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Chloroplast RNA Stability in *Chlamydomonas*: Rapid Degradation of *psbB* and *psbC* Transcripts in Two Nuclear Mutants

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Toward understanding regulation of chloroplast transcript abundance, we have isolated and analyzed nuclear mutant strains of *Chlamydomonas reinhardtii* that lack chloroplast-encoded mRNAs for photosystem II proteins. Mutant 6.2z5 accumulates no transcripts of the *psbC* locus for the 43-kilodalton chlorophyll-binding protein. In mutant GE2.10, transcripts of *psbB*, encoding the 47-kilodalton chlorophyll-binding protein, cannot be detected [Jensen, K.H., Herrin, D.L., Plumley, F.G., and Schmidt, G.W. (1986). J. Cell Biol. 103, 1315–1325]. Also, GE2.10 does not accumulate several low molecular weight transcripts from a region of the chloroplast genome proximal to *psbB*. The levels of mRNAs from other chloroplast genes are not affected in either mutant. Chloroplast transcription was analyzed in permeabilized cells and by in vivo pulse labeling. Although 5′ ribonuclease was found as an artifactual activity of permeabilized cells, the results from both assays demonstrated that wild-type levels of *psbC* transcription occur in mutant 6.2z5 and that chloroplasts of GE2.10 transcribe *psbB* and adjacent genes. Thus, it appears that the nuclear genes that are mutated in 6.2z5 and GE2.10 encode products that, respectively, confer stability to transcripts from the *psbC* and the *psbB* regions of the chloroplast genome.

INTRODUCTION

The relative levels of chloroplast transcripts can vary in response to developmental and environmental cues. In plants with C4 photosynthesis, differentiation of cells with specialized photosynthetic functions is accompanied by increases in the abundance of chloroplast-encoded *rbcL* (Link et al., 1978) and *psbA* mRNAs (Sheen and Bogorad, 1986). In tomato plants, the fruit, roots, stems, and etiolated seedlings accumulate distinct patterns of plastid mRNAs (Piechulla et al., 1986). Light is also a regulatory signal: responses to illumination in barley and maize seedlings include a transient increase in the pool size of chloroplast transcripts as a result of changes in transcriptional activity (Klein and Mullet, 1990). Spinach and mustard seedlings differentially accumulate chloroplast transcripts when grown in light versus darkness (Deng and Gruissem, 1987; Dietrich et al., 1987). Growth of both pea and spinach plants with wavelengths of light that preferentially excite either photosystem I or photosystem II results in different levels of the plastid transcripts encoding photosystem I and photosystem II reaction center polypeptides (Glick et al., 1986; Deng et al., 1989).

Molecular details about the mechanisms for regulating chloroplast RNA transcript abundance have been only partially resolved. Chloroplast gene transcription has been studied both by transcription run-on assays and with extracts capable of accurate transcription initiation. Gruissem and Zurawski (1985a, 1985b) used a chloroplast in vitro transcription system to show that chloroplast promoter sequences for protein coding and tRNA genes possess sequence similarity to canonical *Escherichia coli* −10 and −35 promoter sequences. Although deviation from the prokaryote-like consensus results in differential promoter strength as measured by the in vitro system, it is unclear whether variable promoter strength correlates with in vivo patterns of chloroplast RNA synthesis. To distinguish transcriptional from post-transcriptional regulation of RNA abundance, transcription run-on assays have been used also with purified developing chloroplasts (Deng and Gruissem, 1987; Mullet and Klein, 1987), root cell plastids (Deng and Gruissem, 1988), and chloroplasts adapted to red, yellow, or white light (Deng et al., 1989). Because the ploidy of chloroplast DNA can vary substantially between tissues, mRNA abundance was compared with the gene copy number and the run-on transcription rates. The results from these studies indicate that there is minimal transcriptional regulation in these plastids. Thus, post-transcriptional mechanisms must also strongly affect the

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altered abundance of transcripts observed in some plastid types.

To help elucidate mechanisms in which nuclear gene products affect the abundance of specific plastid mRNAs, we have studied the regulation of *psbB* and *psbC* transcripts in *Chlamydomonas reinhardtii*. These genes encode, respectively, the 47-kD apoprotein of the CP47 and the 43-kD apoprotein of CP43, chlorophyll *a* complexes of the photosystem II reaction center. In *C. reinhardtii*, the apoproteins are designated polypeptides 5 and 6, respectively. The sequence of the *psbB* gene in *C. reinhardtii* has not been reported, but in vascular plants the *psbB* locus exemplifies the complexity of chloroplast gene expression. It is part of a transcription unit that includes *psbH* for the 10-kD phosphoprotein of photosystem II (Westhoff et al., 1986; Rock et al., 1987), *petB* for cytochrome b_6, and *petD* for the subunit 4 of cytochrome b_6/f complex (Alt et al., 1984; Morris and Herrmann 1984). The *petB* and *petD* genes also contain introns that contribute to the large size of the primary transcript (Morris and Herrmann, 1984; Rock et al., 1987). Accumulation of partially processed RNAs leads to a complex pattern of approximately 20 different sized transcripts in leaf tissue (Barkan, 1988; Westhoff and Herrmann, 1988). Transcription also occurs in the opposite direction on the other strand in this region, producing ORF43 RNA from a sequence between *psbB* and *psbH* (Kohchi et al., 1988). In leaf meristem and root tissues, the relative proportion of RNA from the *psbB* operon as well as other chloroplast RNAs that do not undergo exon splicing is much greater than in mature leaf tissue (Barkan, 1989). Whereas intron removal is a prerequisite for *petB* and *petD* translation, processing steps that remove coding sequences for the other components encoded by this operon, whether located either 5′ or 3′, are not requirements for translation of *psbB*, *petB*, or *petD* mRNAs (Barkan, 1988). Thus, regulation of RNA maturation may affect the availability of translatable mRNAs (Barkan, 1989). The mRNAs from this locus may also be translationally or post-translationally regulated because there is accumulation of *petB* and *petD* mRNAs in dark-grown tissue, but an absence of *psbB* mRNA (Herrmann et al., 1985). Also, the photosystem II and cytochrome b_6/f subunit genes of this locus differ in codon bias that may affect differential rates of translation in maize (Rock et al., 1987).

In higher plants, *psbD* and *psbC* are cotranscribed and overlap by approximately 50 bp (Alt et al., 1984; Rasmussen et al., 1984). The transcript is processed in a complex manner in barley: of the six RNA products that accumulate in dark-grown plants, four are not present when the plants are grown in light (Gamble et al., 1988). Whereas the organization of chloroplast genes is highly conserved among higher plants, algae show great variability. In *Euglena gracilis*, *psbC* does not overlap *psbD* and it contains a 1.6-kb intron possessing an open reading frame (Montandon et al., 1986). In *C. reinhardtii*, *psbC* contains no introns and also does not overlap *psbD* (Rochaix et al., 1989).

The goal of this study was to understand how nuclear gene products might be involved in the regulation of *psbC* and *psbB* mRNA abundance. Toward this end, we isolated a mutant of *C. reinhardtii*, 6.2z5, that does not accumulate *psbC* transcripts. We also have analyzed *psbB* gene organization and its expression with the aim of identifying the defect in mutant GE2.10 which accumulates no *psbB* mRNA (Jensen et al., 1986). The abundance of all other plastid mRNAs we have examined is not affected by either of these mutations of a single nuclear gene. Transcription assays were developed to show that the nuclear genes that are mutated in 6.2z5 and GE2.10 affect chloroplast mRNA accumulation at a post-transcriptional level.

RESULTS

Isolation of Mutant 6.2z5

High fluorescent colonies of *C. reinhardtii* were obtained following UV treatment and enrichment for photosynthetic electron transport mutants with metronidazole (Schmidt et al., 1977). To identify strains with defects in photosystem II biogenesis, the isolates were characterized by pulse labeling cells with $^{35}SO_4^{3-}$ in the presence of cycloheximide. Figure 1A shows a fluorograph of the labeled membrane proteins of wild type and one such photosystem II mutant, 6.2z5. During the 30-min labeling, wild-type cells abundantly synthesized two of the core polypeptides of the photosystem II reaction center, D1 and D2, and the chlorophyll *a*-binding apoproteins of CP47 (polypeptide 5) and CP43 (polypeptide 6). Polypeptides 5, D1, and D2 also were labeled during pulse labeling of mutant 6.2z5, but no radiolabeled polypeptide 6 was detectable even when the incorporation of $^{35}SO_4^{3-}$ was limited to 5 min (data not shown).

