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## Relationship between mRNA levels and protein accumulation in a chloroplast promoter-mutant of *Chlamydomonas reinhardtii*

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**Abstract** The photosynthetic chloroplast mutant *G64* of *Chlamydomonas reinhardtii* was shown to contain a single point mutation within the 5' region of the *psbD* gene encoding the D2 protein of the photosystem II reaction center. The mutation affects the sequence element TATAATAT which has previously been hypothesized to function as the *psbD* promoter. Run-on analysis confirmed that transcription of *psbD* in the mutant was reduced to approximately 10% of the wild-type level. However, *psbD* mRNA accumulated to approximately 35%, despite the prominent decrease in RNA synthesis. This suggests that RNA-stabilization effects can compensate to some extent for a reduction in transcriptional activity. Interestingly, a direct correlation between transcript levels and the accumulation of the *psbD* gene product, the D2-protein, was observed in *G64*. The data suggest that posttranscriptionally acting regulatory factors determine the rate-limiting steps of chloroplast *psbD* gene expression.

**Keywords** Chloroplast · Transcription · MRNA/protein relation · *Chlamydomonas*

### Introduction

Chloroplast gene expression is a tightly controlled process in which nucleus-encoded factors play an essential

role. They form a molecular framework which is involved in regulatory events on most hierarchical levels during plastid-gene expression including transcription, RNA metabolism/maturation and translation (for an overview see Allison 2000; Barkan and Goldschmidt-Clermont 2000; Manuell et al. 2004; Herrin and Nickelsen 2004; Rochaix et al. 2004; Nickelsen 2003 and references herein). While the cloning of regulatory factors is progressing, the different rate-limiting steps during the expression of various chloroplast genes are still poorly defined.

There is evidence that chloroplast transcription plays a critical role in light- and development-dependent regulation of plastid-gene expression (Allison 2000). Two RNA polymerases, the phage-like NEP (nuclear-encoded polymerase) and the bacterial-like PEP (plastid-encoded polymerase) are required for transcription in vascular plants (Hess and Börner 1999; Suzuki et al. 2004). In contrast, NEP-like enzymes have not been found within plastids of algae (Smith and Purton 2002) suggesting that here all plastid genes are transcribed by the PEP enzyme.

Insertion of GUS reporter gene constructs into the plastid genome via biolistic transformation has made it possible to characterize the promoter elements of chloroplast genes to a certain extent. In *Chlamydomonas reinhardtii*, two types of promoters were shown to drive transcription within the chloroplast (Klein et al. 1992). The first type, including those of the *rbcL* and *rrn16* genes, resembles a typical bacterial promoter of the *Escherichia coli* sigma70-type, which contains the sequence elements TTGACA and TATAAT at positions –35 and –10, respectively. The other promoter type, which is found upstream of the *atpB*, *psbD* or *psbA* genes for instance, only contains the –10 element. This element has so far been shown to be sufficient for promoting transcription in the case of *atpB* (Blowers et al. 1990; Klein et al. 1992). Furthermore, elements enhancing transcription were identified within inter- and intragenic regions of chloroplast genes (Lisitsky et al. 2001; Anthonisen et al. 2002).

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Transcriptional activity of chloroplast genes in *C. reinhardtii* has been shown to be controlled by circadian rhythm and by light (Leu et al. 1990; Salvador et al. 1993). Nevertheless, a thorough investigation of various chloroplast genes, the transcription of which was modified or inhibited by treatment with either FdUrd or rifampicin, revealed that no direct correlation between transcriptional activity and mRNA accumulation exists (Eberhard et al. 2002). This suggests that posttranscriptional processes determine the steady-state levels of chloroplast mRNAs and that the availability of RNA-stabilizing factors may be one of the important factors involved. Furthermore, these results challenge the role of mRNA levels during chloroplast gene expression: neither protein synthesis nor protein accumulation were compromised by rifampicin-mediated reduction of transcript abundance over a 6-h period.

