Lack of the D2 protein in a *Chlamydomonas reinhardtii* *psbD* mutant affects photosystem II stability and D1 expression

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D1 and D2, two chloroplast proteins with apparent mol. wt of 32 000–34 000, play an important role in the photo-synthetic reactions mediated by the membrane-bound protein complex of photosystem II (PSII). We have isolated and characterized an uniparental, non-photosynthetic mutant of *Chlamydomonas reinhardtii* and show that the mutation is in the chloroplast gene *psbD*, coding for D2. A 46 bp direct DNA duplication in the coding region of the mutant gene causes a frame-shift which results in a *psbD* transcript coding for 186 amino acid residues instead of the normal 352. The truncated D2 peptide is never seen, even after pulse-labeling, suggesting that the mutant protein is very unstable. In addition, little or no D1 protein is detected in this mutant although the gene and normal levels of mRNA for D1 are present in mutant cells. All other core PSII proteins are synthesized and inserted into the membrane fraction, but never accumulate. These results suggest that D2 contributes not only to the stabilization of the PSII complex in the membrane, but also may play a specific role in the regulation of the D1 protein, either at the translational or post-translational level.

Key words: *Chlamydomonas*/photosynthetic chloroplast mutant/ photosystem II assembly/*psbD* nucleotide sequence/thylakoid membrane polypeptides

Introduction

In higher plants, green algae and other photosynthetic eukaryotes, the photosynthetic process by which light energy is converted to chemical energy takes place in the chloroplast. Through a series of complex reactions mediated in part by the thylakoid membrane-bound protein complexes of photosystems I and II (PSI, PSII), electrons are transferred from water to a series of transient and stable electron acceptors, ultimately resulting in the generation of the chemical compounds ATP and reduced NADP. PSII is involved in the photolysis of water and the light-dependent transfer of electrons from the reaction center chlorophyll to the primary and secondary stable quinone electron acceptors, Q_a and Q_b. Active PSII particles purified from thylakoid membranes of *C. reinhardtii* or higher plants contain varying numbers of proteins, depending on the method of purification and the qualities of the resulting particles (Diner and Wollman, 1980; Henry and Møller, 1981; Kuwabara and Murata, 1982; Satoh *et al.*, 1983; Delepelaire, 1984; DeVitry *et al.*, 1984; Ghanotakis *et al.*, 1984; Tang and Satoh, 1985). Five chloroplast-encoded proteins are

found in the core PSII complex. The genes for these proteins have been identified and sequenced. *psbB* (Morris and Herrmann, 1984) and *psbC* (Alt *et al.*, 1984; Holschuh *et al.*, 1984) code for the two largest core proteins of ~ 47–50 kd and 43–47 kd, respectively, which have been identified as chlorophyll *a* binding proteins (Delepelaire and Chua, 1979; Camm and Green, 1982). *psbE* (Herrmann *et al.*, 1984) codes for cytb559, which is ~ 6–10 kd. The two remaining core proteins, called D1 and D2 because of their diffuse migration pattern on SDS-polyacrylamide gels (Chua and Gillham, 1977), are ~ 32 kd each and are encoded by *psbA* (Zurawski *et al.*, 1982; Erickson *et al.*, 1984b) and *psbD* (Rochaix *et al.*, 1984; Rasmussen *et al.*, 1984), respectively. Oxygen-evolving membrane preparations which selectively retain PSII activity contain, in addition to the central core proteins, three nuclear-encoded proteins which can be removed by high salt treatment. One of these peripheral proteins is ~ 33 kd (Kuwabara and Murata, 1979) and is reportedly found in highly purified oxygen-evolving particles (Tang and Satoh, 1985). The other two are ~ 24 and 17 kd, and appear to have a role in oxygen evolution (Bennoun *et al.*, 1981; Åkerlund *et al.*, 1982; Ghanotakis *et al.*, 1984; Henry *et al.*, 1982; Møller and Høj, 1983; see Critchley, 1985 for a review). In addition, numerous other proteins, many of them of low mol. wt, may be associated with PSII (Delepelaire, 1984).