It has been reported previously that most of the photosystem II proteins are rapidly degraded unless the full complement of these polypeptides is synthesized (Jensen et al., 1986; Schmidt et al., 1987; Rochaix et al., 1989). This is also the case for mutant 6.2z5, in which polypeptides 5 and 6 were absent among the thylakoid membrane proteins resolved by SDS-PAGE and stained with Coomassie Blue (Figure 1B). D1 and D2 are normally not detected in stained gels, but immunoblot analyses demonstrated that they also do not accumulate in 6.2z5 (data not shown). In addition, polypeptides 19 and 24, of the water oxidation complex associated with the photosystem II reaction center, were conspicuous among the wild-type thylakoid proteins, but these were absent from purified thylakoids of mutant 6.2z5. Except in the case of mutations affecting D2 synthesis (Kuchka et al., 1989), these extrinsic proteins of the thylakoid lumen usually accumu-

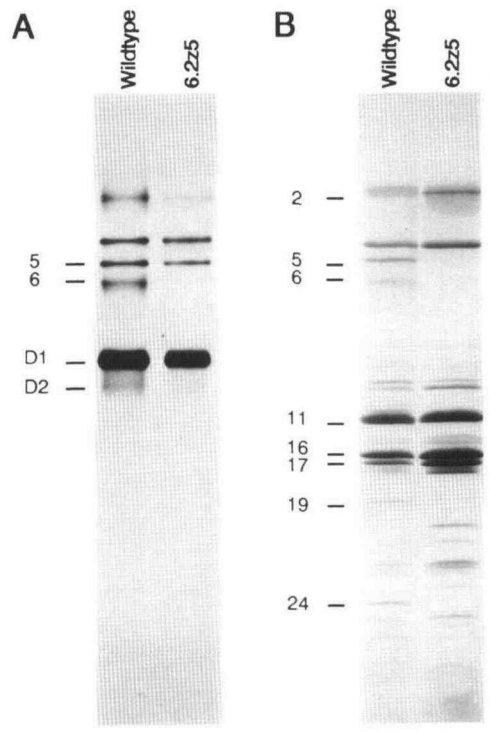

Figure 1. Photosystem II Protein Synthesis and Accumulation in Mutant 6.2z5 and Wild Type (137c GB125).

(A) Fluorograph of electrophoretically separated membrane proteins from cells pulse labeled with $^{35}SO_4^{3-}$ for 30 min in the presence of cycloheximide. The samples for the 10% to 20% polyacrylamide gradient gels contained 40,000 cpm.
(B) Thylakoid membrane polypeptides of mutant 6.2z5 and wild type resolved by SDS-PAGE and stained with Coomassie Blue. Each lane contains thylakoid preparations corresponding to 20 μg of chlorophyll. Samples were heated to 100°C for 45 sec immediately before loading the gel.

late in photosystem II mutants, but they are released from the membranes during the process of cell fractionation (Greer et al., 1986; De Vitry et al., 1989). Finally, other chloroplast-synthesized proteins such as the apoproteins of the photosystem I reaction center (polypeptides 2) and nuclear-encoded polypeptides of light-harvesting complexes (i.e., polypeptides 11, 16, and 17) accumulate to similar levels in the mutant and wild type.

Genetic Characterization of Mutant 6.2z5

The genetic lesion in mutant 6.2z5 was assessed from segregation analyses of back-crosses to wild-type parental strains. The tetrad daughter cells of 86 back-crosses consistently exhibited a 2:2 ratio of the wild type to the high fluorescence phenotype, indicating that there is a single nuclear gene mutation in 6.2z5. An analysis of the thylakoid products of chloroplast protein synthesis in such progeny is shown in Figure 2 (BC1 Tetrad), revealing that the deficiency in polypeptide 6 synthesis is inherited in a Mendelian manner also. Because the parental strain of 6.2z5 was mating type plus, the mutant's chloroplast DNA is transmitted exclusively to all meiotic products (Sager and Ramanis, 1965). To eliminate the possibility that the phenotype of 6.2z5 is due to a double mutation, including one residing on the chloroplast genome of the mutant, the progeny from second generation back-crosses to wild type with mating type minus mutants were also analyzed. Again, the high fluorescence phenotype segregated in a 2:2 ratio among the tetrad products. A pulse-labeling analysis of chloroplast-synthesized thylakoid proteins with the daughter cells of a representative tetrad is shown in Figure 2 (BC2 Tetrad). The segregation of high fluorescence consistently corresponded to the exclusive loss of polypeptide 6 synthesis. Therefore, the phenotype of 6.2z5 results from a single nuclear gene mutation and does not

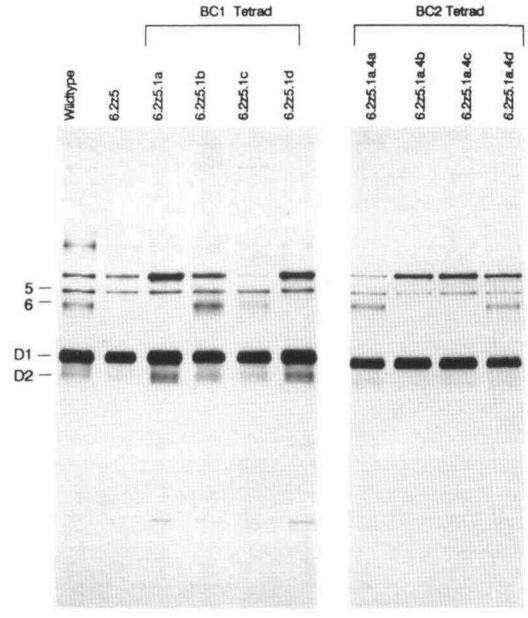

Figure 2. A Single Nuclear Gene Mutation Affects Polypeptide 6 (apo-CP43) Synthesis in Mutant 6.2z5.

For first-generation back-crosses (BC1), gametes of mutant 6.2z5 (mating type +) were mated with those of wild type (137c GB124, mating type −). After zygote germination, colonies of the daughter tetrads were recovered and subjected to pulse labeling in the presence of cycloheximide as in Figure 1A. A subsequent back-cross (BC2) was performed with 6.2z5.1a (mating type −) and, as the maternal parent, 137c GB125 (mating type +). The fluorographs are of thylakoid proteins (40,000 cpm/lane) synthesized in the parental strains and the tetrad products resolved by SDS-PAGE.

involve a cryptic, secondary mutation of the chloroplast genome.

Chloroplast Transcripts of Mutant 6.2z5

To determine whether the absence of polypeptide 6 in mutant 6.2z5 is due to a block in transcription, RNA stability, or translation, chloroplast RNA was analyzed by RNA gel blot hybridization with various chloroplast DNA fragments, as shown in Figure 3. The mutant accumulated wild-type levels of transcripts of the chloroplast genes *rbcL*, *psbA*, *psbD*, *psaA2*, and an array of transcripts from the *psbB* locus. Probes specific for *psbC* mRNA, however, did not hybridize to RNA from mutant 6.2z5. As will be shown later, the defect in polypeptide 6 mRNA accumulation in 6.2z5 is exerted at a post-transcriptional level.

Chloroplast Transcripts of Mutant GE.210

Another photosystem II mutant, GE.210, was previously characterized in our laboratory and was found to possess a phenotype with many similarities to 6.2z5 (Jensen et al., 1986). GE2.10 cannot synthesize polypeptide 5 because *psbB* mRNA for this protein is selectively absent as the result of another nuclear gene lesion. However, the phenotype of GE2.10 is somewhat more complex in that several transcripts from the *psbB* locus, the approximately 2-kb mRNA for polypeptide 5 and a series of transcripts ranging from about 150 bases to about 1200 bases, are not detected by RNA gel blot assays (Jensen et al., 1986). Because no sequence information for the *psbB* gene of *C. reinhardtii* has been available, it seemed possible that the smaller transcripts of wild-type cells that hybridize with large *psbB* probes are excised introns. Alternatively, the low molecular weight transcripts from *psbB* might represent relatively stable breakdown products of the polypeptide 5 mRNA or products of *psbB* cotranscription. Regardless, the phenotype of GE2.10 could be due to either a transcriptional or post-transcriptional defect.

