Plant Physiol. (1994) 106: 673–678

Nuclear Mutation Inhibits Expression of the Chloroplast Gene That Encodes the Large Subunit of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase[1]

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Chlamydomonas reinhardtii **mutant 76–5EN was recovered as a light-sensitive, acetate-requiring strain that failed to complement a chloroplast structural gene mutant of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39). Further genetic analysis revealed that the new mutation was inherited in a mendelian pattern, indicating that it resides within the nucleus. The 76–5EN mutant lacks Rubisco holoenzyme but has wild-type levels of whole-chain electron transport activity and chlorophyll. During a 1-min pulse labeling with $^{35}SO_4^{2-}$, little or no Rubisco large-subunit synthesis occurred in the mutant. Nuclear-encoded small subunits were synthesized to a normal level and were subsequently degraded. When analyzed by northern hybridization, the 76–5EN mutant was found to have a decreased level of large-subunit mRNA. Large-subunit mRNA synthesis also appeared to be reduced during a 10-min pulse labeling with [^{32}P]orthophosphate, but the labeled mRNA was stable during a 1-h chase. These results indicate that a nuclear gene mutation specifically disrupts the accumulation of large-subunit mRNA within the chloroplast. A deeper understanding of the nature of the 76–5EN gene may be useful for manipulating the expression of the agronomically important Rubisco enzyme.**

Rubisco (EC 4.1.1.39) plays a pivotal role in photosynthesis because it catalyzes both the carboxylation and oxygenation of RuBP. The amount of carboxylation and the ratio of carboxylation to oxygenation ultimately determine net photosynthetic CO_2 fixation (reviewed by Spreitzer, 1993). The Rubisco holoenzyme exists within the chloroplasts of plants and green algae as a hexadecamer composed of equal numbers of 55-kD large subunits and 15-kD small subunits. The chloroplast *rbcL* gene encodes the catalytic large subunits, whereas a family of nuclear *RbcS* genes encodes the small subunits (reviewed by Spreitzer, 1993).

As a means for defining the structure/function relationships of chloroplast Rubisco, mutants of the green alga *Chlamydomonas reinhardtii* have been recovered by screening for defects in Rubisco holoenzyme stability or catalysis (Spreitzer et al., 1992). Because *C. reinhardtii* synthesizes a complete photosynthetic apparatus when grown with acetate medium in darkness, photosynthesis-deficient mutants can be analyzed under this growth condition. Most Rubisco-deficient mutants result from mutations in the chloroplast *rbcL* gene (reviewed by Spreitzer, 1993). These mutants have been particularly useful for identifying large-subunit structural interactions that control the carboxylase/oxygenase ratio (Chen et al., 1988, 1991; Chen and Spreitzer, 1989; Thow et al., 1994).

Only several Rubisco mutants have been recovered that arise from mutations in nuclear genes (Spreitzer et al., 1988, 1992). Two of these nuclear mutants have been analyzed in detail. The S52–2B mutation suppresses the temperature-conditional phenotype and altered enzyme properties of the 68–4PP *rbcL* mutant (Chen et al., 1990, 1993). The 68–4PP mutation causes a Leu^{290}-to-Phe substitution in the Rubisco large subunit, which, in the absence of the S52–2B suppressor mutation, decreases the carboxylase/oxygenase ratio and thermal stability of Rubisco (Chen et al., 1988, 1993). A second nuclear mutant, named 68–11AR, was recovered as a temperature-conditional, acetate-requiring strain (Spreitzer et al., 1988). The 68–11AR mutation decreases the carboxylase/oxygenase ratio and thermal stability of Rubisco (Gotor et al., 1994). Each of these mutations resides within a different nuclear gene, neither of which is linked to the Rubisco *RbcS* locus. Thus, it is apparent that both mutations must affect Rubisco at co-translational or posttranslational steps (Chen et al., 1990, 1993; Gotor et al., 1994).