As is true for the other chloroplast-encoded PSII proteins, both D1 and D2 are highly conserved in all species whose genes for these proteins have been sequenced. This includes several higher plant species, the green alga *C. reinhardtii*, *Euglena*, and several cyanobacteria (see Erickson *et al.*, 1985b for a review). The D1 protein has also been called the peak D protein (Eaglesham and Ellis, 1974), the rapidly labeled 32 kd protein (Grebanier *et al.*, 1978), the herbicide-binding protein (Steinback *et al.*, 1981; Pfister *et al.*, 1981) and the Q_B protein (Hirschberg *et al.*, 1984) because of its observed role in herbicide resistance and electron transport. Much less is known about the function of the D2 protein. Marked similarities in the hydrophobic profiles of the D1 and D2 proteins have been noted, as well as the homology of amino acid sequence between certain regions of D1 and D2 (Rochaix *et al.*, 1984). There is a similarity between the reaction center of PSII and of purple photosynthetic bacteria: in both cases, a pheophytin acts as an intermediate electron acceptor and an electron-accepting quinone–iron complex exists (Feher and Okamura, 1978). The structural homology between D1, D2 and L and M subunits of the bacterial reaction center has also been noted (Williams *et al.*, 1983; Rochaix *et al.*, 1984; Hearst and Sauer, 1984; Youvan *et al.*, 1984). Deisenhofer *et al.* (1985) have recently reported their analysis of X-ray diffraction of the crystallized reaction center from *Rhodospseudomonas viridis*. Based on these results and given the structural and functional homology between the bacterial and PSII subunits, it has been proposed that D2 and D1 are the apoproteins of the stable primary and secondary electron acceptors of PSII, respectively, and that both proteins can be folded with five transmembrane domains to form a core with chlorophyll, pheophytin and quinone (Trebst,

Fig. 1. Gel electrophoretic fractionation of thylakoid membrane polypeptides (M) and whole cell polypeptides (C) from wild-type cells (WT) and the FuD47 mutant of *C. reinhardtii*. Cells were labeled for 45 min with [14 C]-acetate in the presence of 6 μ g/ml cycloheximide. Coomassie blue staining of thylakoid membrane polypeptides is shown in the left panel, and autoradiograms are seen in the right two panels. Upper part: 12–18% SDS-polyacrylamide gels with 8 M urea. Lower part: 7.5–15% SDS-polyacrylamide gels, showing only the region of polypeptide 5, which comigrates in the presence of urea with the α subunit of ATP synthase, but is separated in this lower gel from both the α and β subunits, which comigrate in the band labeled 4. The bands that are missing or strongly reduced in FuD47 are indicated by dots. The nomenclature of thylakoid polypeptides is according to Chua and Bennoun (1975), Chua and Gillham (1977), Bennoun *et al.* (1978) and Delepelaire (1984). The PSII proteins corresponding to bands 5, 6, 12, 19, 24, 36, D1 and D2 are as identified in the text. Stained polypeptide 36 corresponds to the L8 band seen on autoradiograms.

1986). This model, however, does not agree completely with data obtained by protease digestion of thylakoid membranes which predicts that the carboxy terminal end of D1 is located on the stromal side and not on the lumen side of the thylakoid (Marder *et al.*, 1984).

Since the study of strains with *psbA* mutations has led to an increased understanding of the role of the D1 protein in herbicide resistance and electron transport (Hirschberg and McIntosh, 1983; Hirschberg *et al.*, 1984; Erickson *et al.*, 1984a, 1985a; Goloubinoff *et al.*, 1984; Golden and Haselkorn, 1985) and in stable assembly of the PSII complex (Bennoun *et al.*, 1986), similar insights into the role of the D2 protein can be expected as a result of an analysis of D2 mutants. Unfortunately, almost all of the *C. reinhardtii* photosynthetic mutants recovered after 5-fluorodeoxyuridine mutagenesis, enrichment and screening procedures are *psbA* null mutants, completely lacking D1 (Bennoun *et al.*, 1986). The isolation of strains with mutations in *psbD* is crucial to a study of the role of the D2 protein in the function and assembly of the core PSII complex, and the relationship of D2 and D1 to each other and to the PSII reaction center.

We report here the isolation and characterization of FuD47, a uniparental, non-photosynthetic mutant of *C. reinhardtii* which contains a mutation in the chloroplast gene *psbD* coding for the D2 protein. Our results suggest that the D2 protein is involved in the stabilization of the PSII complex, and in addition has a surprising role in regulating expression of the D1 protein.

