun and Chua, 1976; Piccioni et al., 1981). Accordingly, their thylakoid membranes were shown to be deficient in the nine constitutive subunits of CF_1 and CF_0 (Lemaire and Wollman, 1989b). Most of these subunits can be viewed only after two-dimensional electrophoresis because of numerous comigrating polypeptides (Lemaire and Wollman, 1989a). However, urea SDS-PAGE allows direct observation of the α, β, and γ subunits. This is shown in Figure 1, where the absence of the α, β, and γ subunits can be noted among the Coomassie blue staining pattern of the F54 thylakoid membrane polypeptides. In contrast, thylakoid membranes from mutant ncc1 only displayed a decreased content in the α, β, and γ subunits (compare with the light-harvesting complex, and P9 and P10 contents in the wild-type strain and ncc1). Consistently, mutant ncc1 grew in phototrophic conditions at the same rate as the wild type (results not shown), whereas mutants thm24 and F54 were acetate-requiring (ac^-) strains. The ncc1 phenotype arose from a single nuclear mutation, as detailed below in Genetic Analysis.

The extent of accumulation of the α and β subunits in whole cells of these various strains can be more accurately compared on the immunoblots shown in Figure 2. As previously reported (Lemaire and Wollman, 1989b), neither the α nor the β subunit was detected in thm24 cells, whereas the β subunit (25% of the wild-type amount), but not the α subunit, was observed in F54 cells (Figure 2A). The cellular accumulation of both the α and β subunits in mutant ncc1 was ~50% of the wild-type amount (Figure 2A). As shown in Figure 2B, these were assembled on the ncc1 thylakoid membranes in the same stoichiometry as in the wild type leading to a membrane binding of half of the α+β subunits found in the wild-type membranes. This indicated that the ncc1 mutation, although changing the extent of cellular accumulation of the α and β subunits, did not alter the assembly and membrane binding of the CF_1 subunits.

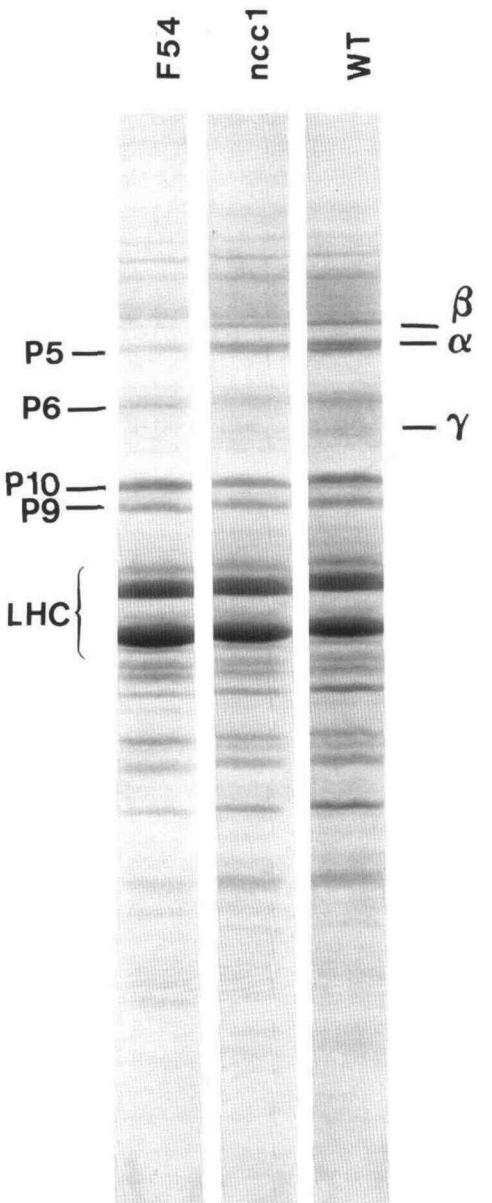


Figure 1. Thylakoid Membrane Proteins from Mutant F54, Mutant ncc1, and the Wild-Type Strain Stained with Coomassie Blue.

Solubilized membrane proteins were separated by denaturing gel electrophoresis (12 to 18% polyacrylamide in the presence of 8 M urea). Equal amounts of chlorophylls were loaded into the wells (compare for example P9, P10, or light-harvesting complex [LHC] bands). Easily detectable bands corresponding to ATP synthase polypeptides are indicated on the right and bands corresponding to PSII polypeptides are indicated on the left as references. WT, wild type.

Rates of Synthesis of the α and β Subunits in the Wild-Type and Nuclear Mutant Strains

We studied the rates of synthesis of the two subunits in vivo by pulse labeling the cells for 5 min with ^{14}C-acetate in the presence of an inhibitor of cytoplasmic protein translation. The rates of synthesis of the α and β subunits can be compared in Figure 3, taking the rate of synthesis of the PSII core subunit P5 as a reference. In the wild type, the band corresponding to the β subunit was labeled less than that corresponding to the α subunit, suggesting that the rate of synthesis of the β subunit was lower than that of the α subunit. F54 cells showed no synthesis of the α subunit but displayed a significant increase in the rate of synthesis of the β subunit when compared

to that in the wild type. The thm24 cells failed to synthesize the β subunit. However, close examination of the P5 region in this mutant showed a faint band above P5, in the position of the α subunit. Therefore, mutant thm24 still showed some synthesis of the α subunit although at a highly reduced rate. The rates of synthesis of the α and β subunits in mutant ncc1 were markedly different from those in the wild type: the rate of synthesis of the β subunit was higher than that of the α subunit in the mutant, in contrast with what we observed in the wild-type cells. Taking as a reference the rate of synthesis of P5, the rate of synthesis of the β subunit increased about four times in mutant ncc1 as compared to that in the wild type. The rate of synthesis of the α subunit decreased in a similar proportion.

Last, we noted a remarkable increase in the rate of synthesis of the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Figure 3) in mutants F54 and thm24 but not in mutant ncc1. We observed a similar increase in the rate of synthesis of the Rubisco large subunit in various photosynthesis mutants lacking either PSII, cytochrome b_6f, or PSI protein complexes (results not shown).

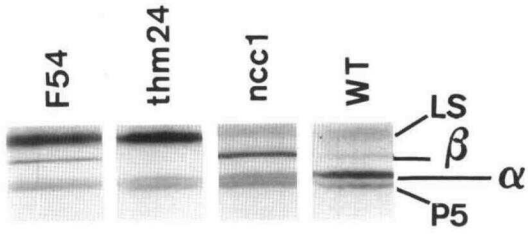


Figure 3. Autoradiogram of Chloroplast Translates (50- to 70-kD Region) from Cells Pulse Labeled with ^{14}C-Acetate for 5 min.

Cells were labeled in the presence of cycloheximide (6.6 μg/mL); polypeptides were separated by SDS-urea gel electrophoresis and visualized by autoradiography. Note the absence of synthesis of the α subunit in mutant F54 and of the β subunit in mutant thm24; rates of synthesis of the α and β subunits vary in opposite directions between mutant ncc1 and the wild type (WT). Also indicated are P5 (a PSII core subunit) and LS (the Rubisco large subunit).