A number of other nuclear mutations have been identified in *C. reinhardtii* that inhibit the expression of specific chloroplast genes. However, these mutations cause a decrease in either the stability, processing, or translation of chloroplast mRNA (reviewed by Rochaix, 1992). If similar mutants could be identified with respect to *rbcL* expression, they might prove useful for exploring ways to increase the expression of Rubisco and, thereby, increase carboxylation under certain conditions. In the present study, we have recovered a photosynthesis-deficient, acetate-requiring mutant of *C. reinhardtii* that lacks Rubisco holoenzyme because of a deficiency in *rbcL* mRNA. This mutant strain, named 76–5EN, results from a mutation in a nuclear gene.

[1] Supported by National Science Foundation grant DCB-9005547 and published as paper No. 10696, Journal Series, Nebraska Agricultural Research Division.

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Abbreviations: *atpB*, ATP synthase β-subunit gene; *mt*, mating-type locus; *pf-2*, paralyzed flagella locus; *psbB*, PSII polypeptide-5 gene; *rbcL*, Rubisco large-subunit gene; *RbcS*, Rubisco small-subunit gene; *Rca*, Rubisco activase gene; RuBP, ribulose-1,5-bisphosphate.

MATERIALS AND METHODS

Strains and Culture Conditions

Wild-type *Chlamydomonas reinhardtii* 2137 mt^+ (Spreitzer and Mets, 1981), centromere-marker strain *pf-2* mt^-, and Rubisco mutant/revertant strains are maintained on medium containing 10 mM acetate and 1.5% Bacto agar at 25°C in darkness (Spreitzer and Mets, 1981). The temperature-conditional, acetate-requiring 68–11AR mutant was described previously (Spreitzer et al., 1988; Gotor et al., 1994). Strain R52–2B was recovered as a photosynthesis-competent revertant of the temperature-conditional 68–4PP *rbcL* mutant (Chen et al., 1988, 1990). R52–2B results from a nuclear mutation, named S52–2B, that suppresses the reduced catalytic efficiency and thermal instability caused by the 68–4PP chloroplast mutation (Chen et al., 1988, 1990, 1993). For biochemical analysis, cells were grown to stationary phase in liquid acetate medium at 25°C in darkness.

Mutant Recovery and Genetic Analysis

Wild-type mt^+ cells were treated with 5-fluorodeoxyuridine, to reduce the ploidy of the chloroplast genome, mutagenized with methyl methanesulfonate, and plated to obtain single colonies on acetate medium in the dark according to standard procedures (Spreitzer and Mets, 1981; Spreitzer et al., 1988). After replica plating colonies to minimal medium (without acetate) in the light, we identified acetate-requiring mutants and subsequently screened them for the lack of ability to recombine or complement with an *rbcL* structural gene mutant (Spreitzer and Ogren, 1983; Spreitzer et al., 1992). Gamete induction in nitrogen-free acetate medium, zygote maturation, and tetrad dissection were performed at 25°C (Spreitzer and Mets, 1981). Phenotypes of progeny were scored by replica plating tetrads to minimal medium in the light and acetate medium in the dark both at 25 and 35°C.

Biochemical Analysis

About 2.5×10^9 cells were harvested by centrifugation and sonicated at 0°C for 3 min in 1 mM DTT, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 50 mM Bicine, pH 8.0. RuBP carboxylase activity was measured in sonicated extracts as the incorporation of acid-stable ^{14}C from $NaH^{14}CO_3$ (Spreitzer et al., 1988). The cell extracts were also subjected to SDS-PAGE (Laemmli, 1970) and Suc gradient fractionation (Spreitzer and Chastain, 1987). Protein was determined by the method of Bradford (1976).

Chl was measured in ethanol extracts of whole cells (Wintermans and DeMots, 1965). Whole-chain electron transport activity was measured as oxygen uptake in the presence of methyl viologen and gramicidin S with a Hansatech (Norfolk, UK) oxygen electrode at 730 $\mu E\ m^{-2}\ s^{-1}$ and 25°C (Spreitzer and Ogren, 1983).