Toward clarifying the nature of the transcripts from the *psbB* locus of *C. reinhardtii*, two adjacent EcoRI fragments of chloroplast DNA (R19 and R5; Harris, 1989) were subcloned, as indicated in Figure 4A. Subsequently, the smaller gene fragments were employed for RNA gel blot hybridization with RNAs from wild-type and mutant GE2.10, as shown in Figure 4B. From these data, it is apparent that the mutation in GE2.10 affects accumulation of transcripts from over 5 kb of the *psbB* region of the chloroplast genome. The mutation in GE2.10 does not affect a small transcript, presumably $tRNA^{Tyr}$ (Bergmann et al., 1985), that hybridizes to subclone A (plasmid 51-3) of the R19 fragment. Similarly, a very large transcript hybridizing with subclones E (plasmid 4-10) and F (plasmid 2-10) is present in wild-type amounts in GE2.10. Woessner et al. (1987) employed a heterologous spinach probe to localize the *atpF* gene (for subunit I of ATP synthetase) to a region of R5 that spans the BamHI site. It is intriguing that the most abundant transcript from this region, if it

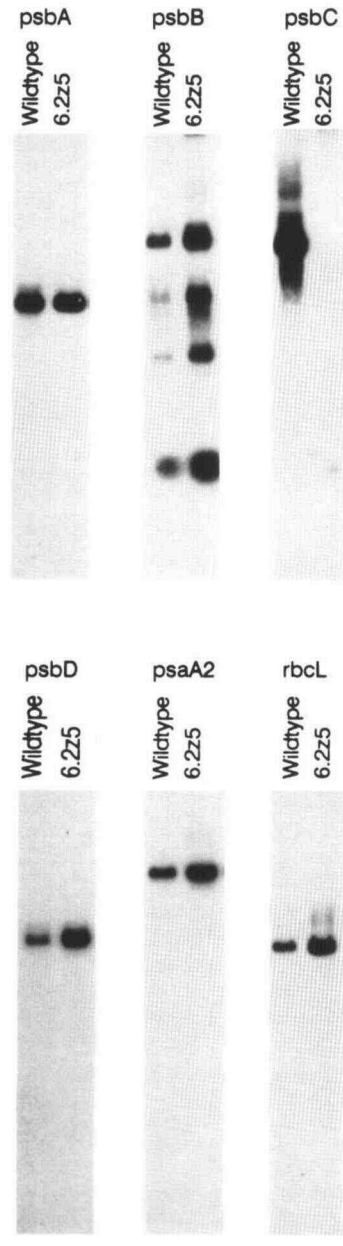

Figure 3. Mutant 6.2z5 Does not Accumulate mRNA for Polypeptide 6 (apo-CP43).

Total RNA from wild type (4 μg) and mutant 6.2z5 (8 μg) was electrophoresed in 1.5% agarose/6% formaldehyde gels. RNA gel blots onto Zeta-probe nylon membranes were prepared and hybridized with random primer-labeled DNA corresponding to the indicated chloroplast genes.

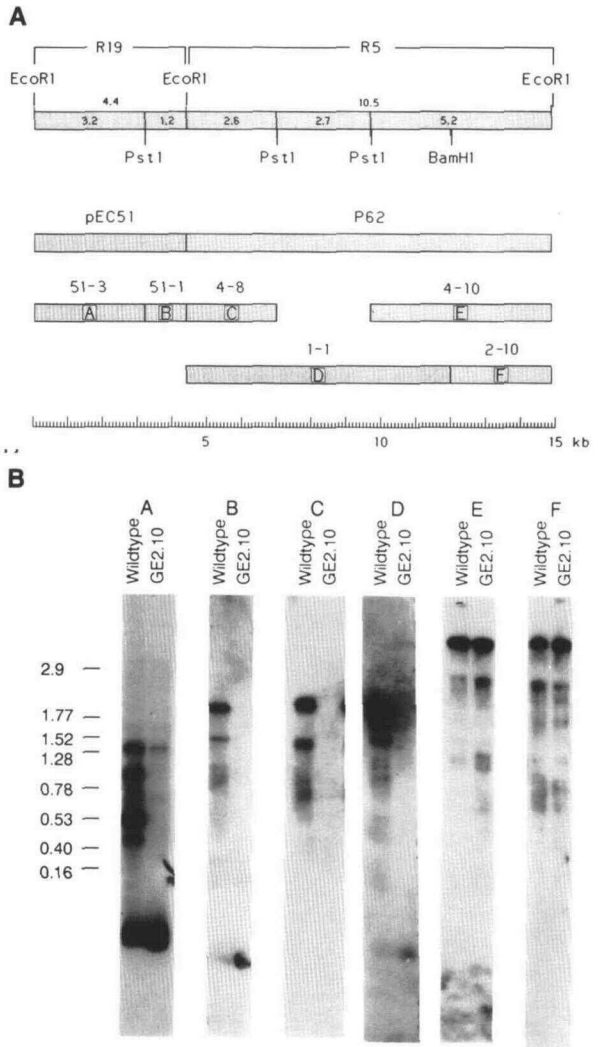

Figure 4. Mapping of the *psbB* Locus.

(A) Restriction map of the *psbB* locus and clones. The *psbB* region of the *C. reinhardtii* chloroplast genome extends across two EcoRI fragments, R19 and R5. These are represented in cloned pEC51 and P62, respectively. pEC51 was further subcloned upon PstI digestion to generate clones 51-3 and 51-1. P62 was subcloned with EcoRI/PstI and EcoRI/BamHI double digestions to generate clones 4-8, 4-10, 1-1, and 2-10.

(B) RNA gel blots of RNA from wild type and mutant GE2.10. RNA was electrophoresed and blotted onto nylon membranes as in Figure 3. Radiolabeled probes were 51-3 (A), 51-1 (B), 4-8 (C), 1-1 (D), 4-10 (E), and 2-10 (F).

corresponds to subunit I of about 20 kD, is so large in *C. reinhardtii*. In vascular plants, *atpF* is a split gene that is part of a complex transcriptional unit that includes *atp*A, *atpH*, *atpI*, and *rps12* for the subunits α, III, and F_0a of the ATP synthetase and a ribosomal protein (Hennig and Herrmann, 1986; Hudson et al., 1987). If there is a parallel, although inexact (Woessner et al., 1987), gene organization in *C. reinhardtii*, perhaps the most abundant, high molecular weight transcript represents an unprocessed precursor for several mRNAs, whereas mature *atpF* mRNA is one of the low abundant transcripts of smaller size.

The selective consequences of the mutation in GE2.10 on transcript abundance are defined by hybridization analyses with probe A (plasmid 51-3), which is specific for transcripts of less than 1.5 kb, and probes B-D (plasmids 51-1, 4-8, and 1-1, respectively) that hybridize to *psbB* mRNA and to a transcript that is slightly larger than 1.5 kb (Figure 4B). The low molecular weight transcripts must not be excised introns of a primary transcript of *psbB* because there is no hybridization of probe A to the polypeptide 5 mRNA of about 2 kb. However, it is possible that the RNAs from the region of probe A are cotranscribed with *psbB* and, therefore, are subject to modes of regulation that are mutual for the mRNA for polypeptide 5.