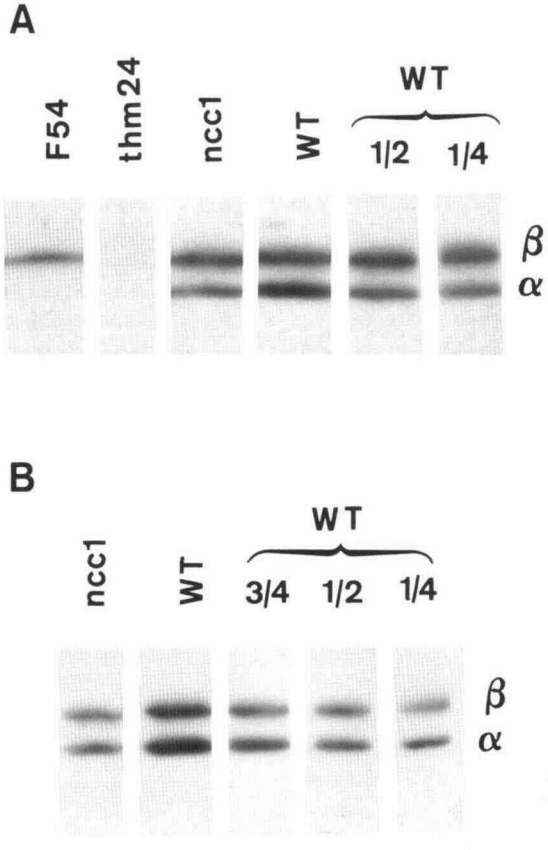


Figure 2. Accumulation of the α and β Subunits in the Cells or Membranes of the Wild Type and Mutants F54, thm24, and ncc1.

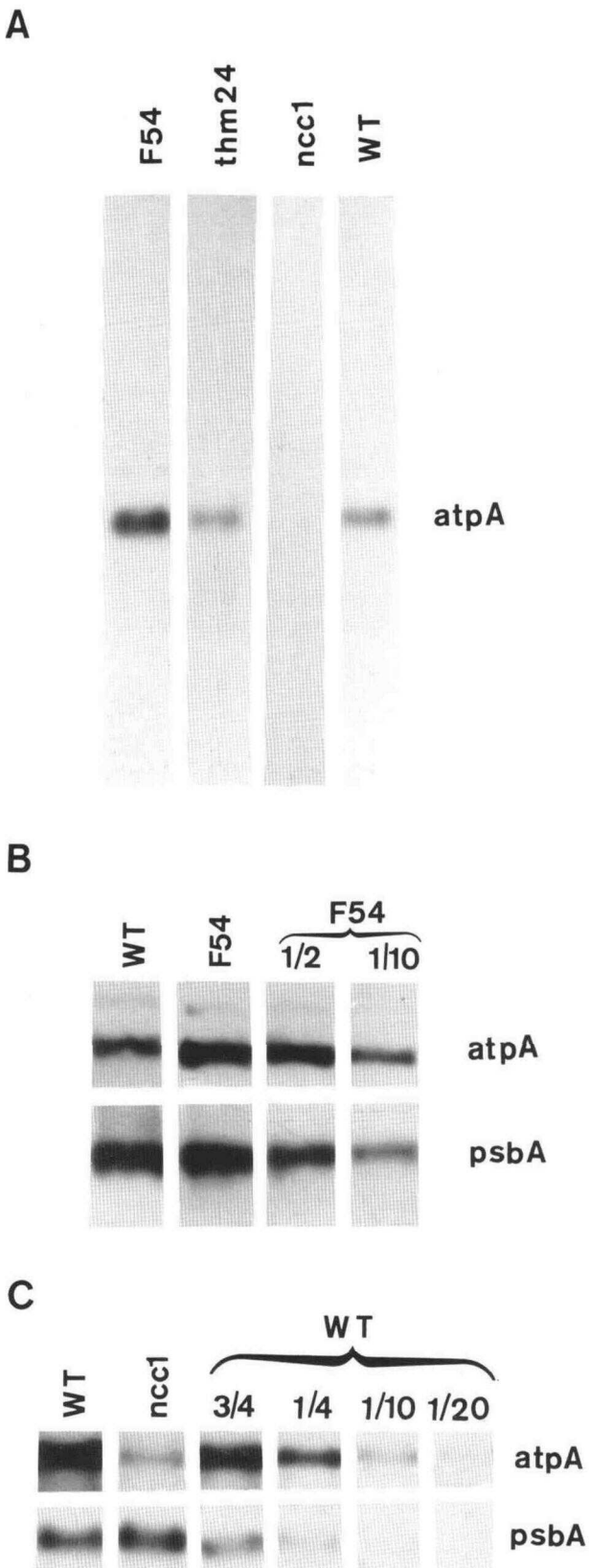
(A) Immunoblots of whole cell protein extracts probed with a mixture of anti-α plus anti-β antibodies. Several dilutions of the wild-type sample were loaded to quantify the decrease in the two subunits in the mutants.

(B) Immunoblots of thylakoid membrane polypeptides from the wild type and mutant ncc1. The conditions are the same as given in **(A)**. WT, wild type.

Accumulation of the *atp*A and *atp*B Transcripts in the Three Mutants

To determine at which step the synthesis of the two subunits was impaired in this set of nuclear mutants, we carried out an RNA gel blot analysis of total RNAs, using *atp*A- and *atp*B-specific probes. The results are shown in Figures 4 and 5. We observed that the 2.2-kb transcript, which corresponds to the α subunit mRNA (Hallick, 1984), was present in similar amounts in mutant thm24 and in the wild type (Figure 4A). The *atp*A transcript was also present in mutant F54 (Figure 4A). Because there was no synthesis of the α subunit in this latter strain, we concluded that mutant F54 displayed a block in *atp*A gene expression at the translational step. It is readily apparent from the data in Figure 4A that the *atp*A transcript was more abundant in mutant F54 than in the wild type. This was further quantified in Figure 4B and corresponded to a fourfold increase in the accumulation of *atp*A transcripts. Thus, the translational block in mutant F54 was accompanied by an increased half-life of the *atp*A transcripts.

Surprisingly, the *atp*A transcript was not detectable in mutant ncc1 at the exposure times given in Figure 4A, although this strain displayed significant synthesis and accumulation of the α subunit. However, after prolonged exposure of the autoradiogram, trace amounts of the *atp*A transcript were detected (Figure 4C). Figure 4C shows that only one-tenth of the mature message present in the wild type remained in mutant ncc1. In contrast, the *psb*A gene transcript for polypeptide D_1 (Figure 4C) and the *rbc*L gene transcript for the Rubisco large



subunit (data not shown) were found in equivalent amounts in the two strains. Thus, the mutation in ncc1 acted on *atp*A gene expression at a transcriptional or post-transcriptional step.

