Biogenesis of Photosystem II Complexes: Transcriptional, Translational, and Posttranslational Regulation

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Abstract. The integral membrane proteins of photosystem II (PS II) reaction center complexes are encoded by chloroplast genomes. These proteins are absent from thylakoids of PS II mutants of algae and vascular plants as a result of either chloroplast or nuclear gene mutations. To resolve the molecular basis for the concurrent absence of the PS II polypeptides, protein synthesis rates and mRNA levels were measured in mutants of *Chlamydomonas reinhardtii* that lack PS II. The analyses show that one nuclear gene product regulates the levels of transcripts from the chloroplast gene encoding the 51-kD chlorophyll *a*-binding polypeptide (polypeptide 5) but is not involved in the synthesis of other chloroplast mRNAs. Another nuclear product is specifically required for translation of mRNA encoding the 32–34-kD polypeptide, D1. The absence of either D1 or polypeptide 5 does not eliminate the synthesis and thylakoid insertion of two other integral membrane proteins of PS II, the chlorophyll *a*-binding polypeptide of 46 kD (polypeptide 6) and the 30-kD "D1-like" protein, D2. However, these two unassembled subunits cannot be properly processed and/or are degraded in the mutants even though they reside in the membrane. In addition, pulse labeling of the nuclear mutants and a chloroplast mutant that does not synthesize D1 mRNA indicates that synthesis of polypeptide 5 and D1 is coordinated at the translational level. A model is presented to explain how absence of one of the two proteins could lead to translational arrest of the other.

In chloroplasts of green algae and higher plants the transfer of electrons from water to plastoquinone in the photosynthetic electron transport chain is accomplished by the photosystem II (PS II)[1] reaction center complex of thylakoid membranes. PS II consists of at least eight proteins and several protein-bound electron carriers and pigments. Recent interest in PS II structure, function, and biogenesis has focused on five integral membrane proteins encoded in chloroplast DNA and three extrinsic proteins encoded by nuclear genes. The extrinsic proteins are involved in the oxidation of water in the thylakoid lumen and the transfer of electrons to the PS II reaction center (9).

The two largest integral membrane proteins of the PS II core complex of *Chlamydomonas reinhardtii*, polypeptides 5 and 6 (7), possess molecular masses of ~51 and 46 kD, respectively, and bind chlorophyll *a* (11). The smaller integral membrane proteins considered to be essential components of PS II are cytochrome b_{559} (3–9 kD), D1 (32–34 kD), and D2 (30 kD) (9). The function of cytochrome b_{559} is unknown, but it may involve cyclic electron flow around the reaction center (9). D1 participates in binding the Q_B species of plastoquinone, a secondary electron acceptor of PS II, and herbicides of the urea and triazine classes (30); D1 has also been called the Q_B protein, the herbicide-binding protein, and the 32-kD protein. D1 is one of the most rapidly synthesized proteins in chloroplasts and also one of the most rapidly degraded (30). In contrast, the function of D2 is not established and, in *Chlamydomonas*, two molecular weight forms of this poorly stained polypeptide have been detected by pulse-labeling studies (10). The nucleotide sequence of the D2 gene of *Chlamydomonas* has been determined (48), and homologues have been identified in chloroplast DNA of spinach (1, 23) and pea (47). However, the D2 gene product in vascular plants has not been characterized. D1 and D2 share sequence homology with each other and with reaction center polypeptides of *Rhodopseudomonas*, especially in regions that include putative quinone-binding sites (9, 58).

The analysis of PS II mutants provides a valuable approach to understanding the biogenesis of the PS II complex; both water oxidation and reaction center mutants have been described in several species. In thylakoids of water oxidation mutants of *Chlamydomonas* (2) and *Scenedesmus* (37), only the extrinsic water oxidation proteins are missing, while PS II reaction center activity is normal when artificial electron donors are provided. Such mutants demonstrate that synthesis and assembly of functional reaction centers occur independently of the extrinsic proteins. Conversely, PS II reaction center deficiency results in the absence of stable water oxidation complexes in thylakoids from mutants of maize (36), barley (24), and *Chlamydomonas* (2, 3).

1. *Abbreviation used in this paper*: PS II, photosystem II.

We have studied PS II mutants of *Chlamydomonas* to identify regulatory processes in the formation of PS II complexes. We report that synthesis of polypeptide 5 and D1 is absent or greatly reduced in three mutants, whereas synthesis and membrane insertion of polypeptides 6 and D2 is not appreciably affected. However, polypeptide 6 and D2 are degraded in the PS II–deficient strains. In addition, a form of D2 that exhibits retarded electrophoretic mobility appears only in wild-type cells. We also present evidence that nuclear control of the synthesis of polypeptide 5 and D1 in the chloroplast is exerted at two levels, mRNA accumulation and translation. Finally, we propose that a translational control mechanism ensures coordinated synthesis of polypeptides 5 and D1. Accounts of this work have appeared in abstract form (26, 27).

Materials and Methods

Culture Conditions and Strains

Chlamydomonas reinhardtii strains were grown in Tris/acetate/phosphate medium (18) containing $MgCl_2$ instead of $MgSO_4$ on orbital shakers under continuous illumination (200 μEin/m^2·s). Wild-type 137c and PS II mutant F34 were obtained from the *Chlamydomonas* Genetics Center, Duke University. PS II mutant 8-36c was provided by Dr. Laurens Mets, University of Chicago (54). Mutant GE2.10 was induced by treatment of wild-type cells (137c) at 5 × 10^6 cells/ml with 0.27 M ethylmethane sulfonate in the light for 3.5 h, followed by growth for 6 d in the dark. The mutant was selected as a metronidazole-resistant colony (52) requiring acetate for growth. When GE2.10 is back-crossed with wild type, an equal ratio of wild type to acetate-requiring progeny is obtained, indicating a nuclear gene mutation. In crosses of GE2.10 and F34, wild-type recombinants are recovered at high frequencies, demonstrating the two mutations reside in different genes (data not shown). GE2.10su was isolated from cultures of GE2.10 that had spontaneously regained wild-type photosynthetic activities as a consequence of either a suppressor mutation or reversion of the mutation.

In Vivo Labeling

In vivo labeling was modified from a method described previously (53). Exponentially growing cells were harvested at 2,500 *g* for 5 min and resuspended in sulfate-free culture medium at 1 × 10^8 cells/ml. Cells were incubated on a rotary shaker at 26°C and illuminated with 200 μEin/m^2·s before the addition of isotope. Unless otherwise indicated, 5 min before the addition of isotope, cycloheximide was added to a final concentration of 10 μg/ml. To initiate labeling, sodium [^{35}S]sulfate (carrier-free, 43 Ci/mg at 100% isotopic enrichment, ICN Radiochemicals, Div. ICN Biomedicals Inc., Irvine, CA) was added to 1 mCi/ml. In pulse-chase experiments, the chase was initiated with 0.1 vol of 1 M sodium sulfate.

Thylakoid Membranes

The preparation of thylakoid membranes was performed as described by Chua and Bennoun (7), except the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), ε-amino-*n*-caproic acid (5 mM), and benzamidine (1 mM) were included in all buffers.