Here, we report on the molecular characterization of the chloroplast mutant G64 of *C. reinhardtii* which harbors a point mutation within the *psbD* promoter. Previous reports on the plastid transcription were based exclusively on the analysis of reporter gene expression. In contrast, using the strain G64 enabled us for the first time to investigate the permanent phenotypic changes induced by a decrease in the transcription of a chloroplast gene, which is essential for photosynthesis.

## Material and methods

### Strains, culture conditions and genetic analyses

*Chlamydomonas reinhardtii* wild-type and mutant strains were maintained on Tris-acetate-phosphate medium (TAP; Gorman and Levine 1965) at 25°C. Mutant strain G64 was obtained after mutagenesis during exponential growth with 0.7 mM FdUrd (Wurtz et al. 1979) followed by an enrichment step with metronidazole (Schmidt et al. 1977). Tetrad analysis was performed as described by Harris (1989). Complementation tests were carried out as described by Bennoun et al. (1980). Chloroplast recombination tests were done on the diploid phase as described by Girard-Bascou (1987). Vegetative diploids were isolated as described by Harris (1989) using complementation between *arg2* and *arg7* mutations.

### Chloroplast transformation

Chloroplasts were transformed with a helium-driven particle gun (Fischer et al. 1996) and the resulting transformants were selected on TAP plates containing spectinomycin (100 µg/ml). The transformants were tested for homoplasmy by Southern analysis (Nickelsen et al. 1999) after a minimum of three consecutive cell transfers onto fresh selective media.

Fluorescence-induction kinetics of dark-adapted cells were determined by using a house built fluorimeter (Bennoun and Delepleaire 1982).

### Plasmid construction

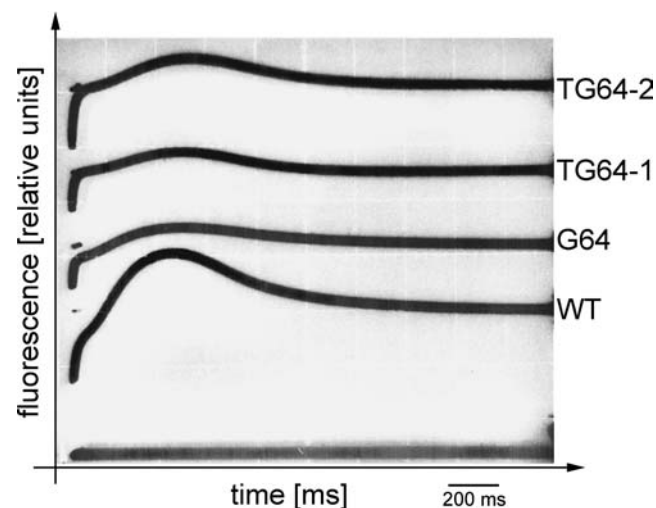
The *psbD* 5' region containing the G64 mutation was amplified by PCR using oligos 1365 and 1963 (see Fig. 1, Nickelsen et al. 1999). The PCR product was digested with enzymes *PvuII* and *ClaI* and was used to replace the *ClaI/PvuII* fragment of plasmid p108.14. The plasmid carries the *aadA* cassette for selection of transformants on media containing spectinomycin (Nickelsen et al. 1999).

### Chloroplast run-on transcription

To measure transcription rates, cells were washed with run-on washing buffer (10 mM HEPES pH 7.5, 250 mM sucrose, 150 mM KCl, 1 mM EDTA, 0.4 mM PMSF freshly supplemented) and permeabilized by repeated freeze and thaw cycles (Gagne and Guertin 1992). Subsequently, 40 µl transcription buffer (50 mM HEPES pH 7.5, 1 M sucrose, 60 mM MgCl<sub>2</sub>, 15 mM DTT, 50 mM NaF, 120 U RNase inhibitors, supplemented with 125 µCi of α-<sup>32</sup>P-UTP, 200 nmol ATP, 100 nmol GTP and CTP) was added and the cells were left to incubate for 12 min at 26°C (Sakamoto et al. 1993). RNA was extracted with phenol/chloroform and precipitated with ethanol. It was then used for hybridization to nylon filter-immobilized PCR fragments of the *psbD* (5 µg) and *atpB* (5 µg) genes. Signals from three independent experiments were quantified in a phosphoimager (Fuji).