Results

Mutagenesis and isolation of FuD47 and phenotypic revertants

Chlamydomonas reinhardtii wild-type cells 137c⁺ were mutagenized with 5-fluorodeoxyuridine (Bennoun *et al.*, 1978) and enriched in the presence of metronidazole (Schmidt *et al.*, 1977) as previously described (Bennoun and Delepelaire, 1982). Mutants deficient in PSII reaction centers were identified by fluorescence screening as those having a high, invariant chlorophyll fluorescence yield upon illumination. When these mutants were further screened by pulse-labeling of proteins in whole cells, several were identified which had no detectable synthesis of D2 after short pulses, and which contained little or no D1. When analysed genetically, two of these mutants had the Mendelian inheritance pattern of nuclear mutations, while at least two exhibited a uniparental inheritance typical of chloroplast mutations. From this latter group, FuD47 was chosen for further study. Figure 1 shows the analysis of polypeptides from FuD47 and wild-type cells. Coomassie blue staining of thylakoid membrane polypeptides (M, left panel) demonstrates the general deficiency in FuD47 of proteins belonging to the PSII complex: the 47–52 kD and 43–47 kD chlorophyll *a* binding proteins (bands 5,6), the three nuclear-encoded proteins of ~ 33, 24 and 17 kD (bands 12, 19, 24) and the small 6–10 kD protein, most likely cytochrome b559 (band 36, equivalent to L8 in autoradiograms) are all present in wild-type membranes, but missing or greatly reduced in FuD47. PSII polypeptides D1 and D2 are stained poorly with Coomassie blue, but are well resolved in SDS-urea acrylamide gel and can be visualized following pulse-labeling of cells, as shown in the autoradiograms of Figure 1. After wild-type and mutant cells were incubated for 45 min with [14 C]acetate in the presence of cycloheximide, isolated thylakoid membrane proteins (M, middle panel) and total cell proteins (C, right panel) were electrophoresed on SDS-acrylamide gels with and without urea (top and bottom gels, respectively).

Fig. 2. These autoradiograms show Northern analysis (see Materials and methods) of RNA from wild-type *C. reinhardtii* (WT), the FuD47 mutant, and its phenotypic revertant FuD47 PR-4. The RNA blot in panel A was hybridized with a 32 P-nick-translated plasmid, pCW-R3, which contains the *EcoRI* chloroplast fragment R3 and serves as a *psbD* probe. After exposure with X-ray film, the filter seen in panel A was rinsed in alkali to remove all traces of labeled *psbD*, and re-hybridized with two other nick-translated plasmid probes, resulting in the pattern seen in panel B. The *EcoRI* fragment R14 cloned in pBR328 contains the first four exons of the D1 gene (Erickson *et al.*, 1984b) and served as the *psbA* probe. The cloned *HindIII* fragment R15.4 contains an internal portion of the gene for the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (Dron *et al.*, 1982) and was used as the *rbcL* probe. The *psbA* and *psbD* messages are each ~ 1.1 – 1.3 kb (Erickson *et al.*, 1984b, Rochaix *et al.*, 1984). The *rbcL* message is ~ 1.6 kb (Dron *et al.*, 1982).

Although they do not accumulate, the chloroplast-encoded PSII proteins corresponding to bands 5, 6 and L8 are in fact synthesized and can be found in the membrane fraction. However, there is no labeled D2 protein, and little if any detectable D1 protein in FuD47. Synthesis of other low mol. wt polypeptides (L5–L7) and of protein 2, an apoprotein of the PSI reaction center, is also decreased. Five-minute pulse-labeling experiments gave similar results (data not shown).

FuD47 PR-1 and FuD47 PR-4 are two phenotypic revertants of FuD47, selected at random from colonies which grew up at a spontaneous frequency of approximately 10^{-4} when FuD47 was plated in the light (3000 lux) on minimal agar plates. Analysis of proteins after pulse-labeling and gel electrophoresis showed that both of these phenotypic revertants synthesize normal amounts of the D1 and D2 proteins (data not shown).

Genetic characterization of FuD47

The inheritance of the FuD47 mutation is uniparental maternal, determined after analysis of at least 12 tetrads from reciprocal

Fig. 3. Restriction map of the *C. reinhardtii* 7.5 kb *HindIII* chloroplast fragment containing *psbD* (black bar) shows the location of the *EcoRI* restriction fragments R5, R3, R06 and R11, and the 1.0 kb *AvaI* fragment that spans the *EcoRI* site between R3 and R06. The expanded map below delineates the coding region of *psbD* (black bar), the location of the 46 bp that are duplicated in FuD47 (hatched bar), and the 5' (open box) and 3' (dotted line) non-translated regions of the gene. The strategy used for Maxam-Gilbert sequence analysis is outlined, giving the restriction sites used for 5' (solid arrow) or 3' (dotted arrow) labeling of ends. Fragments used for S1 mapping are indicated, as is the 300 bp *Fnu4HI* fragment (*) which contains the duplicated DNA in FuD47. Restriction sites for *HindIII* (H▲), *AvaI* (A●), *PvuII* (P▽), *EcoRI* (R○), *HincII* (Hc◊), *HinfI* (Hf□), *TaqI* (Tx), *DdeI* (D♦) and *Fnu4HI* (Fn△) are as indicated. Size bars of 1.0 kb and 100 bp are given for the upper and lower diagrams, respectively.

crosses. Complementation analysis in young zygotes (Bennoun *et al.*, 1980) demonstrated that FuD47 complemented the FuD7 mutant (Bennoun *et al.*, 1986) which lacks both copies of the *psbA* gene coding for D1. Thus, the *psbA* gene in FuD47 appears to be normal.