NaOH Extraction of Whole Cells

1 vol of cells (10^8 cells/ml) was added to 3 vol of 0.15 N NaOH at 0°C and sonicated (Heat Systems-Ultrasonics, Inc., Farmingdale, NY; Cell Disruptor equipped with a stepped microtip; two pulses at 10% duty, output 1). After 30 min at 0°C, the samples were centrifuged 1 min at 13,000 *g*. The pellet of NaOH-insoluble, integral membrane proteins (51) was washed with 5 mM Hepes-NaOH, pH 7.5, 10 mM EDTA, 5 mM ε-amino-*n*-caproic acid, 1 mM benzamidine, and then repelleted before resuspension in the gel sample buffer described below. NaOH-soluble proteins (including extrinsic membrane proteins) were precipitated with TCA (final concentration 10% wt/vol) at 0°C for 1 h and resuspended in gel sample buffer as described below but with 60 mM Tris base instead of Tris-HCl, pH 8.5.

PAGE

Samples were solubilized in gel sample buffer (2% wt/vol LDS, 60 mM Tris-HCl, pH 8.5, 60 mM dithiothreitol, 5 mM ε-amino-*n*-caproic acid, 1 mM benzamidine, 12% wt/vol sucrose) and subjected to electrophoresis at 4°C in 10–20% polyacrylamide gradient gels that were prepared, stained with Coomassie Blue R250, and processed for fluorography as described before (53).

Peptide Mapping

Peptide maps were generated using a modification of a method described previously (51). *Staphylococcus aureus* V8 protease in gel sample buffer was subjected to electrophoresis into the stacking gel of a 15–20% polyacrylamide gradient gel (100 μg protease/cm width of gel). Lanes excised from a first dimension 10–20% polyacrylamide gradient gel were placed horizontally on top of the stacking gel and sealed with 0.05% (wt/vol) agarose in gel sample buffer and electrophoresis was resumed. Silver staining of the partial digests was by the method of Morrissey (44).

Antibody Preparation

Thylakoid membranes were extracted with 0.1 N NaOH before preparative electrophoresis in lithium dodecylsulfate polyacrylamide gels (51). Antibodies to polypeptide 5 were generated by injecting rabbits with pulverized strips excised from the gels after staining with Coomassie Blue (40). The injection and bleeding schedule was described by Plumley and Schmidt (46). The purified IgG fraction precipitated only polypeptide 5 when tested by crossed immunoelectrophoresis (46) against total *Chlamydomonas* thylakoid proteins.

RNA Purification and In Vitro Translation

Total cellular RNA was purified as described by Mishkind and Schmidt (41). In vitro translation was performed in wheat germ extracts prepared as described by Gantt and Key (16). Rabbit reticulocyte lysate translations were performed with materials from Bethesda Research Laboratories (Gaithersburg, MD). Immunoprecipitation of in vitro translation products with antibodies to polypeptide 5 was performed as described by Mishkind et al. (42) except that carboxyamidation was omitted.

DNA Probes

Plasmids pEC23, pEC36, and pEC51 contain Eco RI restriction fragments R14, R9, and R10, respectively, of *Chlamydomonas* chloroplast DNA in the vector pBR325; pEC23 has been described previously (20). Fragment R14 contains four of the five exons of the psbA gene encoding D1 (13). The genes encoding polypeptide 5 (psbB) and polypeptide 6 (psbC) are reportedly located on fragments R10 and R9, respectively (49). Plasmid pCP55 contains fragment R3 encoding most of the D2 gene (psbD) (48) and was obtained from J.-D. Rochaix. Plasmid DNAs were isolated by a rapid-boiling procedure (22). To generate radioactive probes, the plasmids were nick-translated using enzymes from Bethesda Research Laboratories and [^{32}P]dCTP (ICN Radiochemicals, 3,000 Ci/mmol) to a specific activity of 1–5 × 10^8 cpm/μg. Unincorporated nucleotides were removed by spun-column chromatography with Sephadex G-50 (34).

Northern Blot Hybridization

After denaturation of RNA (10 min at 55°C in 6% [wt/vol] formaldehyde, 50% [vol/vol] formamide, 5 mM acetate, 1 mM EDTA, and 20 mM morpholino propane sulfonic acid pH 7.2), the samples were adjusted to 5 mM EDTA, 1.5% (wt/vol) Ficoll, 0.1% xylene cyanol, 0.1% bromphenol blue, and subjected to electrophoresis in 1.5% (wt/vol) agarose gels containing 6% (wt/vol) formaldehyde, 5 mM acetate, 1 mM EDTA, and 20 mM morpholino propane sulfonic acid pH 7.2. The RNA was transferred to nitrocellulose and hybridized to denatured nick-translated probes as described previously (20), except that hybridization was for 24 h. Blots were washed at 50°C in 0.1% SDS, 0.1× saline-sodium phosphate-EDTA (1× = 0.15 M NaCl, 1 mM EDTA, 5 mM NaH_2PO_4, pH 7.4) for 30 min, dried, and exposed to Kodak XAR film at room temperature.

Results

Thylakoid Membrane Polypeptides of PS II Mutants

Some of the characteristics of two of the PS II mutants used in this study have been described before. Thylakoids of F34, a nuclear mutant, lack PS II polypeptides 5, 6, 12, 19, and 24 (2, 7). Mutant 8-36c has a chloroplast gene lesion and is devoid of PS II activity (54).[2] GE2.10 is a separate nuclear mutant, isolated in this laboratory as described in Materials and Methods. Stainable thylakoid polypeptides of the mutants are compared with those of wild-type cells in Fig. 1 (lanes *1–4*). Although the mutants are genotypically distinct, the three strains are similar in that they lack the major polypeptides of the PS II reaction center complex. The abundance of D1 and D2 cannot be assessed in Fig. 1, however, because they usually are not conspicuously stained even when wild-type thylakoids are analyzed. The presence of normal amounts of other thylakoid proteins in the three mutants illustrate that the gene lesions specifically affect the formation of PS II.

Also shown in Fig. 1 are the proteins recovered from the insoluble fraction of cells subjected to extraction with 0.1 N NaOH (lanes *5–8*). NaOH extraction previously has been used to remove extrinsic proteins from purified thylakoid membranes (51), and we show it also can be used with whole cells of *Chlamydomonas*. Polypeptides 5 and 6 and other integral membrane proteins are recovered in the NaOH-insoluble fraction of wild-type cells (lane *5*); extrinsic membrane polypeptides and soluble proteins are released by this treatment. Conventional thylakoid purification procedures are time consuming and involve extensive manipulations during which nonphysiological modifications of proteins may occur. NaOH extraction is a more rapid technique for the enrichment of integral membrane proteins and, in addition, requires a minimal amount of starting material. These factors were critical for the analyses of pulse-labeled cells described below.

Differential Effects of Mutations on Synthesis of PS II Polypeptides

Despite their similar phenotypes concerning staining profiles of thylakoid polypeptides, the mutants differ in their abilities to synthesize PS II proteins. This was established by pulse-labeling cells for 2 min with [^{35}S]sulfate in the presence of cycloheximide and then fractionating the membrane and soluble proteins by the use of NaOH (Fig. 2). When [^{14}C]acetate is used in such experiments, nearly identical labeling patterns are obtained for integral membrane proteins synthesized in chloroplasts (not shown). In the mutants, labeling of polypeptide 5 is either absent (GE2.10) or occurs to such a low extent (F34 and 8-36c) that it is conspicuous only in over-exposed fluorographs. Polypeptide 6 synthesis in all three mutants approaches wild-type levels. D1 synthesis is impaired in GE2.10 and is completely blocked in F34 and 8-36c. There is a faintly labeled polypeptide in F34 and 8-36c that migrates in the vicinity of D1, but thylakoid subfractionation analyses (not shown) indicate that this band is cytochrome *f*, another integral membrane protein synthesized by chloroplast ribosomes (57). Finally, D2 is synthesized in all strains: although its labeling appears to be enhanced in GE2.10 and F34 relative to wild-type cells in Fig. 2, augmented D2 synthesis in these mutants is not consistently observed.