### Analysis of RNA

Total RNA was isolated with hot phenol (Kück et al. 1987), separated electrophoretically, blotted onto nylon membranes and hybridized with radiolabeled *psbD* and



**Fig. 1** Chlorophyll fluorescence induction kinetics of dark-adapted cells of the wild-type, G64 and the chloroplast transformants TG64-1 and TG64-2

*rbcL* DNA probes or a *psaA* exon2 oligonucleotide probe (CCTAATTGACCAAAGTGTGCACTGAA-TAC). Primer-extension analysis of *psbD* mRNA 5' ends using oligonucleotide 3131 was performed as described previously (Nickelsen et al. 1994). Steady-state levels of mRNAs from the three independent experiments were calculated by using the Scion Image software (<http://www.scioncorp.com>) and standardized to the internal *rbcL* RNA control.

#### Analysis of proteins

To obtain polyclonal  $\alpha$ D2 antiserum, a DNA fragment encoding the 55 C-terminal amino acids of the D2-protein from *C. reinhardtii* was inserted into the vector pGEX4-T1. After expression in *E. coli* BL21 the recombinant GST-D2 fusion protein was isolated by affinity chromatography following the manufacturer's instructions (Amersham Biosciences) and injected into rabbits (Biogenes, Berlin). Total cell proteins were separated in a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel with 8 M urea, blotted onto nitrocellulose membranes and decorated with antibodies. D2 protein levels from three independent experiments were calculated with Scion Image software and standardized to the internal RbcL control.

#### Pulse labeling of proteins

Pulse labeling of whole cells in exponential growth phase was carried out in minimal medium with  $^{14}\text{C}$  acetate for 7 min in the presence of cycloheximide (8  $\mu\text{g/ml}$ ) (Kuras and Wollman 1994). Broken-cell preparations were obtained as followed: cells were collected by centrifugation and resuspended in 5 mM HEPES-KOH pH 7.5, 10 mM EDTA in the presence of 0,2 mM PMSF, 1 mM Benzamidine, 5 mM caproic acid. Cells were broken in a French Press (4,000 lb/in<sup>2</sup>) and homogenized with a motor-driven teflon pestle. The homogenate was centrifuged at 39,000 g for 20 min. The pellet was resuspended in 0,1 M Na<sub>2</sub>CO<sub>3</sub>, 0,1 M dithiothreitol. This preparation was fractionated by electrophoresis on a 12–18% sodium dodecyl sulfate (SDS)-polyacrylamide gel with 8 M urea (Piccioni et al. 1981). Signals were

quantitated by using the above-mentioned Scion Image software and standardized to the internal Cytf control.

## Results

The *C. reinhardtii* mutant *G64* was isolated based on its high chlorophyll–fluorescence phenotype (Bennoun and Deleplaire 1982). Its fluorescence induction kinetics were found to be characteristic of leaky photosystem II deficiency (Fig. 1) and accordingly, the cells showed a drastically reduced photoautotrophic growth rate (Table 1). During genetic crossings, the phenotype was uniparentally inherited by the mt+ parent in all the 14 tetrads examined, being indicative of a chloroplast-encoded mutation present in *G64*. Further genetic analysis suggested that the underlying mutation affects the *psbD* gene locus: first, the *G64* mutant was not complemented with a defective *psbD* gene in diploids resulting from a cross with the *FUD47* mutant while it was complemented in control diploids obtained from a cross with the *FUD7* mutant containing a deletion version of the *psbA* gene encoding the D1-protein. Second, in vegetative diploids obtained from a cross involving the *G64* and the *FUD47* mutants a linkage between the mutations was observed. The recombinant frequency was calculated as equal to  $2.7 \times 10^{-2}$ , a value which corresponds to intragenic recombination events (Girard-Bascou 1987).