Using an *atp*B-specific probe, we observed that the β subunit mRNA, a 1.9-kb transcript (Woessner et al., 1986), was present in similar amounts in the wild-type strain and in mutants F54 and ncc1 (Figure 5A). No 1.9-kb *atp*B transcript was detected in mutant thm24, even after prolonged exposure of the autoradiogram (Figure 5B). The situation was similar to that observed in mutant FUD50, a strain carrying a 2.3-kb deletion covering the 3′ end of the *atp*B gene (Woessner et al., 1984) and used here as a control (Figure 5B). Thus, the mutation in thm24 acted on *atp*B gene expression at a transcriptional or post-transcriptional step.

Transcription of *atp*A or *atp*B Genes in Permeabilized Cells of the Wild Type and Mutant ncc1 or thm24

The absence of *atp*B transcript in mutant thm24 as well as the drop in *atp*A transcript in mutant ncc1 could have originated from an impairment of transcription of the *atp*B and *atp*A genes. Therefore, we carried out a 7-min ^{32}P-UTP pulse labeling of RNAs in mutant ncc1 and wild-type strains using toluene-permeabilized cells (see Methods). One hundred nanograms of either an intragenic *atp*A probe or an intragenic *psb*D probe, used as a control, was immobilized on nylon filters and hybridized with transcription products from the two strains. As shown in Figure 6A, no difference was detected between ncc1 and wild-type samples: the *atp*A gene was transcribed at the same rate in the permeabilized cells of the two strains.

A similar RNA pulse labeling experiment was performed with permeabilized cells from mutant thm24. Analyses of the wild type and FUD50 mutant were included as controls. Transcription products from each strain were divided into three aliquots and hybridized with three different regions of the *atp*B DNA fragment immobilized on separate nylon filters: probe 1 was used to detect the 5′ end of the transcript, probe 2 was an intragenic probe located in the middle of the gene, and probe 3 was used to detect the 3′ end of the transcript (Figure 6B). The results are presented in Figure 6C. Probing the transcripts

Figure 4. Accumulation of the *atp*A Transcript in the Wild Type and Mutants F54, thm24, and ncc1.

Equivalent amounts (10 μg) of total RNA were electrophoresed on a formaldehyde-agarose gel, transferred to a nylon membrane, and probed with an intragenic labeled fragment of the *atp*A gene. A specific *psb*A probe was also used as a loading and transfer control.
(A) Comparative analysis was made of the mRNA from the α subunit in the three mutants and the wild type.
(B) A dilution series of total RNA from mutant F54 was made to estimate the increase in *atp*A transcripts in this strain.
(C) A dilution series of total RNA from the wild type was made to estimate the decrease in *atp*A transcripts in mutant ncc1.
WT, wild type.

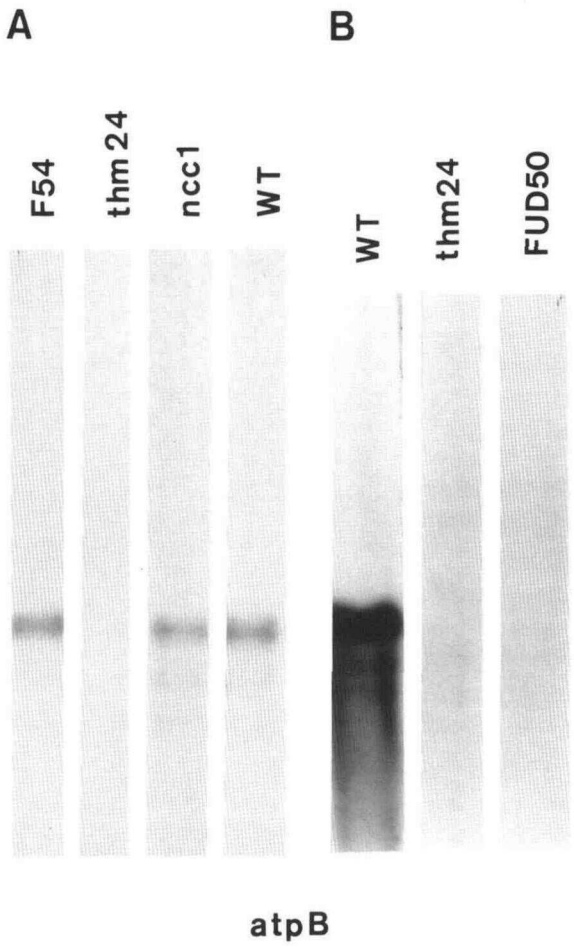


Figure 5. Accumulation of the *atp*B Transcript in the Wild Type and Mutants F54, thm24, and ncc1.

The experimental conditions are as given in Figure 4, using a specific *atp*B probe.
(A) Comparative analysis was made of the mRNA from the β subunit in the three mutants and the wild type.
(B) Mutant FUD50, which bears a deletion in the *atp*B gene, was used as a control. Overexposed film shows that no trace of mature *atp*B transcripts was detectable in either mutant thm24 or mutant FUD50. WT, wild type.

from mutant FUD50 showed that transcription of the *atp*B gene was initiated (Figure 6C, probe 1, lane containing FUD50), but no hybridization was visible with probe 2 or 3, due to the deletion carried by this mutant. In the case of mutant thm24, the hybridization with the three probes was similar to that in the wild type (Figure 6C). Thus, the nuclear mutation in mutant thm24 did not alter the transcription rate of the *atp*B gene in permeabilized cells.

Because we detected no difference at the transcriptional level between the two nuclear mutants ncc1 and thm24 and the wild-type strain, we concluded that there was a selective destabilization of the *atp*A and *atp*B transcripts in mutants ncc1 and thm24, respectively. These phenotypes could have resulted from the absence or inactivation of nuclear *trans*-acting factors binding to the mature transcripts and protecting them from nucleolytic degradation.

Characterization of the Transcript Destabilization by the ncc1 and thm24 Mutations

There is increasing evidence that the 3′ end of chloroplast mRNAs plays a critical role in their stability (Stern et al., 1989, 1991). We wondered whether the destabilization of *atp*A transcript in mutant ncc1 or *atp*B transcript in mutant thm24 was due to an increased access to a 3′–5′ exonuclease. Therefore, we performed S1 nuclease protection experiments using probes hybridizing at the 5′ end of the transcripts. In case of a 3′ end nucleolytic degradation, we expected to trap the mature transcripts as well as shorter transcripts being degraded from their 3′ end in the mutants. This should result in an apparent deficiency in *atp*A or *atp*B messages in the mutants smaller than that observed by conventional RNA gel blot analysis.

Increasing amounts of total RNAs from mutant ncc1 or the wild type were mixed with the labeled R15.0 subfragment of R15 (Dron et al., 1982). This fragment comprises both the *atp*A gene region complementary to the 5′ end of its transcript on one strand and the *rbc*L gene region complementary to the 5′ end of its transcript on the other strand, as illustrated in Figure 7A. Because we used the double-stranded DNA fragment as a probe, both the *atp*A and *rbc*L transcripts could be detected; the latter was used as a control in this experiment. Both *atp*A and *rbc*L probes showed protection from S1 nuclease action when hybridized with total RNAs from the ncc1 and wild-type strains (Figure 7B).