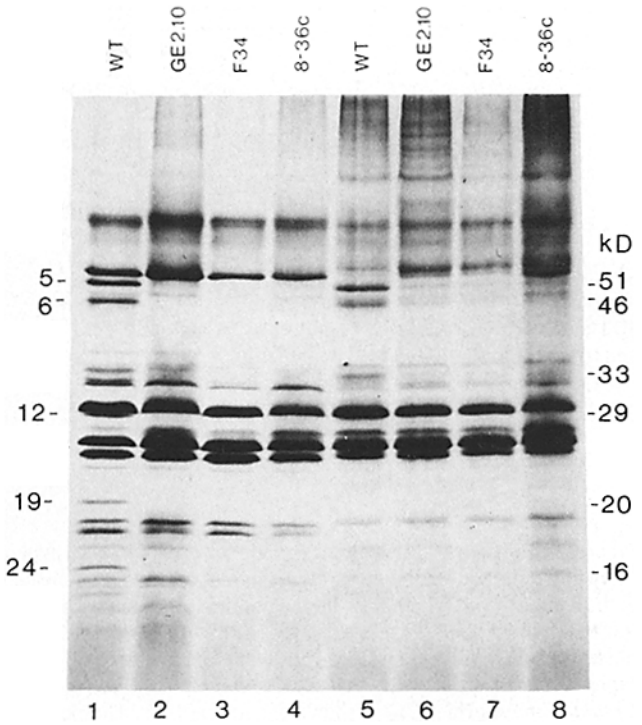

Figure 1. Absence of PS II polypeptides in *Chlamydomonas* mutants. Membrane proteins from wild type (lanes *1* and *5*), GE2.10 (lanes *2* and *6*), F34 (lanes *3* and *7*), and 8-36c (lanes *4* and *8*) were analyzed on a 10–20% polyacrylamide gradient gel and stained with Coomassie Blue. Preparation of thylakoid membranes (lanes *1–4*) and integral membrane proteins of whole cells by NaOH extraction (lanes *5–8*) are described in Materials and Methods. The positions and the approximate molecular masses of PS II polypeptides 5, 6, 12, 19, and 24 are indicated.

Chloroplast-encoded proteins that are subject to rapid turnover in the mutants were identified by supplying the pulse-labeled cells with unlabeled sulfate and analyzing the membrane fractions at intervals (Fig. 2). Because the effectiveness of the chase is not immediate, incorporation of label into the proteins is sustained for 10 min. However, it is apparent that most of the newly synthesized D2, but not polypeptide 6, is degraded during a 1 h chase in all of the mutants. As discussed below, the half-life of polypeptide 6 is shorter in the mutants than in wild-type cells but degradation is not apparent unless the chase is longer than 1 h. The lack of synthesis of polypeptide 5 in GE2.10 and absence of D1 labeling in F34 and 8-36c is also apparent in samples from the later time points, indicating that effects on these two proteins are not simply a reduction of their rate of synthesis. However, Fig. 2 illustrates that the rate of D1 synthesis in GE2.10 is much lower than in wild type as are the rates of polypeptide 5 synthesis in F34 and 8-36c. When label is continuously

2. During revision of this manuscript, a report appeared that showed the chloroplast psbA genes are deleted in 8-36c (Bennoun, P., M. Siere-Herz, J. Erickson, J. Girard-Bascou, Y. Pierre, M. Delosme, and J.-D. Rochaix, 1986, *Plant Mol. Biol.*, 6:151–160).

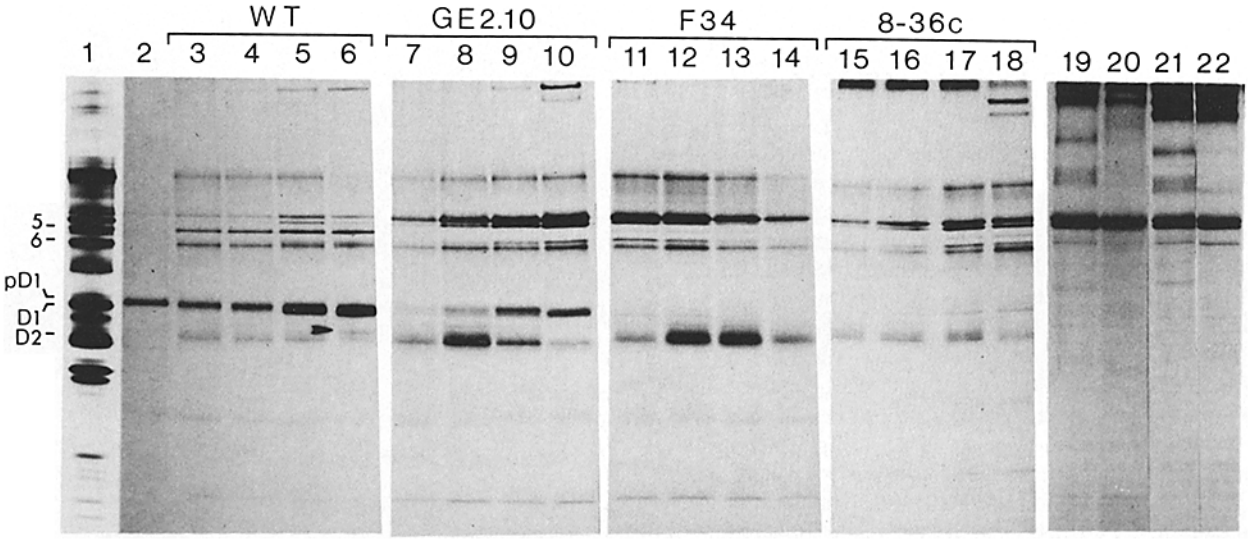

Figure 2. Translational and posttranslational events in wild-type and PS II mutants. Proteins were pulse-labeled in vivo for 2 min with [^{35}S]sulfate in the presence of cycloheximide followed by a chase with unlabeled sulfate. Aliquots were removed after the pulse (lanes *3*, *7*, *11*, *15*, and *19–22*) and after the beginning of the chase at 2 min (lanes *4*, *8*, *12*, and *16*), 10 min (lanes *5*, *9*, *13*, and *17*), and 60 min (lanes *6*, *10*, *14*, and *18*) and extracted with NaOH. Lanes *3–18*, membrane fractions. Lanes *19–22*, NaOH-soluble fraction from 2 min pulse-labeled wild type, GE2.10, F34, and 8-36c, respectively. Samples containing equal radioactivity were analyzed by PAGE and fluorography. In vivo labeled wild-type thylakoid membrane proteins (lane *1*) and proteins synthesized in vitro from total RNA from wild-type *Chlamydomonas* (lane *2*) are shown for comparison. The positions of PS II polypeptides are indicated and D2.2 is indicated by an arrow in lane *6*.

supplied to the PS II mutants for 1 h, similar results are obtained (not shown).