Protein pulse-labeling experiments were performed in darkness with 1.5×10^7 cells mL^{-1} of sulfate-free acetate medium as described previously (Spreitzer et al., 1985). The cells were pulse labeled for 1 min with carrier-free $H_2{}^{35}SO_4$ (0.5 mCi mL^{-1} of culture), followed by a 1-h chase with 10 mM Na_2SO_4. Samples were prepared (Thow et al., 1994) and separated by SDS-PAGE on 7.5 to 15% polyacrylamide gradient gels (Laemmli, 1970). The gels were stained with Coomassie blue and subjected to fluorography (Laskey and Mills, 1975).

mRNA Isolation and Northern Analysis

About 2.5×10^9 cells were lysed by extensive mixing in 2 mL of 6 M guanidine hydrochloride, 0.1 M sodium acetate, pH 5.0, in the presence of 2 g of 3-mm glass beads (Spreitzer et al., 1985). Total RNA was prepared by centrifugation of the lysed cell extracts through a cesium chloride cushion (Sambrook et al., 1989). The purified RNA (10 μg) was then separated on denaturing formaldehyde/agarose gels and blotted to nylon membranes (Sambrook et al., 1989). A 0.8-kb *Hin*dIII subfragment of the chloroplast *rbcL* gene (Dron et al., 1982) and a 1.3-kb *Eco*RI-*Sst*I subfragment of the nuclear *Rca* gene (Roesler and Ogren, 1990) were nick translated with [^{32}P]-dCTP (Rigby et al., 1977) and used to probe the filters. Prehybridization and hybridization were performed in 5× SSPE (750 mM NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, pH 7.4), 50% formamide, 2× Denhardt's reagent (Denhardt, 1966), 0.1% SDS, and 100 $\mu g\ mL^{-1}$ denatured salmon sperm DNA at 42°C (Sambrook et al., 1989). Filters were washed several times with 0.1× SSPE, 0.1% SDS at 50°C, dried, and subjected to autoradiography at −70°C.

mRNA Pulse Labeling

Cells were maintained at a density of 2×10^6 cells mL^{-1} for 4 to 5 d in darkness by dilution in low Pi (65 μM KH_2PO_4/K_2HPO_4, pH 7.2) acetate medium (Baker et al., 1984). The cells were then concentrated into 18 mL of Pi-free acetate medium at a density of 2×10^7 cells mL^{-1} and cultured in darkness for 30 min (Blowers et al., 1990). Pulse labeling was performed for 10 min with carrier-free ^{32}Pi (0.1 mCi mL^{-1} culture). After 2 mL of 130 mM Pi buffer in acetate medium were added, which diluted the ^{32}Pi by more than 1×10^6 times, 10 mL of the pulsed cell mixture were immediately transferred to an ice-cold centrifuge tube and RNA was extracted. After 1 h, the remaining chased cells were harvested and total RNA was isolated. RNA from the pulsed and chased cells was then hybridized with DNA probes (250 ng) that had been immobilized on nylon membranes using a Hybri-Dot manifold (BRL). The DNA probes in this case were the 0.8-kb *Hin*dIII subfragment of the chloroplast *rbcL* gene (Dron et al., 1982) and a 1.5-kb *Hin*dIII-*Pst*I subfragment of the chloroplast *atpB* gene (Woessner et al., 1986). Prehybridization was performed for 12 h, and hybridization was performed for more than 72 h. Filters were then washed, dried, and subjected to autoradiography.

RESULTS

Phenotype and Genotype

Mutant 76–5EN has a light-sensitive, acetate-requiring phenotype that is characteristic of photosynthesis-deficient *C. reinhardtii* mutants (Spreitzer and Mets, 1981). Because 76–5EN failed to complement with an *rbcL* mutant strain

during preliminary screening procedures, it seemed possible that this new mutation also occurred within the *rbcL* gene. Cell extract of mutant 76–5EN lacked RuBP carboxylase activity, and no Rubisco holoenzyme could be detected by Suc gradient fractionation. Furthermore, mutant cells had normal levels of Chl and whole-chain electron transport activity, indicating that the 76–5EN mutation did not cause pleiotropic photosynthesis defects. Altogether, these properties of the 76–5EN mutant are identical with the properties of a number of *rbcL* mutants (Spreitzer et al., 1985; Thow et al., 1994).