Quantification of the amount of mature plus truncated transcripts (still containing their 5′ end region) in the two strains was performed by scanning the autoradiograms. In each set of protection experiments in which we used low amounts of total RNAs from the two strains ($\leq$2.5 μg), we observed that protection of the *rbc*L probe was considerably higher than that of the *atp*A probe. This indicated that *rbc*L transcripts were particularly abundant in the two strains, an observation that is consistent with the much higher transcription rate for *rbc*L than for *atp*A or *atp*B genes (Blowers et al., 1990).

We observed that the labeled *atp*A DNA probe was fully protected from degradation in the presence of 10 to 25 μg of total RNA from the wild type, whereas saturation was not yet reached with 25 μg of total RNA from mutant ncc1. The graph plotted with the samples corresponding to the lowest amounts of total RNA added (corresponding to the linear part of the saturation curve) allowed us to estimate the amount of transcripts with their 5′ ends protected in mutant ncc1 (Figure 7C). The slope of the saturation curve was three times larger with the wild type than with mutant ncc1: there was then a threefold decrease in the amount of mature plus truncated *atp*A

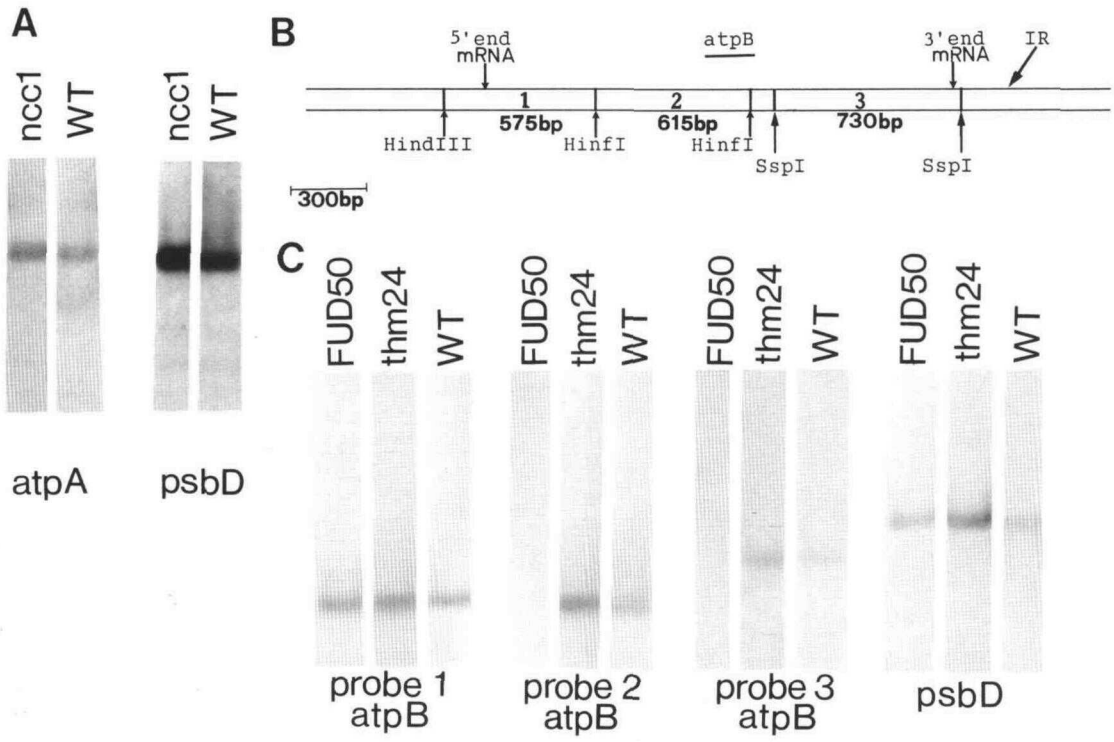


Figure 6. Transcription of *atp*A, *atp*B, and *psb*D Genes in Permeabilized Cells of the Mutant and Wild-Type Strains.

(A) Pulse labeled RNAs from mutant ncc1 and the wild type hybridized with *psb*D and *atp*A DNA fragments. After RNA pulse labeling in vivo for 7 min with α-^{32}P-UTP, total RNAs were extracted from permeabilized cells and hybridized to nylon filters with an excess of immobilized probes.
(B) Schematic representation of the three DNA fragments (parts of the *atp*B gene) chosen for the analysis of the labeled RNAs in mutant thm24, mutant FUD50, and the wild type. IR, inverted repeat.
(C) Pulse labeled RNAs from mutants thm24 and FUD50 and the wild type hybridized with the three different *atp*B probes and the *psb*D probe. Parallel hybridizations using different nylon filters were made with *atp*B DNA fragments complementary to probe 1 (the 5′ end of *atp*B transcript), probe 2 (an internal part of *atp*B transcript), and probe 3 (the 3′ end of *atp*B transcript). The *psb*D probe was used as a control.
WT, wild type.

transcripts in mutant ncc1 as compared to that in the wild-type strain. This must be compared with the 10-fold decrease in *atp*A transcripts when only the mature transcripts were detected by RNA gel blot analysis. S1 nuclease protection experiments, using a probe complementary to the 3′ end of the *atp*A transcript, similarly showed a 10-fold decrease in *atp*A transcripts in mutant ncc1 (results not shown). Thus, we concluded that a degradation process of the *atp*A transcripts occurred from their 3′ end in mutant ncc1.

We also carried out an S1 protection experiment with total RNA from mutant thm24, mutant FUD50, and the wild type using the DNA fragment comprising the *atp*B gene region hybridizing to the 5′ end of the transcript (probe 1, previously described in the in vivo RNA pulse labeling experiment; Figure 6B). Figure 8 shows that as much as 50 μg of total RNA from either mutant thm24 or mutant FUD50 was unable to protect this fragment from S1 nuclease digestion. In contrast, significant protection was detected with only 5 μg of total RNA from the wild type. Extensive destabilization of the truncated *atp*B transcripts in mutant FUD50 was expected to occur due to the absence of the 3′ end stabilizing region (Figure 8). The *atp*B transcripts of mutant thm24 detected in the pulse-labeling experiments were also subjected to a dramatic destabilization that was by far larger than that for *atp*A transcripts in mutant ncc1.

Genetic Analysis: The Three Nuclear Mutations Reside in Three Different Genes

Whereas the two ac$^-$ mutant strains (F54 and thm24) were known to bear a single nuclear mutation (Woessner et al., 1984), mutant ncc1 had not been characterized previously. As described above, the primary effect of mutation ncc1 is the extensive decrease in *atp*A transcript. Evidence for its nuclear origin is presented in Figure 9. The heredity of this mutation

was studied by RNA gel blot analysis of five tetrads obtained in a cross of ncc1 × the wild-type strains. In each tetrad, a Mendelien segregation ratio was observed.