Our findings do not agree with a previously published characterization of F34 in which it was reported that polypeptides 5, D1, and D2 are synthesized, whereas polypeptide 6 is not (10). Although we occasionally observe reduced rates of polypeptide 6 synthesis in F34, the conclusion that polypeptide 6 synthesis is absent in this strain is not clearly supported by the data shown by Delepelaire (Figs. 2 and 3 of reference 10). Because F34 is rather unstable, the different results concerning D1 synthesis can be reconciled partly if the culture used in the earlier report had partially regained wild-type characteristics. During our study, we found it necessary to monitor photosynthetic activities to ensure revertants were absent from the PS II mutant cultures.

Shortly after synthesis and insertion into the membrane, D1 is converted to a more rapidly migrating form in the alga as it is in vascular plants (12, 30, 35). The precursor and mature forms of D1 are most easily distinguished in membranes from GE2.10 subjected to pulse-chase labeling (Fig. 2) or briefly exposed fluorographs of wild-type membranes (not shown). Between 10 (lane *9*) and 60 min (lane *10*) after beginning the chase, the apparent molecular mass of most of the newly synthesized D1 changes from 34.5 to 34 kD. D1 synthesized in a cell-free translation system also has an apparent molecular mass of 34.5 kD (pD1, lane 2)(20). The small difference in electrophoretic mobility of the precursor and mature forms easily accounts for previous failures to detect the D1 precursor in *Chlamydomonas* (56).

The pulse-chase experiment also indicates that D2 undergoes a posttranslational modification in wild-type cells but not in the PS II mutants (Fig. 2). Between 10 and 60 min after the beginning of the chase period, a labeled band migrating slightly slower than D2 appears in wild-type samples, while the labeling intensity of D2 decreases reciprocally (Fig. 2, lanes *4–6*). Structural similarity between the two forms of D2 is indicated by the peptide maps of these polypeptides described below. As the two polypeptides appear to be related, we designate the two forms as D2.1 (slower migrating form) and D2.2 in accordance with the nomenclature used by Delepelaire (10) who also has reported that F34 is missing D2.1. Like F34, GE2.10 and 8-36c do not produce D2.1. Moreover, most of D2 is degraded in the mutants with a $t_{½}$ <1 h.

One explanation for the inability to detect wild-type rates of synthesis of polypeptide 5 and D1 in membranes of the PS II mutants is that the proteins are synthesized but fail to undergo membrane insertion. For this reason, we also analyzed the NaOH-soluble fractions of cells pulse-labeled in the presence of cycloheximide for 2 min (Fig. 2, lanes *19–22*). In none of the strains are there proteins in the soluble fractions that co-migrate with polypeptide 5, D1, or pD1. Therefore, the mutations do not result in failure to integrate the PS II polypeptides into the thylakoid lipid bilayer.

To verify the identities of the PS II polypeptides that are synthesized in the mutants and to ascertain if they are structurally like those of wild-type cells, peptide maps of the integral thylakoid membranes labeled in vivo with [^{35}S]sulfate in the presence of cycloheximide were analyzed (Fig. 3). The PS II proteins synthesized in the mutants yield protease digestion products that are qualitatively identical to those of wild type. The D1 peptide pattern is very similar to those obtained previously with *S. aureus* V8 proteolysis of the polypeptide from several species, including *Chlamydomonas* (21). The peptide maps also demonstrate the mutations do not involve major structural alterations of those PS II polypeptides that the mutants can synthesize.

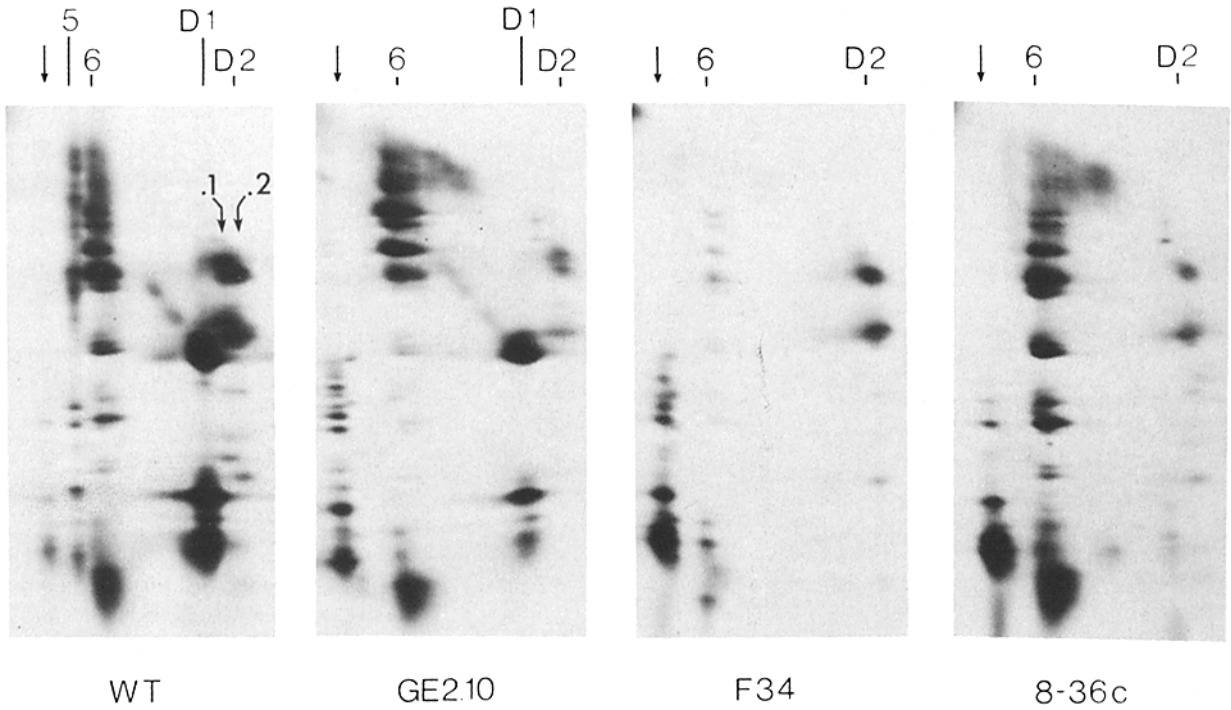

Figure 3. Structural characterization of PS II proteins from mutants and wild-type *Chlamydomonas.* First dimension gel lanes of thylakoid membranes from cells labeled in vivo for 1 h in the presence of cycloheximide and [^{35}S]sulfate were excised and placed on top of a 15–20% polyacrylamide gradient gel. Electrophoresis in the second gel was carried out in the presence of *S. aureus* V8 protease. The location of 5, 6, D1, and D2 in the first dimension gel is indicated above each peptide map. Arrows indicate a polypeptide that is abundantly synthesized in the PS II mutants but is only faintly labeled in wild-type cells (see Fig. 2). In the wild-type samples, the positions of D2.2 and its slower migrating derivative are indicated: the major D2.1 peptides from the partial digestion exhibit a similar pattern to those of D2.2 but have lagging electrophoretic mobilities.

Selective Degradation of Unassembled PS II Polypeptides

The preceding analyses demonstrate that the lack of stainable amounts of polypeptide 5 in the mutants is due to greatly reduced synthesis of this protein. However, the lack of accumulation of stainable amounts of polypeptide 6 in the mutants cannot be similarly explained. Evidence for the selective, proteolytic disposal of polypeptide 6 in PS II mutants is presented in Fig. 4. Membrane proteins of GE2.10 and a derivative (GE2.10su), which spontaneously regained high levels of PS II activity, were analyzed by pulse-chase labeling. In GE2.10, polypeptide 6 is removed from membranes between 1 and 8 h after the beginning of the chase period (Fig. 4 *A*). In contrast, polypeptide 6 is stable for at least 8 h in the reverted strain (Fig. 4 *B*) and in wild-type cells (not shown).