As a next step, the *psbD*-coding region from *G64* as well as 430-bp of its 5' flanking region and 260 bp of its 3' flanking region was amplified by PCR. Sequencing of the PCR product revealed a single point mutation within the *psbD* 5' region. An A residue at position –13 relative to the transcription starting point was replaced by a G residue (Fig. 2). A back-transformation of wild-type cells with a construct harboring this point mutation (see Material and methods; Ossenbühl and Nickelsen 2000) resulted in homoplasmic transformants. Two of these, named *TG64-1* and *TG64-2*, were chosen for further molecular characterization and were shown to exhibit the same phenotype as the *G64* mutant with regard to all physiological and molecular features analyzed (Figs. 1, 2, 3, 4, 5, 6; Table 1). This strongly supports the hypothesis that the identified mutation at position –13 in the *psbD* gene is sufficient to cause the observed *G64*-

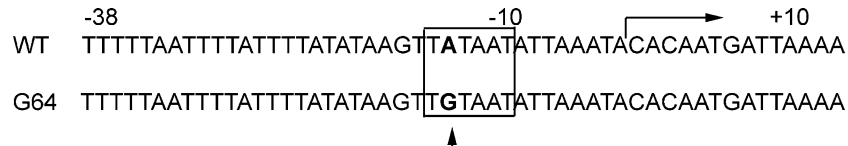
**Table 1** Molecular characterization of strains

Strain	WT	G64	TG64-1	TG64-2
Photoautotrophic growth <sup>a</sup>	30,6 ± 1	45,6 ± 2	48,4 ± 3	43,2 ± 2
Transcription rate of <i>psbD</i> <sup>b</sup>	100	12,5 ± 5	12,25 ± 4	16,75 ± 8
RNA Level <i>psbD</i> <sup>b</sup>	100	38 ± 2	32 ± 7	39 ± 4
Translation rate of D2 <sup>b,c</sup>	100	34	17	29
Protein level D2 <sup>b</sup>	100	36 ± 13	30 ± 7	28 ± 11

<sup>a</sup>Doubling times in hours under photon irradiance of 150  $\mu\text{E m}^{-2} \text{s}^{-1}$

<sup>b</sup>All values are given in percentage relative to the wild-type and represent the average of three experiments except the protein pulse experiment

<sup>c</sup>Translation rate was quantified in relation to the internal amount of Cytf



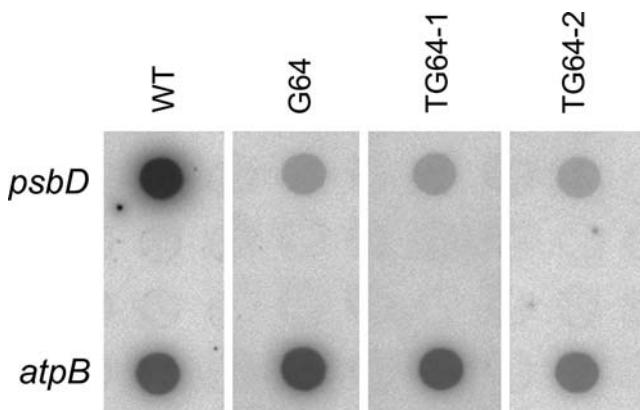
**Fig. 2** Localization of the mutation in *G64*. The sequences of the *psbD* 5' region from wild-type and *G64* are given. The horizontal arrow delineates the starting point of transcription and the  $-10$  promoter element is boxed. The vertical arrow marks the single point mutation in *G64*

phenotype. The affected sequence element TATAATAT ranging from position  $-7$  to  $-14$  had previously been proposed to represent the  $-10$  element of the *psbD* promoter. This promoter is thought to belong to a subgroup of chloroplast promoters lacking a typical  $-35$  element, similar to those of some bacterial genes (Klein et al. 1992).

To test if the mutation does affect promoter activity and thus transcription of the *psbD* gene, run-on transcription assays were performed (Fig. 3). Transcription rates were reduced to approximately 10% of the wild-type rate in the original *G64* mutant and in the two chloroplast transformants *TG64-1* and *TG64-2*. This data confirms that the TATAAT sequence covering position  $-13$  represents an essential *psbD* promoter element.