In an attempt to determine the localization of the three mutations, we performed several crosses between the three mutant strains. In the cross F54 × thm24, 18 tetrads were tested and compared for growth, either in heterotrophic conditions (medium containing acetate as a carbon source) or in phototrophic conditions (minimal medium, acetate omitted). Three types of tetrads were observed and are as follows: 4 ac$^-$ clones (3 tetrads), 3 ac$^-$ clones/1 non–acetate-requiring (ac$^+$) clone (10 tetrads), and 2 ac$^-$ clones/2 ac$^+$ clones (5 tetrads). These were interpreted, respectively, as parental ditype (PD), tetratype (T), and nonparental ditype (NPD) tetrads. Because frequencies of PD and NPD tetrads were similar, we concluded that the two mutations were in two independent genes. In the crosses involving mutant ncc1, the segregation of the ac$^-$ mutation (F54 or thm24) in the progeny was tested on minimal medium, and the segregation of the ncc1 mutation was further tested on ac$^-$ clones by RNA gel blot analysis for the *atp*A transcript. From the cross F54 × ncc1, 5 of 7 ac$^-$ clones were also deficient in *atp*A mRNA. From the cross thm24 × ncc1, 11 of 19 ac$^-$ clones were also deficient in *atp*A mRNA. These results were consistent with an independent segregation of two nuclear genes. We thus conclude that the three mutations were localized in three different nuclear genes that segregated independently.

We then selected three double mutant strains as either ac$^-$ clones in an NDP tetrad from the cross F54 × thm24, or as an ac$^-$ clone deficient in *atp*A mRNA from the crosses F54 × ncc1 and thm24 × ncc1. We found no evidence for an interaction between the nuclear factors mutated in the various single mutants: the three double mutants had mainly the combined phenotypes of the single mutants used in each cross (results not shown).

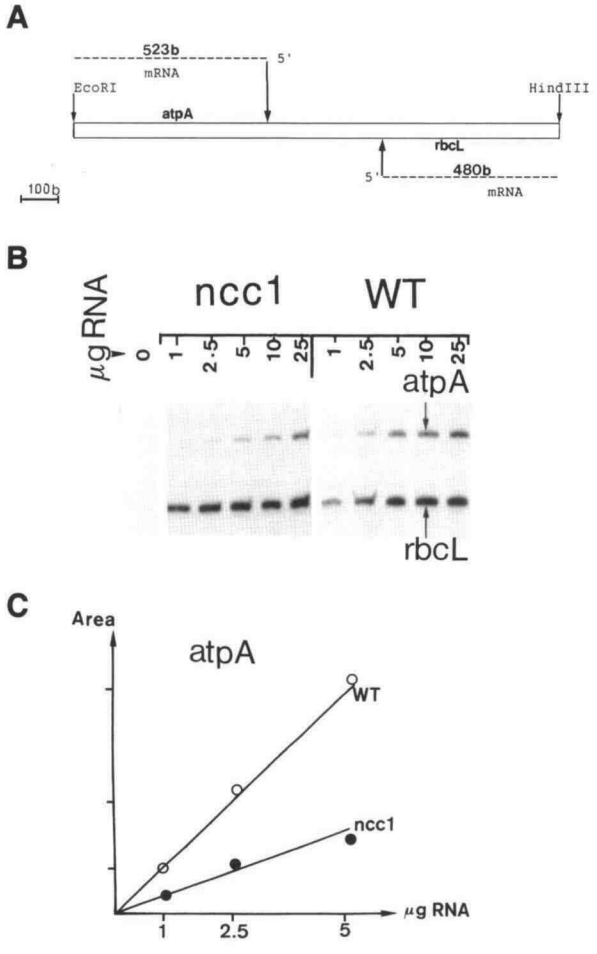


Figure 7. S1 Nuclease Protection of the 5′ End of the *atp*A Transcript from Mutant ncc1 and the Wild Type.

(A) Schematic representation of the DNA fragment (the R15.0 fragment [Dron et al., 1982]) and of the S1-resistant RNA fragments in the experiment shown in **(B)**. b, base pair.

(B) Autoradiogram of the sequencing gel. Labeled R15.0 fragment (10 ng) was mixed with yeast tRNA only (first lane, control), then with increasing amounts of total RNA from each strain (mutant ncc1 or the wild type).

(C) The graph was plotted after scanning the autoradiograms using only samples below saturation for the protection of the labeled fragment. WT, wild type.

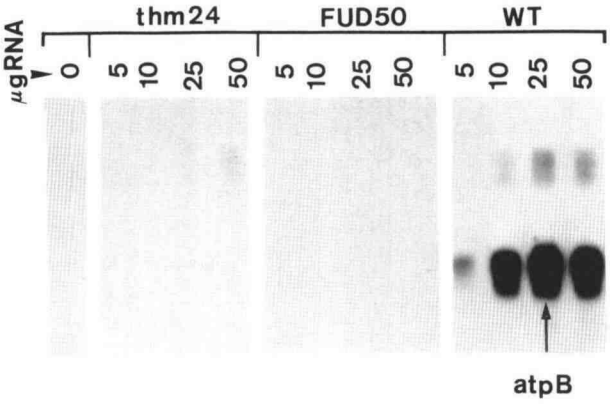


Figure 8. S1 Nuclease Protection of the 5′ End of the *atp*B Transcript in Mutant thm24, Mutant FUD50, and the Wild Type.

The labeled DNA fragment was probe 1 that had already been used in the pulse labeled RNA experiment (Figure 6B). The first lane corresponds to yeast tRNA mixed with the labeled DNA fragment (10 ng); subsequent lanes correspond to increasing amounts of total RNA from each strain (mutant thm24 or mutant FUD50 and the wild type) mixed with the same amount of the labeled DNA fragment (10 ng).
WT, wild type.

DISCUSSION

This study demonstrates the existence of three distinct nuclear products participating in the expression of the chloroplast-encoded *atp*A and *atp*B genes in Chlamydomonas. Tables 1

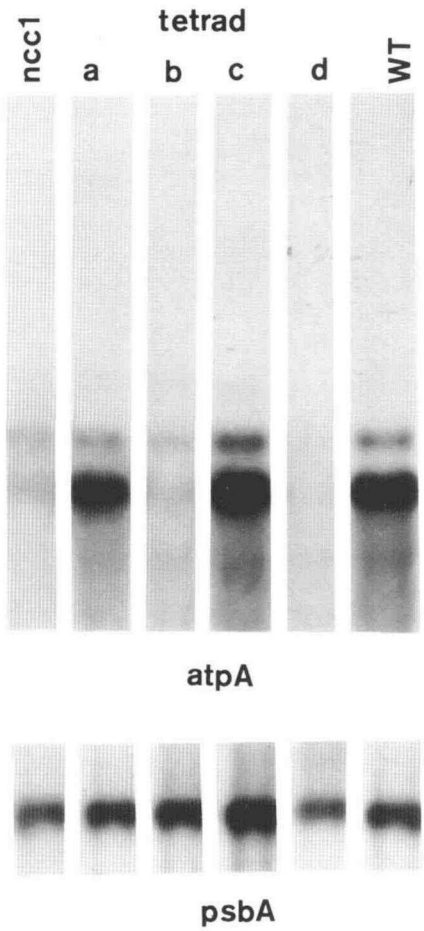


Figure 9. RNA Gel Blot Analysis of Four Clones from a Typical Tetrad Obtained by Crossing Mutant ncc1 and the Wild Type.