The pulse-chase analysis of GE2.10 also shows that most of the labeled D1 is lost within 8 h (Fig. 4 *A*). D1 also undergoes turnover in illuminated revertant (Fig. 4 *B*) and wild type (not shown) but, in these cases, proteolysis is thought to occur after the polypeptide incurs photodamage as a result of photosynthetic electron transport reactions (30).

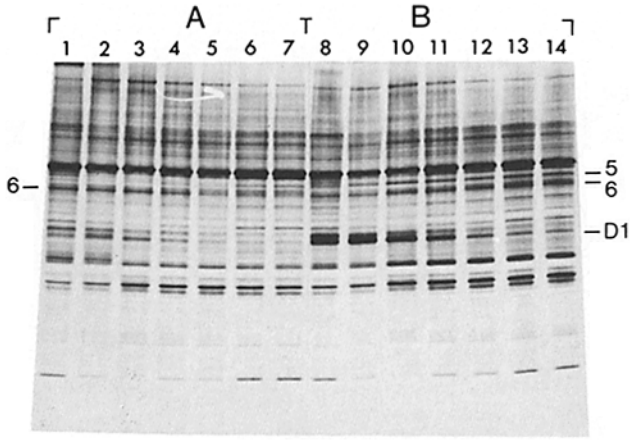

Figure 4. Turnover of polypeptide 6 and D1 in GE2.10. Cells of GE2.10 (*A*) and a derivative strain (GE2.10su) (*B*) that regained full photosynthetic activities were labeled with [^{35}S]sulfate in the absence of cycloheximide for 10 min. Aliquots were removed at the end of the pulse (lanes *1* and *8*) and after the beginning of the chase period at 10 min, 1, 2, 6, and 8 h (lanes *2–7* and *9–14*, respectively). NaOH extraction and membrane analysis were performed as in Fig. 2.

D1 Does Not Accumulate in the Absence of PS II Complexes

Despite the occurrence of turnover and reduced synthesis of D1 in GE2.10, it seemed possible that the polypeptide could accumulate in an unassembled form in the absence of other PS II polypeptides. As a semiquantitative means to measure the abundance of D1, peptide maps of labeled thylakoid proteins from GE2.10 and wild-type cells were generated and stained with silver. Comparison of the stained and fluorographed gels enabled unambiguous identification of D1 fragments. As shown in Fig. 5, D1 peptides are clearly visible

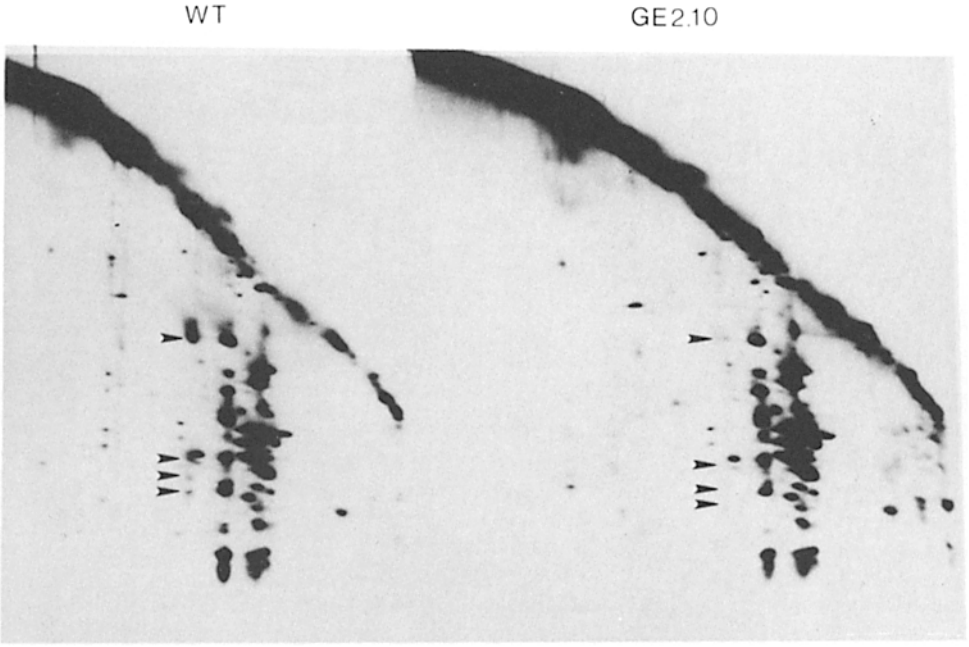

Figure 5. D1 does not accumulate in GE2.10. Thylakoid membranes of wild-type cells (*left*) and GE2.10 (*right*) were analyzed by *S. aureus* V8 peptide mapping as described in Fig. 3 and stained with silver. Arrows indicate the positions of the peptide fragments of D1 in the wild-type sample and the corresponding regions in the GE2.10 sample.

in the digestion profile of wild-type thylakoid proteins and correspond to the fluorographic images of D1 digests presented in Fig. 3. Stainable D1 peptides are barely detected in membranes of GE2.10. We conclude D1 does not accumulate in this mutant for two reasons: its synthesis rate is lower and it undergoes turnover when it cannot assemble into PS II complexes.

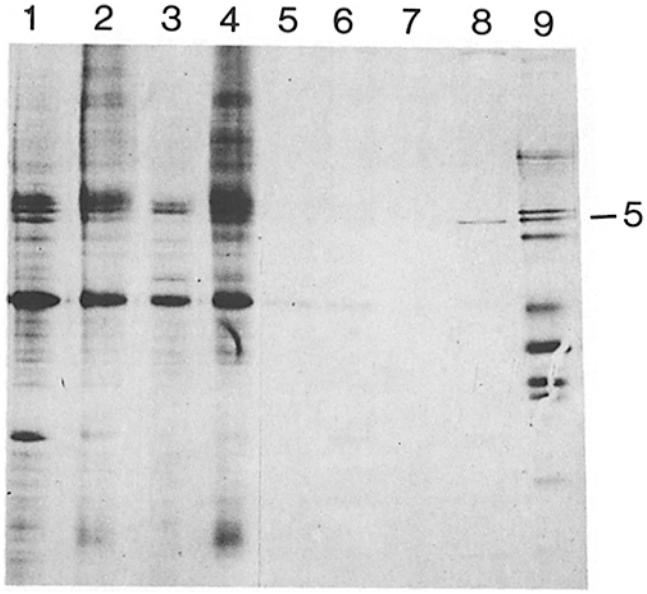

Figure 6. Absence of translatable mRNA for polypeptide 5 in mutant GE2.10. Total RNA from GE2.10 (lanes *1* and *2*) or wild-type cells (lanes *3* and *4*) was translated in the wheat germ (lanes *1* and *3*) or reticulocyte lysate (lanes *2* and *4*) cell-free systems. In lanes *5–8*, antibody against polypeptide 5 was used for immunoprecipitation of the translation products shown in lanes *1–4*, respectively. Thylakoid membrane proteins from wild-type cells labeled in vivo with [^{35}S]sulfate, containing polypeptide 5, are presented in lane *9*. Samples were analyzed by PAGE and fluorography.