Cytochromes of GE2.10 Thylakoids

The complex array of RNAs that hybridize to the chloroplast DNA fragments adjacent to *psbB* is reminiscent of transcript from the *psbB* region of higher plants. As noted previously, the *psbB* operon of vascular plants also contains the *psbH* gene for the 10-kD phosphoprotein of photosystem II and *petB* and *petD* genes that encode cytochrome b_6 and subunit 4 of the cytochrome b_6/f complex (Westhoff et al., 1986). To assess whether expression of *pet* genes is affected in GE2.10, heme-dependent staining of the thylakoid proteins of GE2.10 was used to determine whether these products are absent. Figure 5A shows the profile of chlorophyll-protein complexes that are resolved by SDS-PAGE of nonheated thylakoid samples. The photosystem I chlorophyll-protein complex, CPI, and the light harvesting chlorophyll protein complexes, CPII, are present in both wild type and GE2.10, whereas chlorophyll protein complexes corresponding to the photosystem II polypeptides 5 and 6, CPIII (CP47) and CPIV (CP43), are present only in wild-type thylakoids. Stainings of this gel, first with 3,3′,5,5′-tetramethylbenzidine (TMBZ) to detect heme-associated peroxidase activity and then with Coomassie Blue, are shown in Figures 5B and 5C. An identical cytochrome composition of both heated and nonheated thylakoids of wild type and GE2.10 is apparent. Therefore, the low molecular weight transcripts that are missing in GE2.10 do not encode for thylakoid cytochromes, and the location of chloroplast genes encoding the apoproteins of the cytochrome b_6/f complex must be different in *C. reinhardtii* than in vascular plants. Turmel et al. (1988) have reported that *petB* is adjacent to *rpl2* in *C. reinhardtii*. As demonstrated before (Jensen et al., 1986), photosystem II polypeptides 5 and 6 are apparent among

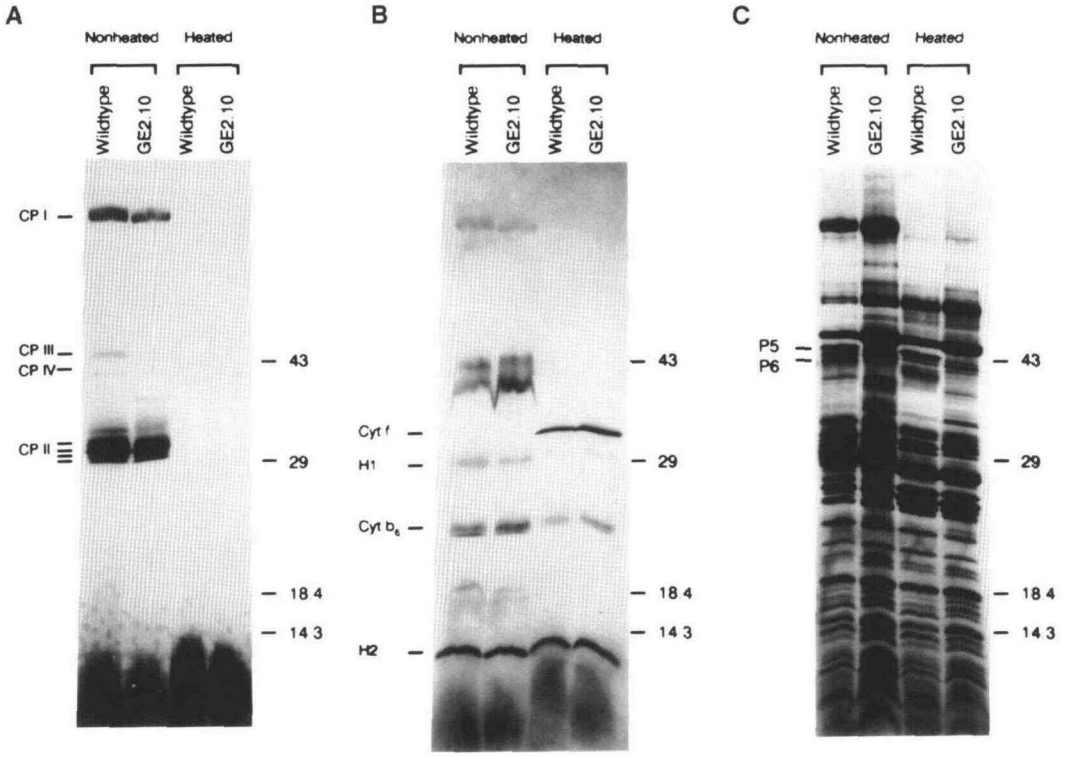

Figure 5. Thylakoid Membrane Polypeptides, Cytochromes, and Pigment-Protein Complexes in Wild Type and Mutant GE2.10.

Thylakoids from wild type and GE2.10 were purified, and samples of 20 μg of chlorophyll were subjected to SDS-PAGE along with the indicated molecular weight markers.

(A) Pigment-protein complexes. In samples that are not heated before electrophoresis, four chlorophyll-protein complexes of wild-type thylakoids can be detected in unstained gels. These are: CPI, the photosystem I reaction center complex; CPII, an array of chlorophyll *a/b* light-harvesting complexes; CPIII, equivalent to CP47 of photosystem II; CPIV, equivalent to CP43 of photosystem II. CPIII and CPIV are absent in GE2.10 thylakoids.

(B) Thylakoid cytochromes. The gel in **(A)** was stained for heme peroxidase activity with TMBZ as described in Methods. The designation of cytochromes is as defined by Lemaire et al. (1986).

(C) Thylakoid polypeptides. The gel in **(A)** and **(B)** was stained with Coomassie Blue.

the wild-type thylakoid proteins but are absent in those of GE2.10.

Transcription in Permeabilized Cells

To distinguish between transcriptional and post-transcriptional effects on the accumulation of *psbB* and *psbC* mRNAs in mutants GE2.10 and 6.2z5, we analyzed chloroplast transcription by hybridization of pulse-labeled RNA to chloroplast DNA fragments. The isolation of intact chloroplasts from *C. reinhardtii* is inefficient and, even in the case of vascular plants, requires manipulations that might alter transcriptional/post-transcriptional processes. As an alternative system, we explored the utility of a permeabilized cell system derived from that described by Guertin and Bellemare (1979). Rifampicin, an inhibitor of chloroplast RNA polymerase in *C. reinhardtii* (Surzycki, 1969), inhibits >90% of the incorporation of ^{32}P-UTP into RNA in permeabilized wild-type cells, but high concentrations of α-amanitin have no effect (Guertin and Bellemare, 1979, and data not shown). Thus, this transcription system is specific for the chloroplast RNA polymerase, but is only active for approximately 10 min (Guertin and Bellemare, 1979, and data not shown). The transiency of RNA synthesis suggests that chloroplast RNA polymerase is capable of elongation but does not reinitiate transcription in the permeabilized cells. An autoradiograph of a denaturing agarose gel resolving the transcription products formed during 5-min, 10-min, and 15-min labelings is shown in Figure 6A. The size of transcripts is heterogeneous, ranging from 700 bases to >1.5 kb at the 5-min pulse labeling, and increasing to 1 kb to >1.7 kb at the 10-min and 15-min time points. Dot blots of cloned chloroplast DNA

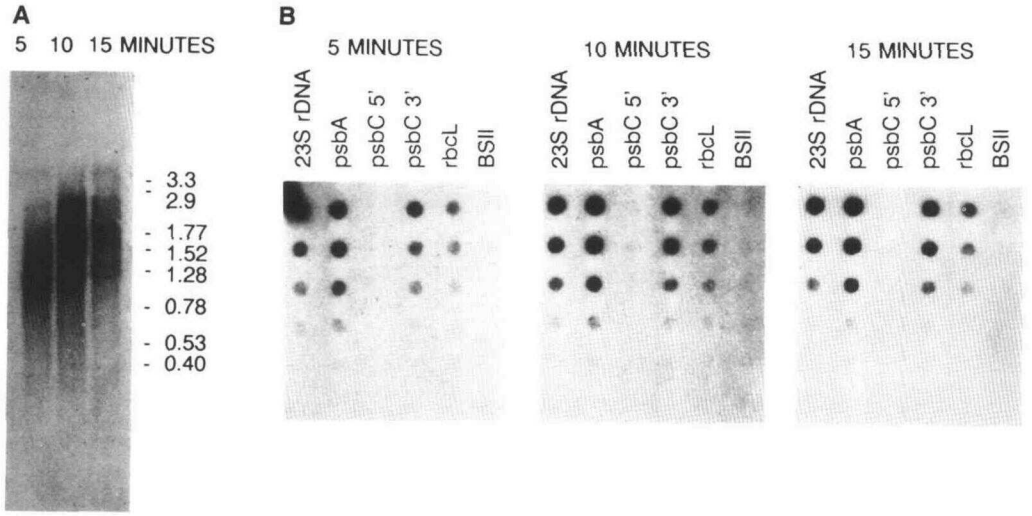

Figure 6. Products of Transcription in Permeabilized Cells.