Note that the overexposure of the autoradiograms reveals a minor band migrating more slowly than the *atp*A transcript clearly visible when minipreparations of RNAs were used. WT, wild type.

and 2 summarize the main phenotypic properties of these mutant strains: mutation in F54 alters primarily a nuclear product acting on the expression of the α subunit at the translational level; mutations in ncc1 and thm24 cause primarily a destabilization of the *atp*A and *atp*B mRNAs, respectively (Table 1). In addition, the three mutations had secondary effects leading to a change in the expression of both the α and β subunits (Table 2). Genetic analysis of the mutant strains showed that the three nuclear mutations were in three different nuclear genes dispersed on the nuclear genome. Thus, our study adds to the growing body of experimental evidence that a number of chloroplast genes are expressed under nuclear controls operating at the level of transcript stability (Jensen et al., 1986; Kuchka et al., 1989; Sieburth et al., 1991) or at the level of translation (Kuchka et al., 1988; Rochaix et al., 1989).

Translational Block in Mutant F54 Is Accompanied by Increased Accumulation of the *atp*A Transcript

We found no evidence for a synthesis of the α subunit in mutant F54. We cannot presently exclude the possibility that the nuclear mutation caused premature termination of translation of the α subunit or an extensive increase in the turnover of the polypeptide. However, two observations argue against these possibilities: (1) no labeled α subunit was detected in pulse labeling experiments, where the duration of the pulse was as short as 5 min; (2) no new translation products, of lower molecular weight than the α subunit, were detected among the chloroplast-encoded polypeptides pulse labeled in this mutant. It is therefore more likely that the nuclear factor mutated in mutant F54 is required for translation of the *atp*A transcripts.

An interesting feature of the nuclear mutant F54 was the increased accumulation of the untranslated *atp*A transcripts. A similar overaccumulation of untranslated transcripts has been previously reported for mutants of Chlamydomonas *nac1-11* and *nac1-18*, which do not synthesize polypeptide D2, a

Table 1. RNA Analysis of the Various ATP Synthase Mutants

			RNA Accumulation			
	RNA Synthesis		S1 Nuclease Protection		RNA Gel Blot	
Strain	*atp*A	*atp*B	*atp*A	*atp*B	*atp*A	*atp*B
WT[a]	1	1	1	1	1	1
F54	ND[b]	ND	ND	ND	4	1
thm24	1	1	1	0[c]	1	0
ncc1	1	1	1/3	ND	1/10	1
FUD50[d]	ND	init.[e]	ND	0	1	0

[a] One was arbitrarily chosen as a reference for each analysis in the wild type.
[b] ND, not done.
[c] 0, absent or below detection.
[d] Chloroplast mutant used as a control in this study.
[e] Initiation of the transcription occurs, but no mature *atp*B transcript is present.

Table 2. Protein Expression in the Various ATP Synthase Mutants

	Synthesis		Accumulation in Cells	
Strain	α Subunit	β Subunit	α Subunit	β Subunit
WT[a]	1	1[b]	1	1
F54	0[c]	3	0	¼
thm24	¼	0	0	0
ncc1	⅓	4	½	½
FUD50[d]	¼	0	0	0

[a] One was arbitrarily chosen as a reference for each analysis in the wild type.
[b] Note that the synthesis of the β subunit is lower than the synthesis of the α subunit in the wild type.
[c] 0, absent or below detection.
[d] Chloroplast mutant used as a control in this study.

polypeptide of the PSII core complex (Kuchka et al., 1988). This is in contrast with *Escherichia coli* where untranslated mRNAs are rapidly degraded (Cole and Nomura, 1986). Therefore, although chloroplast ribosomes are of a procaryotic type, the mechanisms governing transcript stability seem to differ widely between procaryotic cells and the chloroplast. This may be related to the lack of tight coupling between transcription and translation in the chloroplast (Hosler et al., 1989) at variance with the situation in *E. coli.* Klaff and Gruissem (1991) have recently reported that forcing mRNAs in a polysome-bound state in spinach chloroplasts decreased their half-lives, and they discussed the possibility that the polysomes delivered a specific nuclease. The translational block in mutant F54 may therefore originate from a decreased binding of the *atp*A transcripts to polysomes, thereby producing an impairment of the initiation of translation. In other instances, the 5′ untranslated part of the transcripts, implicated in the initiation of translation, has been shown to be the target site for the action of nuclear products: a nuclear mutation in Chlamydomonas, causing a block in the translation of the PSII core subunit P6, was suppressed by a chloroplast mutation located in a stem-loop region of the 5′ untranslated region of *psb*C, the gene encoding P6 (Rochaix et al., 1989). In yeast, three distinct nuclear gene products have been shown to act on the 5′ untranslated region of the mitochondrial *cox*III transcript, where they control translation of cytochrome *c* oxidase subunit III (Costanzo and Fox, 1988).

The thm24 Mutation Leads to a Drastic Destabilization of the *atp*B Transcript

The nuclear mutation in thm24 produced a dramatic depletion in *atp*B transcripts, preventing their detection by RNA gel blot analysis. We looked for a possible decrease in the transcription rate of *atp*B in mutant thm24 by ^{32}P-UTP pulse labeling using toluene-permeabilized cells. This approach, which prevents initiation of transcription and most likely stabilizes newly synthesized mRNAs (Kuchka et al., 1989; Sieburth et al., 1991), nevertheless allows detection of changes in transcription rates (Leu et al., 1990). In this study, we did not detect changes in the transcription rate of *atp*B in mutant thm24 as compared with that in the wild-type strain. Therefore, the thm24 mutation operates by decreasing the stability of the *atp*B message. Nuclear mutations causing such a destabilization of a chloroplast transcript have been previously identified in Chlamydomonas for *psb*D in mutant *nac2* (Kuchka et al., 1989), *psb*C in mutant 6.2z5 (Sieburth et al., 1991), and *psb*B in mutant GE2.10 (Jensen et al., 1986). In this study, S1 protection assays using probes for the 5′ end sequences of the *atp*B transcripts did not reveal any accumulation of such transcript fragments in mutant thm24. This could be indicative of a degradation process of the *atp*B transcripts occurring through their 5′ end. However, we similarly failed to detect 5′ ends of the *atp*B transcripts in S1 protection assays using the FUD50 chloroplast mutant. As demonstrated by RNA pulse labeling experiments using probes hybridizing either to the 3′ or 5′ end of the transcripts, the partial *atp*B gene deletion in FUD50 still allowed expression of *atp*B transcripts with intact 5′ ends but truncated at their 3′ end. The half-life of these truncated *atp*B transcripts was nevertheless short enough to prevent their detection in S1 protection assays using the 5′ end probe. This observation is easily interpreted in light of the study of Stern et al. (1991), which showed that the 3′ untranslated region of the *atp*B transcripts controlled their stability. Thus, the failure to detect 5′ end sequences of *atp*B transcripts in S1 protection assays using mutant thm24 does not preclude a possible destabilization from the 3′ end, as is the case in mutant FUD50.