GE2.10 Lacks Translatable mRNA for Polypeptide 5

Toward identifying the basis for the absence of polypeptide 5 synthesis in GE2.10, total RNA from wild type and GE2.10 was translated in the wheat germ and rabbit reticulocyte lysate cell-free systems (Fig. 6). A band that co-migrates with polypeptide 5 is specifically immunoprecipitated from the products of wild-type RNA translated in reticulocyte lysates (Fig. 6, lane *8*). In contrast, there is no product that corresponds to polypeptide 5 when GE2.10 RNA is used (Fig. 6, lane *7*). The translation analysis also indicates that polypeptide 5 is not synthesized in vitro as a higher molecular weight precursor. In addition, it is apparent that polypeptide 5 mRNA is translated more efficiently by reticulocyte lysate than wheat germ systems. A similar finding has been observed previously in cell-free translations of the chloroplast mRNA for the large subunit of ribulose bisphosphate carboxylase (6).

Abundance of Accumulated Transcripts

Molecular defects in the mutants were studied further by assessing steady-state mRNA levels for the PS II proteins with cloned fragments of *Chlamydomonas* chloroplast DNA (13, 48, 49). In RNA from wild-type cells, three major transcripts hybridize to a DNA fragment, which has been indicated by Rochaix (49) to contain the polypeptide 5 gene (psbB) (Fig. 7 *A*). Although the identities of each of the transcripts is unknown, the largest is long enough, ~2 kb, to be the mature mRNA for polypeptide 5 and is the same size as the major transcript from this region reported by Rochaix (49). Of the several minor bands detected, two co-migrate with 18S and 16S ribosomal RNA and may represent a low level of cross-hybridization with these abundant RNA species. The 18S and 16S RNAs are the only transcripts of GE2.10 that hybridize with the polypeptide 5 gene, whereas such hybridization profiles with RNA from mutants F34 and 8-36c are qualitatively and quantitatively like that of wild type. The data indicate that mutant GE2.10 lacks hybridiza-

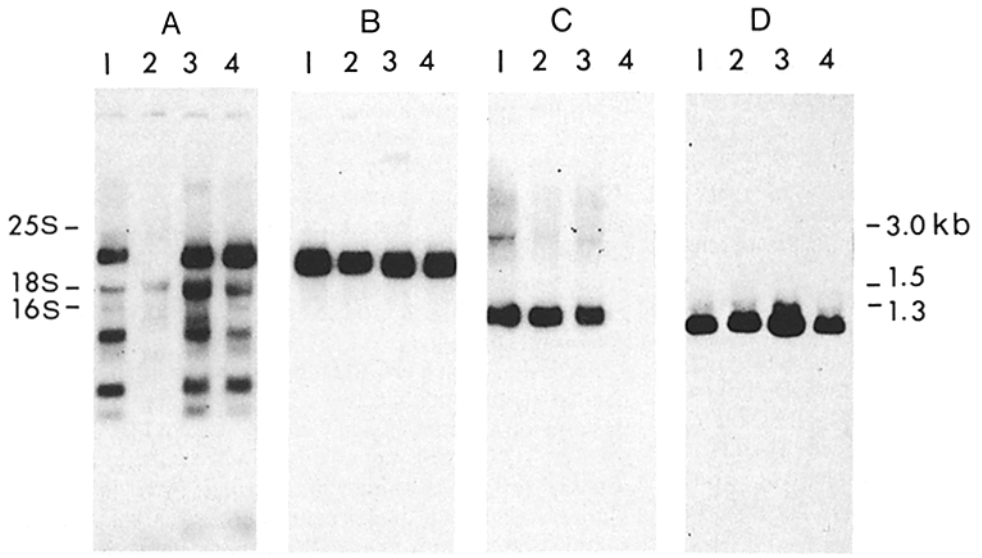

Figure 7. Chloroplast transcripts in PS II mutants of *Chlamydomonas*. Total RNA from wild-type and mutants GE2.10, F34, or 8-36c was separated by formaldehyde agarose gel electrophoresis, transferred to nitrocellulose, and probed with nick-translated pEC36 (*A*, polypeptide 5 gene, psbB), pEC51 (*B*, polypeptide 6 gene, psbC), pEC23 (*C*, D1 gene, psbA), or pCP55 (*D*, D2 gene, psbD). Migration of ribosomal RNAs, visualized by methylene blue staining of adjacent lanes of the blots (34), is indicated in the left margin and their approximate sizes in kilobase pairs to the right.

ble polypeptide 5 mRNA and the other major transcription products of the psbB region of the chloroplast genome, whereas wild-type levels of these RNAs accumulate in F34 and 8-36c.

The D1 gene (psbA) probe hybridizes to a single major transcript in wild-type RNA of ~1.2 kb, the size of the mature D1 mRNA in *Chlamydomonas* (13, 20). D1 mRNA is also present in the nuclear mutants GE2.10 and F34, but not in the chloroplast mutant 8-36c (Fig. 7 *C*). Although there are four large introns in the psbA gene in *Chlamydomonas* (13), the mutants and wild-type cells accumulate only minor amounts of D1 mRNA precursors or processing intermediates.

All three PS II mutants synthesize polypeptide 6 and D2. Thus, it was not unexpected that hybridization analyses would show the presence of substantial amounts of transcripts for these proteins in the mutants. Indeed, the RNA preparations from all strains contain a major psbC (polypeptide 6) transcript of ~1.8 kb (Fig. 7 *B*). The 1.8-kb transcript appears to be mature polypeptide 6 mRNA since it is the same size as that described by Rochaix (49) and is slightly smaller than the polypeptide 5 mRNA. Finally, normal amounts of D2 mRNA are present in all strains (Fig. 7 *D*).

D1 mRNA in Mutants GE2.10 and F34 Is Translatable

D1 synthesis is greatly reduced in GE2.10 and absent in F34 yet these cells contain abundant D1 transcripts. It was of interest to determine the extent to which the D1 mRNA in these mutants is translatable in vitro. The precursor to D1 (pD1) is one of the major products of translation of total RNA from wild-type cells of *Chlamydomonas* in cell-free translation systems. The amount of pD1 synthesis directed by RNA from GE2.10 is similar to that obtained with wild-type RNA, whereas somewhat less pD1 synthesis is observed with RNA from F34 (Fig. 8, lanes *2* and *3*). No pD1 is made upon in vitro translation of 8-36c total RNA (Fig. 8 *A*, lane *4*), which is consistent with the hybridization analysis (Fig. 7) and genetic studies that suggest the D1 gene of 8-36c is structurally altered (15).

Verification of the identity of the D1 precursor synthesized in vitro was achieved by peptide mapping (Fig. 8 *B*). The D1 precursors synthesized in vitro from GE2.10 and F34 RNAs are indistinguishable from pD1 produced from wild-type RNA. The proteolytic digestion products of pD1 are only slightly different from those of mature D1 synthesized in vivo (Fig. 3). No peptides corresponding to pD1 are seen in the peptide map of products synthesized with RNA from 8-36c (not shown). We conclude that GE2.10 and F34 contain high levels of mature, translatable D1 mRNA.