However, in all three mutant strains *psbD* mRNA accumulated to levels of approximately 35% of wild-type mRNA indicating that no direct correlation between transcription rates and the level of transcript within a cell exists (Fig. 4a). At least in part, this could be explained by posttranscriptional processes which are able to increase RNA stability thus compensating for the defect in RNA synthesis. Such posttranscriptional processes therefore appear to represent the rate-limiting steps for RNA accumulation.

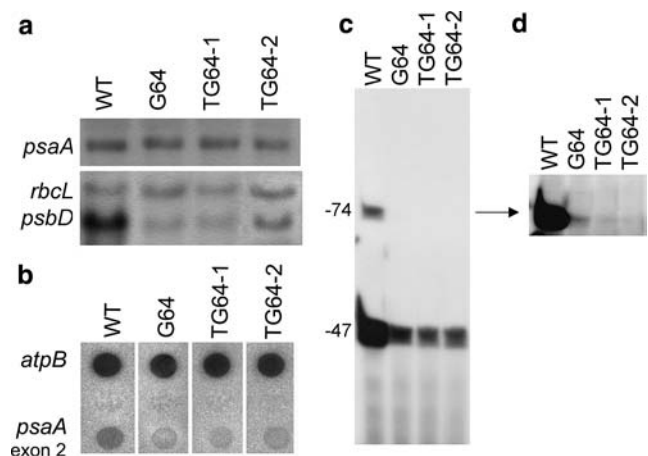
In *C. reinhardtii*, the *psbD* gene is cotranscribed with the downstream exon 2 of the tripartite *psaA* gene whose mRNA is generated via two transsplicing reactions



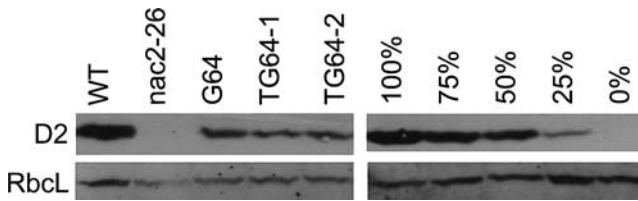
**Fig. 3** Chloroplast run-on transcription analysis. Radioactively labeled run-on transcripts from the strains indicated at the top were hybridized with PCR-amplified fragments of the *psbD* and *atpB* genes ( $5 \mu\text{g}$  each) which were transferred to nylon membranes by using the dot blot technique. Transcription rates relative to the wild-type are given in Table 1

involving exons 1, 2 and 3 (Kück et al. 1987). Although the fluorescence data (Fig. 1) did not indicate any effect of the *G64* mutation on photosystem I, we tested whether the *G64* mutation had any effect on *psaA* mRNA accumulation. As shown in Fig. 4a, *psaA* transcript levels were the same in the wild-type and all mutant strains despite the low *psaA* exon 2 transcription rate which is in the same range as *psbD* sequences (Fig 4b). This indicates that the availability of *psaA* exon 2 precursor RNA is not rate-limiting for *psaA* mRNA maturation. Most likely, the strong *psbD* promoter produces an excess of *psaA* exon 2 precursor transcripts as compared to *psaA* exon 1 and exon 3 precursor RNAs.

Moreover, the *psbD* mRNA has previously been shown to exist in two forms. The difference lies in the length of their 5' untranslated region and is most likely due to a 5' processing event (Nickelsen et al. 1999; Herrin and Nickelsen 2004). Both transcription initiation at position  $-74$  and 5' maturation were not compromised in *G64* nor the chloroplast transformants *TG64-1/2* as proven by primer-extension analysis (Fig. 4c, d). Hence, only quantitative changes in *psbD*