(A) Autoradiograph of radiolabeled transcripts from permeabilized cells. RNA (100,000 cpm) from wild-type cells labeled for 5 min, 10 min, and 15 min was electrophoresed in a 1.5% agarose-6% formaldehyde gel along with marker RNA of the indicated sizes (in kilobases).
(B) Hybridization of RNA transcribed in vitro to dot blots of chloroplast genes. Each column is a fourfold dilution of cloned chloroplast DNA fragments, ranging from 2 μg (top) to 2 ng (bottom). In addition to those chloroplast loci described in Methods, the Bluescript plasmid vector (BSII) was also employed for hybridization with radiolabeled RNA synthesized during 5 min, 10 min, and 15 min in permeabilized wild-type cells.

fragments and the plasmid vector probed with products from the pulse labelings are shown in Figure 6B. Nearly equivalent hybridization signals were obtained with *psbA* and a 23S rDNA fragment; these exhibited a parallel increase between 5 min and 10 min of incorporation and then ceased to change in intensity. In contrast, synthesis of *rbcL* mRNA for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase appeared to be arrested within 5 min. No hybridization to dot blots containing vector DNA (BSII) was detected, demonstrating hybridization specificity.

Because there were no conspicuous qualitative changes in the newly synthesized RNA between 10 min and 15 min of incubation, it appears that endogenous RNases generally do not affect the spectrum of transcripts synthesized in permeabilized cells. Moreover, the inclusion of RNase inhibitors (Inhibitase, 5 Prime-3 Prime, Inc.; human placental RNase inhibitor) in the reaction mixtures did not alter hybridization signals, transcript sizes, or the duration of incorporation (data not shown). However, the permeabilized cell system was not free of artifacts. The synthesis in wild-type cells of mRNA for polypeptide 6 was not detected with a 5′ DNA fragment of corresponding to 935 nucleotides that are present in the mature transcript of *psbC*. On the other hand, a clone for approximately 600 bp of the 3′ portion of the gene indicated that the rate of *psbC* transcription is equivalent to that of *psbA* and 23S rDNA. Reproducible hybridization signals with the *psbC* 5′ fragment were not obtained by using shorter pulse labelings or by modifying the buffer conditions for transcription in the permeabilized cells. Hence, upon cell permeabilization, either an RNase with specificity for the 5′ region of the *psbC* transcript is activated or factors that normally protect this RNA from 5′ digestion are dissociated. We do not know whether additional RNAs are similarly subject to degradation, but we recommend cautious interpretation of transcription profiles obtained with permeabilized cells. Nevertheless, it is probable that a chloroplast gene is transcribed in vivo if it is determined that its corresponding RNA is synthesized in permeabilized cells.

In Figure 7, DNA gel blots containing digests of purified chloroplast DNA were hybridized with transcription products from permeabilized mutant and wild-type cells. Very similar patterns of hybridization for run-on products derived from wild type and the mutant were obtained and nearly all regions of the genome appeared to undergo transcription. The restriction fragments that were intensely labeled corresponded to the inverted repeat regions of the chloroplast genome. In contrast, very little hybridization was associated with the BamHI fragment 8, indicated by the arrow in Figure 7. So far, only an unidentified tRNA gene has been mapped to this region of the *C. reinhardtii* chloroplast genome (Bergmann et al., 1985). It was previously shown that the spacer regions between chloroplast

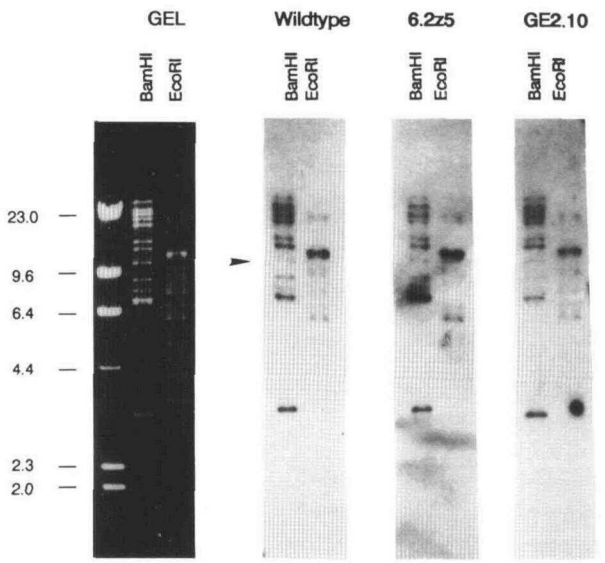

Figure 7. Chloroplast Transcription Profiles in Mutant and Wild-Type Strains.

Purified chloroplast DNA, digested with either EcoRI or BamHI, was employed for DNA gel blot hybridization with labeled RNA synthesized in permeabilized cells. The arrow indicates the position of BamHI fragment 8.

genes are not transcribed in the permeabilized cell system (Herrin and Schmidt, 1988). Thus, the pattern of RNA synthesis in permeabilized cells faithfully reflects the arrangement of transcription units.

To assess gene-specific transcription, DNA gel blots with gene-specific fragments were hybridized with the transcription products from permeabilized cells, as shown in Figures 8 and 9. Although mutant 6.2z5 accumulated no *psbC* mRNA, the *psbC* locus (as measured with a 3′ probe) and several other chloroplast genes were transcribed at wild-type rates in permeabilized cells of the mutant. This finding has been confirmed by hybridization of the labeled transcripts to dot blots of increasing amounts of *psbC*-3′ DNA: comparable hybridization signals were obtained with the wild-type and mutant RNAs as the immobilized DNA approached or achieved excess (data not shown). If it is assumed that chloroplast RNA polymerase has bound to the *psbC* gene before permeabilization, the nuclear mutation in mutant 6.2z5 does not affect the transcription of this gene. Similarly, Figure 9 shows that permeabilized cells of GE2.10 synthesize *psbB* mRNA as well as the low molecular weight transcripts from adjacent genes. Based upon gene sequence analysis, probe 4-8 corresponds to almost all of the coding sequence and a small 5′-untranslated region of *psbB* (S.B. Lowe and G.W. Schmidt, manuscript in preparation). Thus, the data of Figure 9 do not show whether there is a ubiquitous effect of cell permeabilization on the degradation of chloroplast RNA 5′ sequences such as is observed with *psbC* transcripts.

Transcription in Vivo

It seems possible that in vivo the RNA polymerase is stalled at the *psbC* or *psbB* genes in mutants 6.2z5 and GE2.10, respectively, and that permeabilization results in release of a transcriptional elongation block. Also, it is possible that artifactual initiation of transcription can occur in the permeabilized cells. For a definitive test of transcription of the affected genes in the mutants, we analyzed chloroplast RNA synthesis in vivo. Cells were phosphate depleted and then were pulse labeled with $^{32}PO_4^{3-}$ for 5 min or 10 min. Purified RNA was used as a hybridization probe for DNA gel blots, shown in Figures 10 and 11, and DNA dot blots (data not shown). The patterns of chloroplast gene transcription in the mutants were nearly identical to those obtained with wild-type cells. Specifically, *psbC* was transcribed at similar rates in 6.2z5 and wild type. It is noteworthy that the *psbC*-5′ probe could be used to detect in vivo transcripts from wild-type and mutant chloroplasts (Figure 11). Furthermore, when very brief labeling periods were employed, no defects were observed in GE2.10 in the transcription of *psbB*, as determined with probe 4-8, or the nearby genes for the low molecular weight transcripts, hybridizing to probe 51-3. We conclude that the absence of chloroplast mRNAs in the two mutant strains

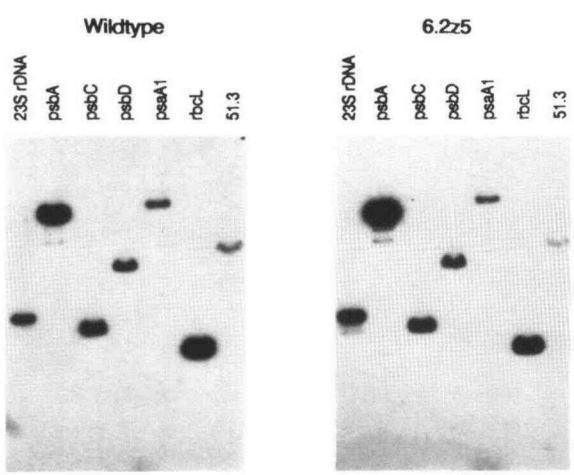

Figure 8. Transcription of Specific Genes in Permeabilized Cells of Mutant 6.2z5 and Wild Type.