Whereas *rbcL* (chloroplast) mutations are inherited in a uniparental pattern (Spreitzer et al., 1992), genetic analysis revealed that the phenotype of mutant 76–5EN mt^+ followed a mendelian (nuclear) pattern of inheritance when crossed with an mt^- strain. In other words, the 76–5EN acetate-requiring phenotype segregated 2:2 with the wild-type phenotype among progeny in tetrads. Mendelian inheritance of the 76–5EN mutation was confirmed in a reciprocal cross between wild-type mt^+ and mutant mt^- progeny obtained from the original cross (Table I, cross 1). Because the *pf-2* centromere marker was included in this cross, the distance between the 76–5EN mutation and its centromere could be calculated and was found to be about 19 map units (Table I, cross 1).

Two other nuclear mutations have been investigated that appear to affect Rubisco at the posttranslational level. Mutant S52–2B was recovered as a suppressor of the temperature-conditional, acetate-requiring 68–4PP *rbcL* mutant (Chen et al., 1990, 1993), whereas mutant 68–11AR was recovered directly as a temperature-conditional, acetate-requiring strain (Spreitzer et al., 1988). The S52–2B and 68–11AR mutant genes (named *rbc-1*-S52–2B and *rbc-2*-68–11AR, respectively) are not linked with each other or with the *RbcS* locus (Chen et al., 1990; Gotor et al., 1994). Although the *rbc-1* and *rbc-2* centromere distances are quite large (Gotor et al., 1994), crosses were performed to confirm that the 76–5EN mutant gene was not an allele of *rbc-1* or *rbc-2* (Table I, crosses 2 and 3). In both cases, the frequencies of parental and nonparental ditype tetrads were nearly equal, indicating that the 76–5EN mutation resides in a different gene that is not linked with either *rbc-1* or *rbc-2*. We have named this new gene *rbc-3* and formally denote the mutant allele as *rbc-3-76–5EN*.

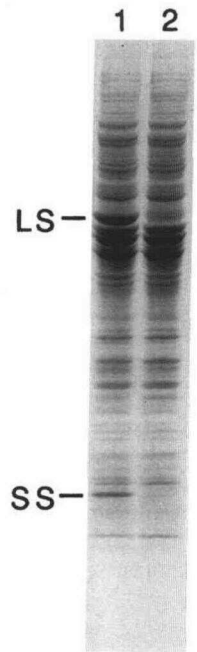

Figure 1. SDS-PAGE of total soluble cell proteins from wild type (lane 1) and mutant 76–5EN (lane 2). Each lane received 60 μg of protein extract. The gel was stained with Coomassie blue after electrophoresis. LS, Large subunit; SS, small subunit.

Synthesis of Rubisco Subunits

Rubisco large and small subunits were found to be absent or greatly diminished when an extract of mutant 76–5EN cells was fractionated by SDS-PAGE (Fig. 1, lane 2). No other protein bands were absent or altered in mobility, further indicating that the 76–5EN mutation is quite specific for the decrease in Rubisco accumulation. Protein pulse-labeling experiments were performed to determine whether Rubisco large and small subunits are synthesized in mutant 76–5EN.