The Destabilization of the *atp*A Transcript in Mutant ncc1 Allows Efficient Translation of the α Subunit

The nuclear mutation ncc1 also acted on transcript stability. In this mutant, the transcription rate of *atp*A was unaltered, but the half-life of the *atp*A transcripts was decreased by a factor of 10, as judged from RNA gel blot analysis. The destabilization of *atp*A transcripts in mutant ncc1 most likely occurred

through a nucleolytic degradation of the 3′ to 5′ ends. This conclusion arises from our comparison of the relative amounts in mature *atp*A messages (RNA gel blots) versus mature plus truncated *atp*A messages still containing their 5′ end (S1 nuclease protection experiments): the latter experiments yielded a decrease of only three times the amount of DNA fragments protected from S1 nuclease in mutant ncc1.

Interestingly enough, this extensive decrease in the accumulation of mature transcripts still allowed efficient translation of the α subunit. About half of the ATP synthase complexes present in the wild-type strain were assembled in mutant ncc1. This nuclear mutation is particularly remarkable because the transcript stability is dramatically altered with moderate consequences on the expression of the corresponding polypeptide. This type of mutation cannot be easily selected due to the rationale generally used for the isolation of photosynthesis mutants, which are selected as ac^- strains. In contrast, we obtained the ncc1 mutant strain from a spontaneous mutation while growing the wild-type strain in the laboratory. Mutant ncc1 showed the same growth rate as the wild type in phototrophic conditions. Although containing less chloroplast ATP synthase complexes on a chlorophyll basis, it had no detectable phenotype from a functional point of view.

The changes in *atp*A gene expression in mutant ncc1 resemble those observed for *atp*B in transformed cells of Chlamydomonas by Stern et al. (1991). Having made a series of deletions in the flanking region downstream of the *atp*B gene, these authors reported that some deletions, preventing formation of a stem/loop secondary structure in the 3′ untranslated region of the *atp*B transcripts, reduced the transcript amount by 80%, but still allowed 50% accumulation of the β subunit. Thus, the nuclear product altered by the ncc1 mutation could be an RNA binding protein that protects the 3′ untranslated region of the *atp*A transcripts from exonuclease digestion. The mutation in ncc1 may cause an increased turnover of the mRNA binding protein complex on its binding site, thus preserving the mature size of *atp*A transcripts when bound and delivering them to degradation when unbound. In this respect, it would differ from the mutation in mutant thm24, in which the mRNA binding site for a stabilizing nuclear-encoded protein may be entirely unprotected from nucleolytic attack.

It is presently unclear why a mutation of a 3′ end mRNA binding protein, decreasing transcript stability, improves translational efficiency of the remaining *atp*A transcripts. More than half of the *atp*A transcripts present in the wild type could be in a nontranslatable form, as has been suggested by Hosler et al. (1989). In this hypothesis, the stabilizing RNA binding protein mutated in ncc1 would be required for this conversion in a nontranslatable form of the transcripts. However, according to the hypothesis discussed in the next section, the presence of regulatory elements limiting translation of both *atp*A and *atp*B transcripts in the wild-type strain would provide an explanation for the improved translation of *atp*A transcripts in mutant ncc1.

Evidence for a Translational Coupling of the α and β Subunits

The rates of synthesis of the α and β subunits in the wild type were markedly different. Our pulse labeling studies indicated that the α subunit was synthesized in large excess over the β subunit. This cannot be accounted for by differences in transcript availability as both the rates of transcription and steady-state levels of the transcripts are similar for the two genes (Blowers et al., 1990; Leu et al., 1990). Therefore, this difference in the rates of protein synthesis rather points to a regulation at the translational level. Further regulation also occurs at the post-translational level because the relative amounts of α and β subunits accumulated in the wild-type cells are similar. Indeed, we have shown previously that the α subunits were less stable than the β subunits in whole cells of Chlamydomonas (Lemaire and Wollman, 1989b). Thus, in contrast to the suggestion of Stern et al. (1991), the α subunit rather than the β subunit has the highest turnover. In a previous study, we failed to detect the oversynthesis of the α subunit (Lemaire and Wollman, 1989b). What we have now identified as mutant ncc1 was at that time misused as the wild-type strain. The actual rates of synthesis of the α and β subunits in the wild-type strain are then similar to that in the various mutants studied so far, lacking either CF_0 or other CF_1 subunits, at variance with the conclusion we had reached previously (Lemaire and Wollman, 1989b).

In contrast, mutations altering the expression of either of the two main CF_1 subunits, α or β, had pronounced effects on the rate of synthesis of the other subunit. Upon 5 min of pulse labeling of the chloroplast-encoded proteins, the α subunit was barely visible in mutant thm24, which lacks synthesis of the β subunit. Pulse chase experiments provided no evidence for an increased turnover of the α subunit in this mutant (data not shown). Thus, we conclude that there is only marginal synthesis of the α subunit in the absence of the β subunit. A similar concerted expression of several chloroplast-encoded subunits of a same protein complex has been reported in Chlamydomonas for D2, D1, and P5, the three subunits of a protein complex intermediate in the biogenesis of the PSII core (Erickson et al., 1986; Jensen et al., 1986; de Vitry et al., 1989). In a thorough analysis of the rate of D1 translation in barley, Kim et al. (1991) recently showed that the ribosomes paused at specific sites during D1 synthesis. Ribosome pausing may facilitate cotranslational binding of cofactors, as suggested by these authors. It may also allow proper folding of the nascent chain interacting with neighboring subunits. The presence of the β subunit may then control the rate of translation of the α subunit through an interaction between the nascent α polypeptide chain and a β subunit nearby.

Surprisingly, mutants F54 and ncc1 that show impaired synthesis of the α subunit displayed a stimulation in the synthesis of the β subunit. Here again, the regulation in the expression of the β subunit took place at the translational level because

we observed no modification in the accumulation of the *atp*B transcripts in these mutant strains. That translation of the α subunit down regulates the synthesis of the β subunit could be related to the higher rate of translation of the α subunit than of the β subunit in the wild-type strain. We suggest that, at some stage of translation, the two transcripts compete for regulatory elements that are present in limiting concentration in the wild type and have a higher affinity for *atp*A transcripts. A larger proportion of regulatory elements would be available for translation of *atp*B transcripts both in mutant ncc1, deficient in *atp*A transcripts, and in mutant F54, where *atp*A transcripts would not associate with polysomes. This would provide the molecular basis for the increase in the rate of translation for the β subunit in the two mutants.

The presence of such regulatory elements that limit the translation of *atp*A and *atp*B transcripts in the wild-type strain would readily explain why there was an improved translation of the *atp*A transcripts remaining in mutant ncc1: being in lower amount than in the wild type, their translation would no longer be limited by the availability in these regulatory elements.

Conclusion

Besides a major post-translational regulation process, by which unassembled subunits of a thylakoid-bound protein complex are rapidly degraded, there are a number of upstream regulation steps that contribute to a fine-tuning of the expression of each chloroplast gene. This study illustrates the need for a concerted expression of different subunits of the same complex: the much higher rate of synthesis of the α subunit versus that of the β subunit makes up for the lower stability of the α subunit in cells, thereby allowing optimization of the assembly of the chloroplast ATP synthase. The fact that mutant ncc1, in which the rates of synthesis of these two subunits tend to be similar, accumulates less ATP synthase complexes than the wild type illustrates how the production of an oligomeric protein results from the interaction between regulations at the translational and post-translational steps. In addition, each chloroplast-encoded subunit is produced under the control of its own set of nuclear genes. Isolation of chloroplast suppressor mutations of the F54 and thm24 nuclear mutations should provide decisive information on the transcript regions where the mutated nuclear factors act.