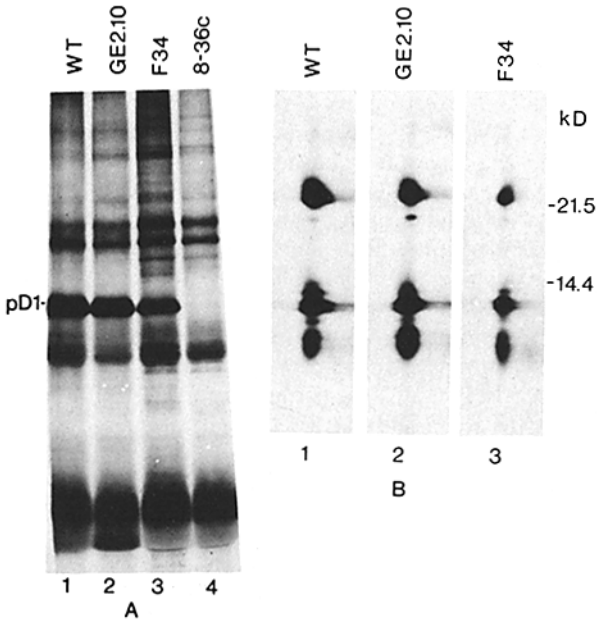

Figure 8. In vitro translation products and peptide maps of pD1 from PS II mutants and wild-type *Chlamydomonas*. Total RNA from wild-type cells (lane *1*), GE2.10 (lane 2), F34 (lane *3*), or 8-36c (lane *4*) was translated in the wheat germ system. (*A*) Translation products were visualized by 10–20% PAGE and fluorography. The position of the precursor to polypeptide D1 (*pD1*) is indicated. (*B*) *S. aureus* V8 peptide maps of the pD1 region from gel lanes similar to those shown in *A*. The molecular mass markers indicated are soybean trypsin inhibitor (*21.5 kD*) and lysozyme (*14.4 kD*).

Discussion

We have analyzed three PS II mutants of *Chlamydomonas* to define the extent to which they can synthesize four integral membrane proteins of the PS II reaction center. Thylakoids from all three are missing two of the PS II integral membrane proteins, the chlorophyll *a*-binding polypeptides 5 and 6, and the extrinsic proteins of the water oxidation complex, polypeptides 12, 19, and 24. Although the mutants are phenotypically identical at the level of protein deficiencies, they differ in their ability to synthesize proteins of the PS II core complex. In nuclear mutant GE2.10, synthesis of polypeptide 5 is completely blocked, while synthesis of D1 is greatly impaired. In contrast, F34, the other nuclear mutant, and the chloroplast mutant 8-36c do not synthesize D1, and the rates of synthesis of polypeptide 5 are greatly reduced. Therefore, the absence of PS II complexes results from at least three distinct genetic defects, indicating there are multiple sites for the regulation of formation of this multisubunit component of thylakoids. Furthermore, the ultimate characteristics of the PS II mutants arises because of regulatory mechanisms that operate at the posttranscriptional and posttranslational levels in the chloroplast.

Posttranslational Regulation

The three PS II mutants we have studied synthesize polypeptides 6 and D2 at nearly normal rates. Thus, some of the polypeptides of the PS II complex can be expressed independently in the chloroplast. When they cannot assemble with other core components of PS II, namely polypeptide 5 and/or D1, the reaction center polypeptides are degraded even though they have been inserted in the thylakoid membrane lipid bilayer. The proteolytic disposal of D2 is nearly complete within 1 h, whereas D1 and polypeptide 6 have longer half-lives. D1 is also subject to degradation in GE2.10, but turnover of this protein also occurs in wild-type membranes. It has been suggested that degradation of D1 in normal chloroplasts ensues after it is damaged by plastoquinone radicals that form in high light and highly oxygenic conditions (30). Since no photosynthetic oxygen evolution occurs in GE2.10, turnover of D1 in this strain undoubtedly is due to reasons other than photodamage. The inability of GE2.10 to accumulate D1 indicates the protease that acts on unassembled D1 is quite efficient but we do not know if the protease is the same for the turnover of photodamaged D1 in wild-type cells.

Recently, Leto et al. (31) partially characterized a PS II mutant of maize and also concluded that chloroplast products homologous to polypeptide 6 and D1 are synthesized and inserted into thylakoids but then are degraded when they cannot assemble into a complex. Our studies also demonstrate that formation of PS II complexes requires, at least, the concurrent synthesis of both polypeptides 5 and D1. In their absence, synthesis of other PS II proteins is either attenuated or the unassembled polypeptides are rapidly degraded. Therefore, the fine control of accumulation of the PS II reaction center subunits in general appears to be achieved by selective proteases in the chloroplast. This posttranslational mechanism can ensure stoichiometric accumulation of the PS II subunits but also may be a means for regulating the amounts of PS II during plastid differentiation. For instance, bundle sheath cells of some C_4 plants exhibit reduced PS II activity (5), and plants grown with light preferentially absorbed by PS II appear to produce less PS II than PS I (17). Differential gene expression in these cases might be exerted on only one of the PS II coding regions, while the other polypeptides are controlled posttranslationally.

Other circumstances in which specific proteins are degraded in chloroplasts have been described; all are like the PS II polypeptides in that selective proteolysis of components of multisubunit complexes occurs in response to subunit or prosthetic group imbalances (39, 53). Thus, posttranslational regulation of chloroplast biogenesis appears to be of general importance in the fine control of development of the photosynthetic apparatus.

Our analyses also provide information on other posttranslational modifications incurred by the PS II polypeptides. It was previously reported that *Chlamydomonas* lacks a precursor form of D1 (56), but our data indicate the biosynthetic pathway for D1 is similar in vascular plants and algae. Marder et al. have provided evidence that a carboxy terminal peptide is removed from the D1 precursor of *Spirodela* (35). The proposed amino acid sequence where proteolysis occurs is conserved in the D1 precursor of *Chlamydomonas* as deduced from the nucleotide sequence of the algal gene (12, 13). Our studies show that the posttranslational processing of D1 is not dependent on a functional PS II complex, since D1 achieves its mature size in mutant GE2.10.

There is a report that D1 in *Chlamydomonas* is phosphorylated (45). We have observed phosphorylation of polypeptide 6 and D2 in wild-type thylakoids, in accordance with the report of Delepelaire (10), but not of D1 (Ikeuchi, M., F. G. Plumley, and G. W. Schmidt, manuscript in preparation). It has been proposed that phosphorylation causes reduced electrophoretic mobility of these polypeptides, generating derivatives D2.1 and polypeptide "6'" (10). Additionally, Delepelaire provided evidence that conversion of D2 to D2.1 does not occur in mutant F34 and suggested that lack of this modification might be directly responsible for the absence of PS II complexes in this strain (10). However, our studies on GE2.10, F34, and 8-36c indicate that failure to produce D2.1 is the result and not the cause of PS II complex deficiency; complete PS II complexes appear to be required for the formation of D2.1.

Transcript Abundance

In GE2.10, polypeptide 5 mRNA is not detected, accounting for the lack of synthesis of this protein in this strain. There are many reports indicating that the chloroplast genome may be differentially expressed (5, 28, 32, 33) and, in maize chloroplasts, a protein called "S factor" stimulates transcription of the gene for the large subunit of ribulose bisphosphate carboxylase (28). The characterization of GE2.10 demonstrates, for the first time, that a nuclear gene product is involved in accumulation of specific chloroplast transcripts.