Table I. *Genetic analysis of Chlamydomonas mutation rbc-3-76-5EN*

No.	Cross	Tetrads[a]			Map Distance
		PD	NPD	T	
1.	$++mt^+ \times rbc\text{-}3\ pf\text{-}2\ mt^-$	11	14	15	19[b]
2.	$rbc\text{-}2 + mt^+ \times + rbc\text{-}3\ mt^-$	3	3	8	Not linked[c]
3.	$rbcL\ rbc\text{-}1 + mt^+ \times ++ rbc\text{-}3\ mt^-$	9	9	6	Not linked[d]

[a] With respect to two markers in a cross, tetrads were scored as parental ditype (PD), nonparental ditype (NPD), or tetratype (T). [b] Gene-centromere distance was calculated relative to the pf-2 centromere marker as: 100 × 0.5T/(PD + NPD + T). [c] A temperature-conditional, acetate-requiring phenotype (*rbc-2*-68-11AR) was scored relative to a nonconditional, acetate-requiring phenotype (*rbc-3*-76-5EN), and *rbc-3*-76-5EN was epistatic to *rbc-2*-68-11AR. [d] A "wild-type" phenotype (*rbcL*-68-4PP/*rbc-1*-S52-2B) was scored relative to a nonconditional, acetate-requiring phenotype (*rbcL*-68-4PP/*rbc-3*-76-5EN), and *rbc-3*-76-5EN was epistatic to both *rbcL*-68-4PP and *rbc-1*-S52-2B.

Rubisco large subunits were not readily apparent in mutant cells during a 1-min pulse with $^{35}SO_4^{2-}$ (Fig. 2, lane 2), but Rubisco small subunits, as well as all other proteins, were synthesized at near-normal rates in comparison with wild-type cells (Fig. 2, lanes 1 and 2). However, the Rubisco small subunits synthesized in the mutant cells were subsequently degraded during a 1-h chase (Fig. 2, lanes 2 and 4), which is a common attribute of a number of chloroplast *rbcL* mutants that fail to accumulate large subunits (Spreitzer et al., 1985; Chen et al., 1988; Thow et al., 1994).

Synthesis of *rbcL* mRNA

Because mutant 76–5EN failed to synthesize Rubisco large subunits in protein pulse-chase experiments, it seemed reasonable to consider whether the 76–5EN nuclear mutation inhibited the accumulation of *rbcL* mRNA. When northern analysis was performed, mutant 76–5EN appeared to have substantially less than the normal amount of *rbcL* mRNA, and no degradation products were detected (Fig. 3). Because this decrease in mRNA could result from a defect in either mRNA synthesis or stability, we performed mRNA pulse-chase experiments in an attempt to discriminate between these possibilities. During a 10-min pulse with ^{32}Pi, 76–5EN cells appeared to synthesize substantially less *rbcL* mRNA than did wild-type cells (Fig. 4). Furthermore, the *rbcL* mRNA that was synthesized in mutant 76–5EN appeared to be stable during the 1-h chase period (Fig. 4). Wild-type and 76–5EN mutant cells synthesized similar amounts of mRNA from the chloroplast *atpB* gene during the 10-min pulse labeling, and this mRNA was also stable during the 1-h chase (Fig. 4).

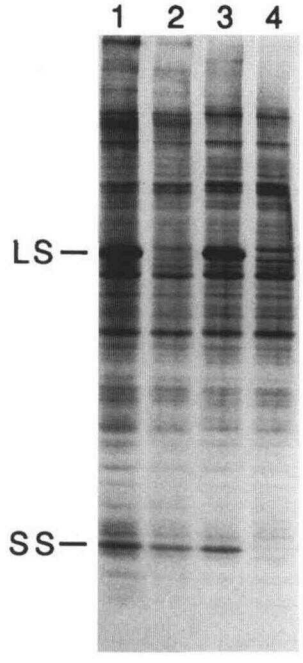

Figure 2. Pulse labeling of soluble cell proteins in wild type and mutant 76–5EN grown in darkness. Cells were labeled with $^{35}SO_4^{2-}$ for 1 min (lanes 1 and 2) and chased with 10 mM Na_2SO_4 for 1 h (lanes 3 and 4). Samples were extracted, and equal amounts of radioactivity were subjected to SDS-PAGE, followed by fluorography. Lanes 1 and 3, Wild type; lanes 2 and 4, mutant 76–5EN. LS, Large subunit; SS, small subunit.