The indicated cloned chloroplast DNA fragments were electrophoresed in agarose gels, transferred to nylon membrane, and hybridized with radiolabeled RNA that was synthesized in the permeabilized cells.

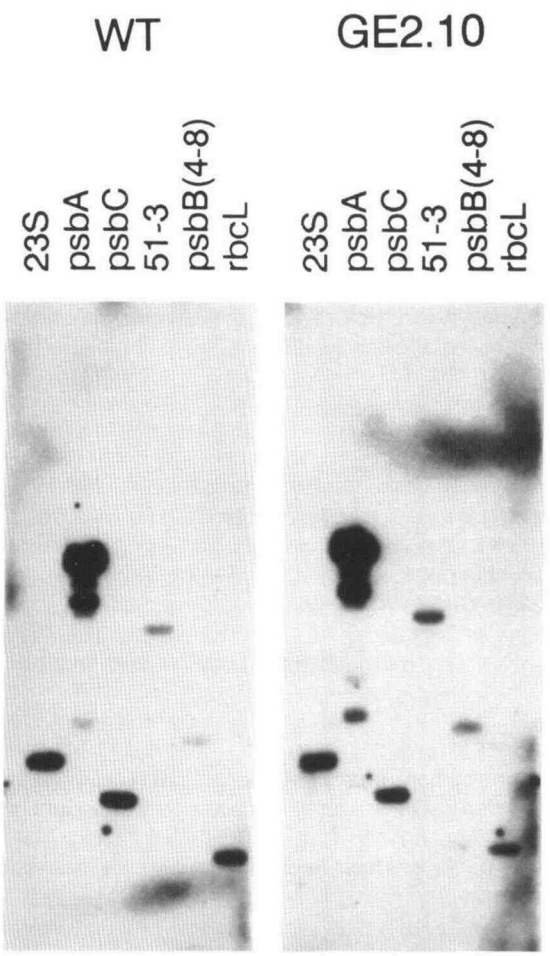

Figure 9. Transcription of Specific Genes in Permeabilized Cells of Mutant GE2.10 and Wild Type.

Electrophoresis, blotting, and hybridization were as in Figure 8.

is due to defects in factors required for stabilization of the affected transcripts.

DISCUSSION

Photosystem II Biogenesis

In vivo pulse labeling of organelle polypeptides (Figure 1) revealed no synthesis of the CP43 apoprotein, polypeptide 6 encoded by *psbC*, in mutant 6.2z5. In contrast, the photosystem II polypeptides 5, D1, and D2 were synthesized at approximately wild-type rates. This pattern of chloroplast polypeptide synthesis contrasts with other photosystem II mutants, which show pleiotropic effects exerted at the translational level for the synthesis of photosystem II polypeptides. For example, mutant 8-36c, a plastid *psbA* deletion mutant that cannot synthesize D1, synthesizes polypeptide 5 at greatly reduced rates (Jensen et al., 1986). Similarly, mutant GE2.10, which does not synthesize polypeptide 5, also shows a strong reduction in the translation of *psbA* mRNA (Jensen et al., 1986). Hence, the synthesis of D1 and polypeptide 5 appears to involve a translational coupling. We have isolated another mutant, 6.2u, which cannot synthesize D2 but also shows a strong reduction in the translation of both polypeptide 5 and D1 mRNAs (Schmidt et al., 1987; L.E. Sieburth and G.W. Schmidt, manuscript in preparation). The analysis of these mutants has revealed that the expression of the photosystem II polypeptides 5, D1, and D2, is regulated in a concerted manner. The absence of translational coupling of these three photosystem II components with synthesis of polypeptide 6 in mutant 6.2z5 may be related to the proposed role of this chlorophyll *a*-binding polypeptide as a peripheral antenna component of photosystem II (Choquet et al., 1988b) and the presumed order of assembly of photosystem II complexes (De Vitry et al., 1989). As shown in Figure 1, polypeptide 5 does not accumulate in

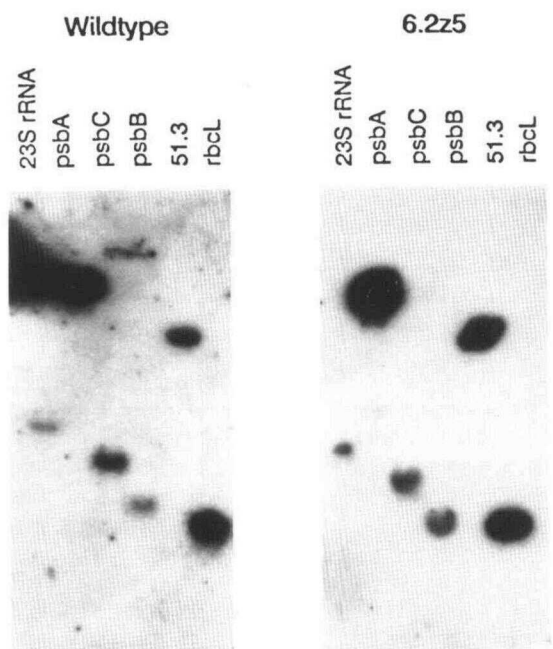

Figure 10. In Vivo Synthesis of the *psbC* Transcript in Mutant 6.2z5.

Wild-type and 6.2z5 cells, grown medium reduced in phosphate, were employed for pulse labeling of RNA with $^{32}PO_4^{3-}$ for 10 min, as described in Methods. Purified RNA was then employed for hybridization to DNA gel blots of the indicated cloned chloroplast DNA fragments.

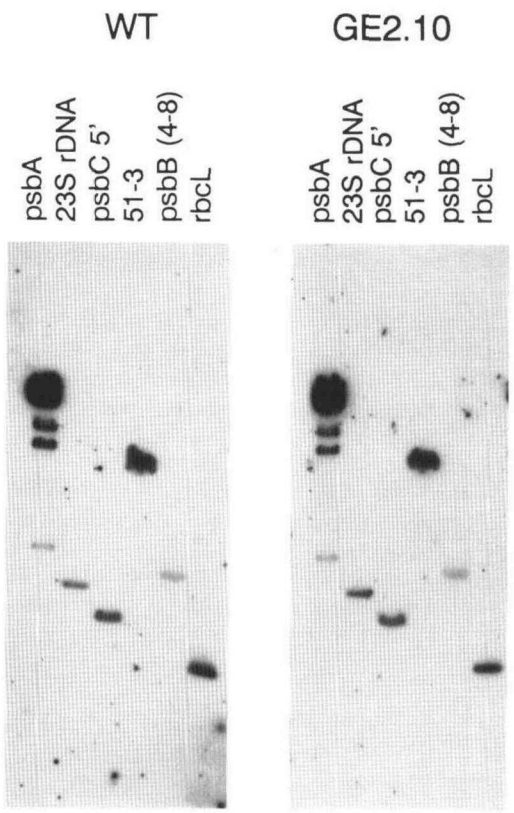

Figure 11. In Vivo Synthesis of the *psbB* Transcript in Mutant GE2.10.

Except that in vivo labeling of RNA was for 5 min instead of 10 min to detect rapidly degraded *psbB* mRNA in mutant GE2.10, hybridization to DNA gel blots of cloned chloroplast DNA fragments and other procedures were performed as in Figure 10.

mutant 6.2z5. Destabilization of other photosystem II components because of the loss of one integral membrane component has been documented for mutants whose primary lesions affect polypeptide 5, D1 (Jensen et al., 1986), D2 (Schmidt et al., 1987; Kuchka et al., 1988), and other mutants affecting synthesis of polypeptide 6 (Rochaix et al., 1989). Although polypeptide 6 synthesis is not required for translation of mRNAs for other photosystem II subunits, it is necessary for the stabilization of the photosystem II polypeptides in the thylakoid membrane.