METHODS

Growth Conditions, Strains, and Genetic Characterization

Wild-type and mutant strains were grown at 300 lux in Tris-acetate phosphate medium, pH 7.2. Mutants used in this work were mutant F54 isolated by Bennoun and Levine (1967), mutant thm24 isolated by G. W. Schmidt (University of Georgia, Athens, GA), mutant FUD50 isolated by Woessner et al. (1984), and mutant ncc1 obtained from a spontaneous mutation that occurred in the 137c strain in our laboratory.

Induction of gametes, crosses, maturation of zygotes, and dissection of tetrads were carried out according to Levine and Ebersold (1960).

Protein Analysis

Pulse labeling experiments were carried out according to Delepelaire (1983); the inhibitor of cytoplasmic translation was cycloheximide (6.6 μg/mL), and 5 μCi/mL of ^{14}C-acetate (50 mCi/mM; Commissariat à l'Energie Atomique, Saclay, France) was added; the 5-min incubation was stopped by the addition of unlabeled sodium acetate (50 mM). Cells were stored at −70°C in 0.1 M Na_2CO_3/0.1 M DTT. Solubilized total cell proteins were separated by denaturing gel electrophoresis (12 to 18% polyacrylamide gel in the presence of 8 M urea). Labeled polypeptides were detected by autoradiography of the dried gels.

Thylakoid membranes, which were used for immunoblotting experiments and for a preliminary characterization of the mutants by staining the protein gels with Coomassie Brilliant Blue G, were prepared as described by Chua and Bennoun (1975). Immunoblotting experiments were performed as in de Vitry et al. (1989). Anti-α and anti-β antisera were kindly provided by C. Lemaire (Centre de Génétique Moléculaire, Gif/Yvette, France).

RNA Isolation and RNA Gel Blot Analysis

Total RNA was extracted from cultures at 1.10^6 cells/mL with guanidium hydrochloride as described by Rochaix et al. (1988). In some experiments, when a series of RNA extractions were to be done (screening double mutants or strains carrying the ncc1 mutation) or in RNA pulse labeling experiments, we performed quick mini-preparations of total RNA from 20-mL cell cultures following the procedure of Goldschmidt-Clermont et al. (1990). RNA was separated on 1.2% agarose-formaldehyde gels, transferred under vacuum to nylon membranes (Hybond-N; Amersham International) in 20 × SSPE (1 × SSPE is 0.15 M NaCl, 1 mM EDTA, 5 mM NaH_2PO_4, pH 7.4) that were then exposed to UV light. Prehybridizations and hybridizations were in 50% formamide, 5 × SSPE, 0.1% SDS, 0.5% low-fat milk at 42°C. DNA probes were labeled using the random-primed DNA labeling kit from Boehringer Mannheim. Membranes were exposed to Agfa-Gevaert (Rueil-Malmaison, France) industrial P films at −70°C using intensifying screens. After hybridization, the labeled probe could be washed off the membrane by boiling the blot in 0.1% SDS for 1 min, allowed to cool to room temperature in the solution, and then rehybridized with another probe.

Plasmids carrying chloroplast DNA fragments with *atp*A, *atp*B, and *psb*A sequences were kindly provided by J. D. Rochaix (University of Geneva, Geneva, Switzerland). The *atp*A probe was a part of the R7 chloroplast DNA fragment according to the nomenclature of Rochaix (1978): the 0.9-kb EcoRI-PstI fragment was internal to the coding sequence of the *atp*A gene (Dron et al., 1982; Hallick, 1984). The *atp*B probe was the 2.9-kb EcoRI-KpnI fragment (containing the *atp*B gene) of the Ba5 chloroplast DNA fragment (Woessner et al., 1986). To control the loading and the transfer of RNAs, filters were rehybridized with the R14 chloroplast DNA fragment including the *psb*A gene (Erickson et al., 1984).

In Vivo Pulse Labeling of RNA

Experimental conditions were the same as in Kuchka et al. (1989): cells were permeabilized by a treatment with toluene (Guertin and Bellemare, 1979), and 150 μCi of α-^{32}P-UTP (400 Ci/mM; Amersham International) was used to label 7 × 10^7 permeabilized cells (1 mL) for 7 min. Labeled RNA was hybridized to DNA gel blots. Prehybridization, hybridization, and detection were the same as for RNA gel blots. For studies with mutant ncc1, DNA fragments immobilized on filters were the *atp*A DNA fragment already described in the RNA gel blot analysis procedure and the fragment SR0.9, internal to the *psb*D gene, described by Choquet et al. (1988) and used here as a control.

For mutants thm24 and FUD50, three fragments of the *atp*B gene were used: a 0.9-kb HindIII-HindfI fragment, a 0.6-kb HinfI-HinfI fragment adjacent to the former fragment, and a 0.7-kb SspI-SspI fragment complementary to the 3′ end, the intragenic part, and the 5′ end of the *atp*B transcript, respectively (Figure 5B). The *psb*D DNA fragment described above was used as a control.

S1 Nuclease Protection

Experimental conditions were as given in Weaver and Weissmann (1979): DNA (~1 μg) was dephosphorylated with the alkaline phosphatase (EC 3.13.1; Boehringer Mannheim), and the 5′ ends of the fragments were labeled with T4 polynucleotide kinase (EC 2.7.1.78; Stratagene) and γ^{32}P-ATP (3000 Ci/mM; Amersham International); for hybridization, total RNA of the wild-type or mutant strains (from 1 to 25 or 50 ng) was mixed with the labeled DNA fragment (10 ng), and yeast tRNA was used as a carrier; the samples were then denatured by heating and hybridized in 80% formamide, 40 mM Pipes, pH 6.4, 1 mM EDTA, 0.4 M NaCl. The hybridization temperatures were determined following the conditions of Casey and Davidson (1977), where the formation of DNA/RNA hybrids was promoted rather than DNA/DNA hybrids (as we used double-stranded DNA probes). Hybridizations were stopped by dilution, and endonuclease S1 (EC 3.1.30.1; Boehringer Mannheim) was added; samples were incubated for 45 min at 37°C for the digestion of single-stranded DNA or RNA and analyzed by electrophoresis on 6% polyacrylamide gels containing 7 M urea followed by autoradiography.

DNA fragments were chosen as follows: for the detection of the *atp*A and *rbc*L transcripts, the R15-0 fragment was used (Figure 4A); for the detection of the *atp*B transcript, the 0.7-kb HindIII-HinfI subfragment of the Ba5 fragment was used. The DNA fragments were prepared after a separation on 5% polyacrylamide gels in 0.04 M Tris-HCl, pH 8.0, 1 mM EDTA followed by an electroelution in the same buffer from gel slices with an electro-eluter (Bio-Rad).

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