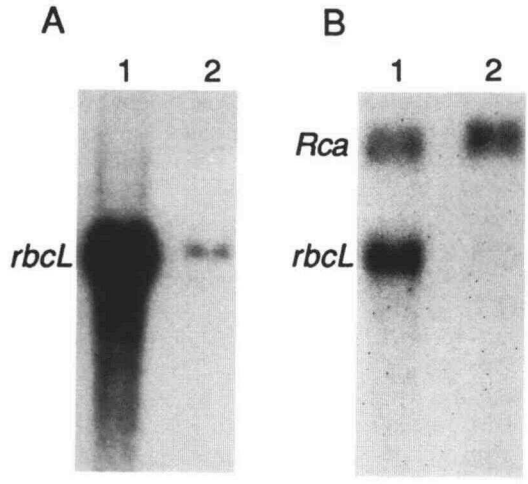

Figure 3. Northern analysis of *rbcL* and *Rca* mRNAs in wild type and mutant 76–5EN. A, Total RNA (10 μg) was separated by electrophoresis on formaldehyde/agarose gels, blotted to nylon membrane, and hybridized with an *rbcL* gene probe. Blots were exposed to x-ray film with an intensifying screen for 24 h at −70°C. Lane 1, Wild type; lane 2, mutant 76–5EN. B, After an autoradiogram was obtained, the same filter was treated with 0.1× Denhardt's reagent, 1 mM EDTA, 1 mM Tris, pH 8.0, at 75°C to partially remove the *rbcL* DNA probe. The filter was then hybridized with an *Rca* gene probe to verify that equal amounts of total RNA had been present in both lanes. Autoradiography was performed with an intensifying screen for 72 h at −70°C. Lane 1, Wild type; lane 2, mutant 76–5EN.

DISCUSSION

Nuclear-encoded proteins must be involved in the expression of organellar genes because the genomes of chloroplasts

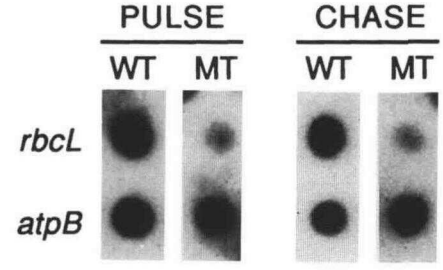

Figure 4. Pulse labeling of total RNA in wild type (WT) and mutant 76–5EN (MT) grown in darkness. Cells were pulse labeled with carrier-free ^{32}Pi for 10 min and chased with 13 mM Pi for 1 h. Wild-type and mutant 76–5EN cells were collected at the two times, and RNA was extracted. An equal amount of radioactivity from each of the four samples was then hybridized with *rbcL* and *atpB* gene probes immobilized on nylon membranes (250 ng of DNA per each spot). After hybridization, the four filters were exposed to a single x-ray film for 72 h. A 1.3-kb *Eco*RI-*Sst*I subfragment of the nuclear *Rca* gene was also included in these experiments, but the abundance of the labeled *Rca* mRNA was too low to allow adequate detection.

and mitochondria encode only a limited collection of structural proteins and RNAs (reviewed by Attardi and Schatz, 1988; Sugiura, 1992). What is perhaps more interesting is that many nuclear mutations have been identified that specifically affect the expression of individual organellar-encoded proteins. These mutations have been found to affect expression at posttranscriptional, translational, or posttranslational levels (reviewed by Costanzo and Fox, 1990; Rochaix, 1992). Because nuclear and organellar genes follow different patterns of inheritance, one can expect that continued genetic approaches will prove fruitful for identifying the nature of putative regulatory proteins encoded by the nuclear genes.

In the present study, we have identified a nuclear gene mutation of *C. reinhardtii*, named *rbc-3-76–5EN*, that appears to specifically block the expression of the chloroplast-encoded Rubisco large subunit. Large subunits are not synthesized to any appreciable extent in the mutant, but Rubisco small subunits are synthesized at a near-normal rate (Fig. 2). Because small subunits are subsequently degraded in the absence of large subunits (Fig. 2; Spreitzer et al., 1985), the mutant fails to accumulate either subunit (Fig. 1). However, mutant 76–5EN has normal levels of Chl and whole-chain electron transport activity, and it synthesizes and accumulates other cellular proteins to the same levels as wild type (Figs. 1 and 2). Thus, this mutant is clearly different from maize or *C. reinhardtii* mutants that are defective in chloroplast translation in general (Harris et al., 1974; Barkan, 1993). Such mutants have pleiotropic deficiencies in a number of photosynthetic proteins, including Rubisco.