Organization and Expression of the *psbB* Locus in *C. reinhardtii*

It was shown previously that GE2.10 is impaired concurrently in the accumulation of *psbB* mRNA and several low molecular weight transcripts (Jensen et al., 1986). Although the simple hypothesis that the nuclear lesion in this strain affects *psbB* transcription turned out to be incorrect, our studies on the transcripts from this region in the mutant versus wild-type cells enabled a more refined characterization of this region of the chloroplast genome. By correlating transcripts missing from GE2.10 with the restriction map of the regions surrounding *psbB*, it is clear that the low molecular weight transcripts are not excised introns of the primary transcript of *psbB*. They encode no sequences found in the mature *psbB* mRNA and are transcribed from loci adjacent to *psbB*. Their absence in GE2.10, however, indicates that the low molecular weight RNAs probably are cotranscribed with *psbB*. Sequencing of this region has revealed that the additional transcripts are encoded by sequences located toward the 3′ region of *psbB* (S. Berry-Lowe and G.W. Schmidt, manuscript in preparation).

The nature of the transcripts that arise from the chloroplast DNA region adjacent to the *psbB* gene of *C. reinhardtii* has not been determined. Of the genes for *psbH*, *petB*, and *petD*, which are cotranscribed with *psbB* in vascular plants, only *petB* has been mapped in *C. reinhardtii* (Turmel et al., 1988). In addition, Turmel et al. (1989) have reported that the *petD* of *C. eugametos* is not linked to *psbB*. The absence of the low molecular weight transcripts from this region of the chloroplast genome in GE2.10 has provided a means to show that *psbB* gene organization in *C. reinhardtii* is also different from that of higher plants. Thus, if the *petB* and *petD* were among the RNAs that do not accumulate in GE2.10, heme staining of thylakoid proteins would have revealed an absence of cytochrome b_6. The heme staining pattern in GE2.10, however, is identical to wild type, and both cytochromes b_6 and *f* accumulate.

Chloroplast RNA Stability

We have found that it is necessary to employ two methods to show that mutants 6.2z5 and GE2.10 have nuclear gene defects that affect the stability of transcripts from the *psbC* and the *psbB* loci of the chloroplast genome. Permeabilized cells retain chloroplast transcriptional activity for a short time (Guertin and Bellemare, 1979, and data not shown), indicating that chloroplast RNA polymerase elongates but does not initiate RNA synthesis. It should be noted, however, that a system for regeneration of nucleotide triphosphates to sustain transcription has not been included in the permeabilized cell system. Thus, correct or artifactual initiation of transcription could occur and the transient nature of RNA synthesis simply could be due to energy and/or substrate depletion. Also, we do not know whether termination of transcription is correct in permeabilized cells. Nevertheless, the hybridization patterns obtained with restriction fragments of the entire chloroplast genome seem to reflect the distribution of known coding sequences (Figure 6), and transcription from

intergenic regions has not been detected (Herrin and Schmidt, 1988). The permeabilized cell system does exhibit an artifact in that we cannot detect transcription of *psbC* if a probe for the 5′ sequences of the mRNA is used for hybridization (Figure 6). We assume that 5′ ribonucleases become activated or gain access to the substrate when cells are treated with the permeabilization buffer. In spite of these problems, we demonstrated the synthesis of mRNA from *psbC* in permeabilized 6.2z5 and RNAs of the *psbB* locus of permeabilized GE2.10 even though these strains are impaired in the accumulation of these transcripts. Because of the uncertainties about the fidelity of the permeabilized cell system, we resorted to in vivo RNA pulse labeling to demonstrate conclusively that transcription of the two genes in fact does occur in the mutant strains to the same extent as in wild-type cells. Therefore, the absence of *psbC* mRNA in 6.2z5 and the absence of the low molecular weight transcripts and *psbB* mRNA must reflect rapid and specific turnover.

Our findings are congruent with the conclusions of Kuchka et al. (1989) in their study of a *C. reinhardtii* nuclear gene mutation, nac2-26, that causes loss of chloroplast *psbD* mRNA. Although transcription in permeabilized cells provided the basis of their conclusion that *psbD* mRNA is unstable in the mutant, it is most likely that the nac2-26 mutation also affects specific plastid mRNA stability. The primary transcript of *psbD* also contains at its 3′ region a spacer region and then exon-2 of the tripartite *psaA* gene. The mutation affecting *psbD* mRNA does not affect accumulation of the *trans*-spliced *psaA* mRNA unless maturation of the primary transcript is impaired in double mutants that also are blocked in *psaA trans*-splicing of exon-2. The authors suggest that a large inverted repeat in the 5′ end of the *psbD* gene may be the stabilizing target of the nuclear gene product identified by their mutant. We have detected the activity of a 5′ ribonuclease for *psbC* mRNA in chloroplasts of permeabilized cells (Figure 6). The study of Kuchka et al. (1989) and our studies with 6.2z5 and GE2.10 indicate that distinct nuclear gene products specifically stabilize plastid mRNAs. Control of the abundance of the nuclear products could be a pervasive means by which the half-life of chloroplast transcripts is regulated.

Genes whose mRNA levels are regulated post-transcriptionally have been described in a variety of systems. One commonly observed feature is the binding of a factor to the 3′ end of the regulated mRNA. For instance, AU-rich sequences at the 3′ ends of transiently expressed human lymphokine genes interact with a protein that confers high turnover rates (Shaw and Kamen, 1986). This mechanism appears to differ from that of 6.2z5 and GE2.10, where the absence of nuclear gene products results in specific mRNA instability. An example that is more similar to our mutants is provided by the human transferrin receptor mRNA. Specific sequences on the 3′ end of this transcript provide a binding site for a polypeptide that, when bound, confers increased stability to the mRNA (Liebold and Munro, 1988; Mullner and Kuhn, 1988; Casey et al., 1989; Koeller et al., 1989). The interaction of an mRNA with ribosomes can also affect its stability. Translational repression leads to a rapid decrease of transcripts encoding ribosomal proteins (Cole and Nomura, 1986). It is unlikely that the destabilization of *psbC* mRNA in 6.2z5 results from a lack of its translation. Two nuclear mutants of *C. reinhardtii*, F34 and F64, fail to translate *psbC*, but they accumulate wild-type levels of the mRNA (Rochaix et al., 1989).

Determinants of chloroplast RNA stability have been investigated by Stern and Gruissem (1987) and Stern et al. (1989). Their studies show an importance of inverted repeat (IR) structures, commonly found at the 3′ ends of chloroplast transcripts, for stability of RNAs in chloroplast lysates. Interestingly, stability within a chloroplast extract is high for transcripts containing the IR sequence, but otherwise identical sequences are very unstable. However, the stability of RNAs with different IRs at their 3′ ends varies in a manner that is not proportional to the free energy of the stem/loop. This suggests that either other elements of these transcripts mediate stability or that stability is conferred by the association of factors with the IR. Proteins that bind to the 3′ end of the *petD* and *rbcL* transcripts have been detected by mobility shift assays (Stern et al., 1989). Moreover, other chloroplast mRNAs were unable to compete for binding, implying that some components of the binding proteins are transcript specific. UV cross-linking has allowed identification of polypeptides binding to *petD*, *rbcL*, and *psbA* transcripts. A subset of these polypeptides binds to all of the RNAs examined, whereas others exhibit apparent transcript specificity. The authors suggest that these polypeptides function to stabilize specific transcripts, and variation in chloroplast transcript abundance may be modulated by the IR-binding proteins. Similar proteins might be missing in mutants 6.2z5, GE2.10, and nac2-26. However, it is also possible that there are stabilization requirements for proteins that bind to the 5′ region of chloroplast mRNAs. In yeast, a nuclear gene product (CBP1) has been identified that binds to a 5′ IR of the mitochondrial *cob* mRNA and is required for its stability (Weber and Dieckmann, 1990).

The mechanism by which the single nuclear gene mutation in 6.2z5 results in specific destabilization of *psbC* transcripts is unknown. In addition to a 5′ IR in the *psbC* mRNA implicated by Rochaix et al. (1989) to promote translation, we observe that there also is a large (36-bp) perfect inverted repeat at the 3′ end. An attractive hypothesis is that the nuclear gene product identified by the lesion in 6.2z5 binds to this sequence and that the interaction with this polypeptide is required for stabilization. Alternatively, a failure either to terminate transcription accurately or to mature a primary transcript could also lead to RNA instability.

The considerations above do not explain the loss of the low molecular weight RNAs from the *psbB* locus in

GE2.10. Perhaps these transcripts share a structural motif with the *psbB* mRNA. On the other hand, if *psbB* mRNA and the adjacent transcripts are cotranscribed, it is possible that the lesion in GE2.10 affects RNA processing and the primary transcript is subject to rapid turnover. This would be unusual, however, because RNA processing intermediates accumulate from the *psbB* operon in higher plants (Barkan 1988) and in *trans*-splicing mutants of *C. reinhardtii* (Choquet et al., 1988a; Herrin and Schmidt, 1988).