We find three major transcripts from the polypeptide 5 gene region in RNA from wild-type *Chlamydomonas*, all of which are missing from GE2.10. We have not detected any other deficiencies of chloroplast transcripts in GE2.10 (unpublished results). In spinach, the gene for the larger of the two PS II chlorophyll *a*-binding proteins (the equivalent of polypeptide 5 in *Chlamydomonas*) is transcribed initially as a large RNA molecule that also contains mRNAs for two other proteins (43). In mutant GE2.10, lack of accumulation of polypeptide 5 mRNA could be due to a specific lack of

transcription of the polypeptide 5 region of the genome, or to rapid degradation of the primary transcript in the absence of a specific factor required for transcript processing. Nuclear gene products are necessary components for maturation of mRNA precursors transcribed from mitochondrial genomes (8).

Translational Regulation

Three examples of translational control of specific chloroplast proteins are apparent from our analyses. They include: (*a*) the complete arrest of D1 mRNA translation in F34; (*b*) the partial arrest of D1 mRNA translation in GE2.10; (*c*) the severe arrest of polypeptide 5 mRNA translation in F34 and 8-36c. In each case, high levels of mature mRNA for the severely affected protein are present. Because the transcriptional block of the polypeptide 5 gene in GE2.10 is likely to be the primary effect of the nuclear mutation in this strain, the partial arrest of D1 mRNA translation is a secondary result of the inability to synthesize polypeptide 5 or to form PS II complexes. Similarly, the primary defect in the nuclear mutation in F34 appears to cause a complete block in translation of D1 mRNA; secondary effects in this strain are the partial arrest of polypeptide 5 mRNA translation. In 8-36c, where D1 synthesis and loss of D1 mRNA appears to result from a structural defect of the D1 gene (15), translation of polypeptide 5 mRNA is also strongly attenuated. Together, these data indicate that synthesis of polypeptide 5 and D1 are tightly coupled at the level of mRNA translation.

We offer a hypothesis and working model to account for the coordinated synthesis of polypeptide 5 and D1 as deduced from our analysis of PS II mutants. Another basis of the model (Fig. 9) is the synthesis of D1 by thylakoid-bound ribosomes (20) and assumes the same is true for polypeptide 5. Hence, we suggest translational coordination is achieved by a mechanism similar to that which directs synthesis of precursors of secretory proteins on the rough endoplasmic reticulum as was initially described in the signal hypothesis (50). The signal hypothesis has been subsequently modified to include the activity of a ribonucleoprotein complex called the signal recognition particle (55). Synthesis of secretory proteins is initiated on free ribosomes in the cytoplasm and elongation occurs until it is inhibited by the signal recognition particle binding to the nascent polypeptide. Translation resumes when the complex encounters a membrane receptor (the docking protein) and the signal recognition particle is released (55).

We propose that translation of mRNAs for integral thylakoid proteins is initiated in the chloroplast stroma but translation then is stopped by an arrest factor analogous to the signal recognition particle. Translation is resumed when arrest factor–ribosome complexes encounter unoccupied membrane receptors specific for D1 and polypeptide 5. Normally, receptors for D1 and polypeptide 5 are available because the newly synthesized polypeptides are rapidly recruited in assembly of PS II complexes (Ikeuchi, M., and G. W. Schmidt, manuscript in preparation). Proposed functions of polypeptide 5 and D1 imply that these two proteins may be closely associated with one another in mature PS II complexes (9). It is reasonable to suppose that they interact during or shortly after synthesis. When polypeptide 5 is absent, as in GE2.10, D1 remains bound to its receptor, blocking continued D1 mRNA translation. The low level of D1 synthesis in GE2.10 can be explained on the basis of a mechanism for degrading proteins that remain attached to receptors for long times, thus slowly releasing receptors. In addition, a small amount of D1 synthesis could occur as new receptors are produced. In a similar manner, translation of polypeptide 5 would require a specific membrane receptor with which newly synthesized polypeptide 5 would remain associated until D1 is encountered. This would account for attenuated polypeptide 5 synthesis in F34 and 8-36c, which cannot synthesize D1. In contrast, D2 and polypeptide 6 are synthesized in all of the mutants. Assuming they also are synthesized by thylakoid-bound ribosomes (1), D2 and polypeptide 6 appear to dissociate from their receptors without forming PS II complexes.

The simplest explanation for the absence of D1 synthesis in the nuclear mutant F34 is that D1 receptors are missing or are defective in this strain. F34 presumably possesses fully functional arrest factors that effectively block the translation of mRNAs for D1 and polypeptide 5. It has been reported that isolated maize chloroplasts synthesize the pD1 equivalent but it is not properly inserted into membranes (19). This situation could result from loss of activity of the arrest factor during chloroplast purification. D1 synthesis could then occur to completion in the stroma, followed by its aberrant association with thylakoids.

The model differs from the signal hypothesis in that, in the case of D1 and polypeptide 5, the membrane receptor is not released from the newly synthesized protein until another protein subunit of the complex is available for binding. Another difference is that removal of an amino terminal signal sequence, usually observed in eukaryotic and prokaryotic systems (50), does not appear to occur in the PS II proteins. As shown in this paper, polypeptide 5 does not appear to be synthesized as a higher molecular weight precursor. Moreover, the amino acid extension in pD1 is reported to be at the

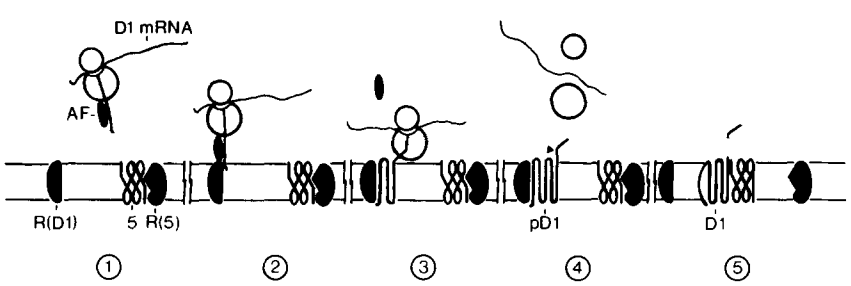

Figure 9. Proposed translational control mechanism. (*1*) Translational arrest factor (*AF*) binds to nascent polypeptide; translation is arrested. (*2*) AF and nascent polypeptide interact with membrane receptor (*R*). (*3*) AF is released; translation resumes. (*4*) Translation is complete; ribosome is released; R, still attached to newly synthesized protein, cannot bind new ribosome-AF complexes. (*5*) R is released. In some cases, this occurs only when the newly synthesized protein interacts with a specific thylakoid protein.

carboxy rather than amino terminus (35). However, amino terminal processing of a putative signal sequence has been proposed for the cytochrome *f* precursor in pea chloroplasts (57).

Both the synthesis and degradation of D1 are stimulated at high light intensities (30). The increase in degradation is thought to result from enhanced damage to D1, whereas high rates of its synthesis are required for maintenance of electron transport activities. We suggest that replacement synthesis of D1 is coordinated with degradation by the same translational control mechanism that couples D1 synthesis with that of polypeptide 5: a PS II complex from which damaged D1 has been removed can release newly synthesized D1 from its membrane receptor. Consequently the receptor becomes available for interacting with the nascent chain of another D1 molecule.

Translational arrest of the synthesis of several chloroplast encoded proteins, including D1 and the large subunit of ribulose bisphosphate carboxylase, has been reported to occur as the result of darkness, low ATP levels, and heat shock (4, 14, 25, 29, 38). Our data provide evidence that translational arrest of specific mRNAs in chloroplasts is also a means of coordinating the synthesis of components of a multisubunit complex.

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