Northern analysis revealed that mutant 76–5EN accumulates only a small amount of *rbcL* mRNA (Fig. 3). One might propose that the 76–5EN mutation causes a primary defect in translation, which subsequently leads to the destabilization of the *rbcL* mRNA. However, *C. reinhardtii* nuclear mutations that inhibit translation or accumulation of specific chloroplast proteins do not cause an associated decrease in the respective mRNAs (Kuchka et al., 1988; Rochaix et al., 1989; Girard-Bascou et al., 1992; Drapier et al., 1992). Furthermore, an *rbcL* nonsense mutant, in which large subunit synthesis is terminated at residue 65, also accumulates a normal amount of *rbcL* mRNA (Spreitzer et al., 1985).

Pulse-chase experiments with ^{32}Pi indicated that mutant 76–5EN is unable to synthesize *rbcL* mRNA at the wild-type rate (Fig. 4). One could argue that the mutant synthesizes *rbcL* mRNA at a normal rate, but the mRNA is so unstable that it is substantially degraded during the 10-min pulse. Such may be the case for one *C. reinhardtii* nuclear mutant that fails to accumulate any chloroplast *psbB* mRNA (Monod et al., 1992). However, the amount of *rbcL* mRNA synthesized by mutant 76–5EN during the 10-min pulse is stable during a 1-h chase (Fig. 4), and the mutant strain appears to accumulate a similar level of *rbcL* mRNA when analyzed by northern hybridization (Fig. 3). Thus, no *rbcL* mRNA instability is apparent. Mutant 76–5EN is also quite different from other *C. reinhardtii* nuclear mutants that are known to be defective in chloroplast mRNA processing or stability (Herrin and Schmidt, 1988; Kuchka et al., 1989; Goldschmidt-Clermont et al., 1990; Sieburth et al., 1991). Those mutants synthesize mRNA at a normal rate, but none of the mature mRNA accumulates.

Although no nuclear mutation has previously been found to reduce the transcription of a specific chloroplast or mitochondrial gene, our results appear to favor the idea that the 76–5EN mutation causes a specific reduction in *rbcL* transcription. Perhaps transcription mutants are rare because there are few nuclear-encoded proteins involved in the transcription of specific organellar genes. Furthermore, because many chloroplast and mitochondrial genes are polycistronic (reviewed by Attardi and Schatz, 1988; Sugiura, 1992), mutations that block transcription may often eliminate a number of gene products. It may be difficult to analyze such pleiotropic mutants, or they may be difficult to recover if one of the gene products is essential (e.g. a chloroplast tRNA). Such is not the case for the *C. reinhardtii rbcL* gene, which is not associated with a polycistronic transcription unit (Dron et al., 1982).

Although further studies will be required to determine the exact nature of the 76–5EN mutation, the mutant strain may be of immediate use for selecting photosynthesis-competent suppressor mutations that increase the level of Rubisco expression. It is apparent that changes in the Rubisco carboxylase/oxygenase ratio are often accompanied by changes in Rubisco holoenzyme stability (Chen et al., 1988; Thow et al., 1994). Perhaps a catalytically improved Rubisco will also have an associated structural instability. Mutant cells harboring such an enzyme may benefit from an increase in *rbcL* expression.

ACKNOWLEDGMENTS

The technical assistance of Kathryn Harter is gratefully acknowledged. We thank N.W. Gillham (Duke University) and W.L. Ogren (University of Illinois) for providing plasmid clones of *atpB* and *Rca*, respectively.

Received May 18, 1994; accepted July 7, 1994.

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