**Fig. 4** a, b, c, d Analysis of *psbD* mRNA. a Total RNA of the strains indicated at the top was separated by gelelectrophoresis, blotted onto a nylon membrane and hybridized with radiolabeled DNA probes of the *rbcL* and *psbD* genes as well as exon 2 of the *psaA* gene. The *rbcL* signal served as internal standard control. Relative *psbD* mRNA accumulation as compared to the wild-type is given in Table 1. b Run-on transcription analysis of *psaA* exon 2 sequences (for explanation see Fig 3) c Total RNA from strains indicated at the top was hybridized to oligonucleotide 3131, reverse transcribed, and the products were separated by denaturing PAGE. Arrows mark the *psbD* mRNA 5' ends at positions  $-74$  and  $-47$ . d Overexposure of the autoradiogram shown in c revealed the *psbD* mRNA 5' ends at position  $-74$  also in the mutant strains



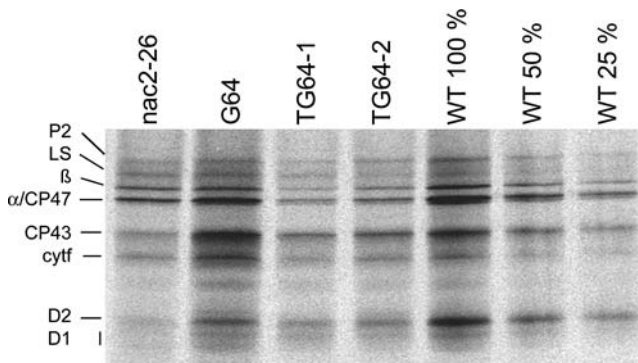
**Fig. 5** Western Analysis of the D2-protein. Total proteins of the strains indicated at the *top* were separated by denaturing SDS-PAGE, transferred to nitrocellulose filters and decorated with antibodies against either D2 or RbcL. At the *right*, serial dilutions of wild-type proteins with that of the *nac2* mutant which accumulates no D2 were coanalyzed. Thus, in these samples constant protein contents were maintained. The relative accumulation of D2 as compared to the wild-type is given in Table 1

mRNA accumulation but not changes in mRNA quality were observed in the mutant strains.

As the next step, the accumulation of the D2-protein in the different strains was monitored by a comparative Western analysis using a D2-specific antiserum. As shown in Fig. 5, D2-protein accumulated to levels corresponding to approximately 35% of the levels found in the wild-type in *G64* as well as in the two chloroplast transformants *TG64-1/-2*. Thus, these data revealed a direct correlation of *psbD* mRNA levels and D2-protein levels suggesting that the mRNA level is a rate-limiting factor for efficient protein synthesis in the chloroplast (Table 1). Consistently, a more than twofold reduction of D2 synthesis as compared to the wild-type was observed in the mutant strains in protein pulse labeling experiments with  $^{14}\text{C}$  acetate (Fig. 6; Table 1).

## Discussion

Here, we report on the molecular analysis of the chloroplast mutant *G64* from *C. reinhardtii*, which harbors a



**Fig. 6** Pulse labeling of chloroplast proteins. Proteins from the indicated strains were labeled with  $^{14}\text{C}$  for 7 min in the presence of cycloheximide and separated on a 12–18% sodium dodecyl sulfate (SDS)-polyacrylamide gel with 8 M urea. On the *left margin* reference electrophoretic bands are indicated, i.e., P2 (*psbA* gene product), LS (Rubisco large subunit, *rbcL* gene product), CP47 and CP43, (*psbB* and *psbC* gene products),  $\alpha$  and  $\beta$  subunits of ATP synthase (*atpA* and *atpB* gene products), *cyt f* (*petA* gene product), D2 and D1 (*psbD* and *psbA* gene products). For the WT 50 and 25%, broken labeled cells were diluted (1/2 and 1/4 respectively) with nonlabelled cells

point mutation within the 5' region of the *psbD* gene. Our findings show that the mutation results in a drastic reduction of transcriptional activity confirming an earlier hypothesis that the sequence element TATAAT is part of the *psbD* promoter (Klein et al. 1992).