In this study, we have identified nuclear genes whose products are required for the accumulation of the chloroplast *psbC* and *psbB* mRNAs. Together with the study of Kuchka et al. (1989), it appears possible that most, if not all, chloroplast mRNAs have a corresponding nuclear gene product that is essential for the stability of the transcript. By regulation of their abundance, specific mRNA-binding proteins derived from the nuclear compartment could constitute a mechanism for controlling mRNA accumulation during cell differentiation and in response to environmental influences.

METHODS

Cell Culture, Mutagenesis, and Genetic Analysis

Chlamydomonas reinhardtii wild-type strains 137c mt^+ and 137c mt^- were obtained from the *Chlamydomonas* Genetics Center, Duke University. Cells were grown in liquid media containing Tris/acetate/phosphate (TAP) (Gorman and Levine, 1965). UV light was used to mutagenize the 137c mt^+ strain of *C. reinhardtii*. A dose yielding 10% survival was administered, followed by a 2-day dark incubation to prevent photoreactivation. Surviving cells were then grown for 2 days in dim light, followed by a 2-day incubation in bright light in the presence of 12 mM metronidazole to enrich for photosynthetic electron transport mutants (Schmidt et al., 1977). Cells were then plated onto TAP-agar. After 16 days, high fluorescent colonies were identified by visualization under long-wavelength UV light and were isolated by three successive rounds of single colony isolation. Genetic crosses between mutant 6.2z5 and wild type were performed as outlined in Harris (1989).

In Vivo Protein Labeling and Thylakoid Membrane Isolation

In vivo labeling of membrane proteins in the presence of cycloheximide, SDS-PAGE, and fluorography were performed as described previously (Schmidt and Mishkind, 1983) except $^{32}SO_4^{3-}$ was used at 0.5 mCi/mL. Labeling was terminated and cells were fractionated into membrane and soluble components as described by Jensen et al. (1986). Preparations of thylakoid membranes were obtained by flotation in sucrose density gradients (Jensen et al., 1986). Cytochrome staining with TMBZ was performed as described by Höyer-Hansen (1980) except that sodium acetate, pH 4.6, was employed as the buffer.

Chloroplast Clones

The *psbA* gene probe, plasmid CP62, contains the EcoRI fragment 15 [nomenclature of Harris (1989)] of *C. reinhardtii* chloroplast DNA. The *psbC* probe used for RNA gel blots is CP83, which corresponds to the EcoRI fragment 6. CP83 was utilized to generate p83-7, a 1.1-kb EcoRI-SalI Bluescript plasmid that encompasses approximately 600 bp of the 3′ region of *psbC* (Rochaix et al., 1989). To obtain a clone corresponding to 935 transcribed bases of the 5′ terminus of *psbC* (Rochaix et al., 1989), a HindIII fragment from a clone of EcoRI fragment 20 was subcloned into pUC18. Plasmid pC55 contains the EcoRI fragment 27, which includes most of the *psbD* gene, and was obtained from Dr. J.-D. Rochaix. The plasmid pEC51 contains the EcoRI 19 fragment and was used as a probe for *psbB* and the neighboring genes encoding low molecular weight transcripts. Clone 51-3 is a 3.2-kb EcoRI-PstI subclone of pEC51, and it contains sequences hybridizing to unidentified transcripts smaller than *psbB*. Clone 51-1 is the other 1.2-kb PstI-EcoRI subclone of pEC51. P62 is a clone of EcoRI fragment 5 and was provided by Dr. E.H. Harris. To obtain additional probes for *psbB*, subclones 4-8, 4-10, 1-1, and 2-10 were generated by PstI, BamHI, and EcoRI digestions of P62 and ligation into BlueScript plasmid. The plasmid pEC50 contains the EcoRI fragment 12, which includes sequences corresponding to *psaA1*. The clone corresponding to *rbcL*, pEC15.4, is a HindIII subclone of the EcoRI fragment 14. The probe for *psaA2* is an 8-kb BamHI fragment derived from the EcoRI fragment 14 in plasmid pEC8, also obtained from Dr. J.-D. Rochaix. The 23S rDNA clone is a BamHI-HindIII subclone of the BamHI fragment 11. Clones pEC51, pEC50, pEC15.4, and those for 23S rDNA and *psaA2* were provided by Dr. D.L. Herrin.

RNA Gel Blot Analysis

Total RNA was isolated as described previously (Herrin and Schmidt, 1988). Electrophoresis in 1.5% agarose, 6% formaldehyde gels of 4 μg of total RNA per lane, blotting, and hybridization were performed as described by Jensen et al. (1986) except that nylon membrane (Zeta-Probe, Bio-Rad) was used. Probes used for hybridization were random-primer labeled inserts (Feinberg and Vogelstein, 1983).

Chloroplast Transcription in Permeabilized Cells

The method for transcription in permeabilized cells of *C. reinhardtii* was based on a method described by Guertin and Bellemare (1979). Permeabilization was performed on ice after the resuspension of harvested cells to a concentration of 1×10^8/mL in a buffer containing 10 mM Tris, pH 8.0, 10 mM KCl, 20 mM $MgCl_2$, 1 mM DTT, and 0.5% toluene. After 30 min, cells were pelleted by microcentrifugation for 10 sec, and the transcription reactions were initiated by resuspension of cells in a 25°C buffer containing 50 mM Tris, pH 8.0, 50 mM KCl, 100 mM $MgCl_2$, 375 mM sorbitol, 0.1 mM DTT, 2 mM ATP, 2 mM CTP, 2 mM GTP, 0.01 mM UTP, and 100 μCi of α-^{32}P-UTP (3000 Ci/mmol). Transcription reactions were performed at room temperature for up to 15 min and were followed by RNA purification as described above except that LiCl precipitation was omitted. Incorporation was evaluated for cells

pretreated with or without RNA polymerase inhibitors (350 μg/mL rifampicin or 1 mM α-amanitin) by spotting aliquots onto Whatman DE81 paper. The filters were subjected to extensive washing with 0.5 M K_2HPO_4, followed by water and ethanol rinses, before they were dried. Incorporation was measured with a scintillation counter (Maniatis et al., 1982).

In Vivo Transcription Assays

Cells were grown for 6 days in TAP medium but with a 50-fold reduction of phosphate. Cells were harvested by centrifugation at 2.5*g*, resuspended at 1×10^8 cells/mL in TAP minus phosphate, and incubated with shaking for 45 min. Aliquots of 10 mL were labeled for 10 min with 1 mCi/mL $^{32}PO_4^{3-}$. RNA was purified as described above and DNA was removed by treatment with RQ1 DNase (Promega Biotech).

DNA Gel and Dot Blots

DNA gel blots were generated after electrophoresis of digested plasmids in 0.8% agarose-Tris/borate/EDTA (TBE) gels (Maniatis et al., 1982). Vacuum blot transfer to nylon membrane (Duralon, Stratagene) was performed as recommended by the manufacturer (American Bionetics). The DNA was UV cross-linked to membranes using a Stratalinker (Stratagene). Hybridizations were performed at 50°C in 5 × SSPE (1 × SSPE = 0.15 M NaCl, 50 mM NaH_2PO_4, pH 7.4, 5 mM EDTA), 0.5% nonfat dry milk, 10% dextran sulfate, 100 μg/mL sheared herring sperm DNA, 1% SDS, 0.05% sodium pyrophosphate for 48 hr. The membranes were washed at 50°C as follows: 1 × SSPE, 0.5% SDS for 15 min; twice in 0.5 × SSPE, 0.5% SDS for 15 min; and 0.2 × SSPE, 0.5% SDS for 20 min.

Dot blots were prepared from CsCl-purified plasmid DNA that had been denatured by addition of NaOH to 0.4 M and incubation at 65°C for 60 min (Costanzi and Gillespie, 1987). Subsequently, an equal volume of 2 M ammonium acetate, pH 7.0, was added and fourfold serial dilutions were prepared with 1 M ammonium acetate. After UV cross-linking, hybridizations were performed as described above.

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