To date, in vivo analysis of chloroplast promoter regions has only been carried out in algae and higher plants using reporter gene constructs (Blowers et al. 1990; Klein et al. 1992; Allison and Maliga 1995; Shiina et al. 1998). The advantage of this approach is that the process of transcription is assessed in relative isolation such that some additional regulatory effects on gene expression, for instance, feed-back control mechanisms involving the respective endogenous chloroplast gene products cannot interfere with the assay. However, the phenotypic changes resulting from mutations affecting the promoter regions cannot be monitored because in this experimental set-up, transcription as well as other steps involved in gene expression are uncoupled from the regulatory network which links gene expression to the physiological condition of the cell. The availability of the mutant *G64* has enabled us to analyze these phenotypic effects of reduced transcription of the chloroplast *psbD* gene from *C. reinhardtii* for the first time. When we quantitated the different levels of *psbD* gene expression, i.e., transcription, RNA accumulation and protein accumulation, two major conclusions could be drawn.

First, the reduction of the *psbD* transcription rate to ca. 10% of the wild-type level led to mRNA accumulation equivalent to ca. 35% of the wild-type level. This clearly demonstrates that no direct correlation exists and that, instead, mainly processes involved in stabilizing RNA appear to determine transcript levels. Interestingly, the work of Eberhard et al. (2002) revealed that a complete inhibition of chloroplast transcription by rifampicin treatment results in the accumulation of approximately 24% *psbD* mRNA over a 6-h period. This level is in the range of the one found in the *G64* mutant. It has therefore been suggested previously that the availability of regulatory factors acting on the posttranscriptional level is rate-limiting for the expression of chloroplast genes including the *psbD* gene (Eberhard et al. 2002). One obvious candidate for a regulatory factor controlling *psbD* mRNA accumulation is represented by the Nac2 protein, which has been shown to be specifically required for *psbD* mRNA accumulation (Boudreau et al. 2000). In a recently proposed model, Nac2 protects *psbD* transcripts against degradation from the RNA 5' end. In addition, it is involved in subsequent steps during the initiation of translation by directing the RNA-binding protein RBP40 to its cognate-binding site, a process required for D2 synthesis (Ossenbühl and Nickelsen 2000; Nickelsen 2003). Hence, this model is based on a tight association between RNA-stabilization events and the initiation of translation on a molecular level. Interestingly in a *nac2* mutant, *psbA* gene expression is not compromised resembling the situation found for *G64* (Kuchka et al. 1989). This suggests that—despite the

cotranscription of *psbD* and the *psaA* exon 2 regions—no coupling between the expression of these genes exists.

Our second finding further supports this hypothesis because a direct correlation between *psbD* mRNA accumulation and D2-protein accumulation has been demonstrated in *G64*. This correlation of RNA levels to protein accumulation was unexpected because it contradicts with the previous results (Eberhard et al. 2002). As mentioned above a fourfold reduction of the *psbD* mRNA steady-state level was measured over 6 h after the inhibition of chloroplast transcription in *C. reinhardtii* wild-type cells by rifampicin treatment. However, similar to other chloroplast proteins, the D2 level was not affected by the drug treatment during that period. Secondary effects caused by the inhibitor rifampicin or differences in the experimental set-ups used may explain the apparent discrepancy between the results. For example, the duration of inhibitor treatment had to be restricted to 6 h because longer drug exposure would have been lethal for the cells. In contrast to the genetic approach used in this study, it was impossible to perform long-term measurements covering several generations. Another explanation to be considered is the possibility of a chloroplast-encoded translational repressor of D2-synthesis, which could be affected by the transcriptional inhibitor rifampicin.

In summary, the analysis of the *C. reinhardtii* chloroplast-promoter mutant *G64* determined for the first time the long-term relationship between transcription, RNA accumulation and protein accumulation inside a living cell. In the case of the *psbD* gene from *C. reinhardtii*, the data indicate that if transcription was reduced due to a genetic defect, i.e., a point mutation, alterations in RNA stability could partially compensate for the suboptimal RNA synthesis. Moreover, the level of *psbD* mRNA was directly proportional to the level of D2-protein accumulation suggesting that regulatory factors like Nac2 catalyze the rate-limiting steps for *psbD* gene expression. Current approaches to further elucidate this hypothesis include the targeted deregulation of Nac2 and the analysis of the resulting molecular and physiological changes.

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