Analysis of RNA

The size and amount of transcripts homologous to the D1 and D2 genes are seen in the autoradiogram in Figure 2. RNA prepared from wild-type cells 137c, FuD47 and one phenotypic revertant of FuD47 was electrophoresed on a denaturing formaldehyde gel, transferred to a nitrocellulose filter and hybridized with a radio-labeled *psbD* DNA probe. As seen in Figure 2 panel A, FuD47 contains RNA homologous to *psbD* which is slightly larger in size than the RNA from wild-type cells or from the phenotypic revertant. In addition, the amount of *psbD* message in FuD47 is less than that seen in wild-type cells. This same Northern blot was subsequently rinsed in alkali to remove the labeled *psbD*, and re-hybridized with labeled probes homologous to *psbA* and *rbcL* (described in legend to Figure 2). The latter was used as a control for calibration of the amount of RNA on filters. Results of this second hybridization are shown in panel B of Figure 2. The quantity and size of D1 mRNA in FuD47, grown under the conditions described in Materials and methods, is essentially identical to that of the wild-type cells. Analysis of RNA from FuD47 PR-1 (not shown) gave results similar to those seen for FuD47 PR-4.

Comparative sequence analysis of *psbD* from FuD47 and wild-type cells

A 7.5 kb *HindIII* fragment of chloroplast DNA from FuD47 was

Fig. 4. DNA sequence of the *C. reinhardtii* *psbD* gene and flanking regions. Amino acid residues starting with the second methionine in the open reading frame are indicated below the sequence by the three letter code, and numbered at the left margin. Amino acid residues coded by DNA between the first and second methionine codons in the open reading frame are indicated below the sequence in the single letter code. DNA residues are numbered above the sequence, with 1 as the first base in the second methionine, and -1 as the base immediately preceding this. Boxed-in regions in the 5' flanking region correspond to 25 short sequences that are conserved, in order, between the 5' flanking regions of *psbA* (Erickson et al., 1984b) and *psbD* from *C. reinhardtii*. The putative ribosomal binding site (Shine and Dalgarno, 1974) is marked with a dashed box, and a region with homology to the Pribnow transcription initiation box (Rosenberg and Court, 1979) is marked PB. S1 sites are marked (S1), and those corresponding to sites shown in Figure 6 are indicated as S1a-d. The stars mark regions in which faint S1 sites are seen in Figure 6. Inverted repeats in the 3' flanking region are marked with arrows. The 46 bp outlined in the coding sequence are those that are directly duplicated in FuD47.

Fig. 5. This diagram compares the 352 amino acid (aa) residues coded for by the *cw15* (WT) *psbD* gene to the 186 residues in *C. reinhardtii* mutant FuD47. Thick white bars represent the extent of the *psbD* open reading frame. The 46 bp in the wild-type gene which are duplicated in the mutant are shown by hatched lines. 5' and 3' transcribed, non-translated regions are shown by thin white bars. Scale is given by the 100 bp size bar. The FuD47 DNA sequence corresponding to the bracketed region in the diagram is given below, along with the amino acid residues 140–186 coded for by this region. Residues also found in the wild-type strain are indicated in italics, while the new residues in FuD47 which result from the frame shift are non-italicized. The boxed-in region of the sequence delineates the duplicated 46 bp.

cloned into the λ vector NM1149, resulting in the recombinant λ FuD47-H3. This *Hind*III chloroplast fragment contains the entire *Eco*RI restriction fragment R3, as well as portions of the flanking fragments R5 and R11 (Rochaix, 1978; Rochaix *et al.*, 1984). In addition, an *Eco*RI restriction fragment of 1.05 kb, that had not previously been placed on the physical chloroplast map, was found between R3 and R11. This fragment, called R06, contains the 3' end of *psbD* as shown in Figure 3. The 7.5 kb *Hind*III fragment, the 2.85 kb R3 fragment and the 1.05 kb R06 fragment from both the FuD47 and *cw15* strains of *C. reinhardtii* were each transferred into the pUC9 vector resulting in the recombinant plasmids described in Materials and methods.

psbD from FuD47 was sequenced according to the Maxam–Gilbert sequencing strategy outlined in Figure 3. Sequence analysis of the isolated 1.0 kb *Ava*I fragment allowed for an unambiguous verification of the sequence across the *Eco*RI site between R3 and R06. The entire D2 gene from FuD47, including ~ 400 bp 5' and 250 bp 3' of the coding region, was sequenced.

The 5' portion of *psbD* contained in R3 had been previously sequenced from *C. reinhardtii* strain *cw15* (Rochaix *et al.*, 1984). In order to compare the mutant gene to the entire gene from a photosynthetically wild-type strain, the sequence of R06 from *cw15* was also determined. The only difference found in *psbD* between the wild-type strain and the FuD47 mutant was a 46 bp direct duplication of DNA. In Figure 4, which gives the wild-type *psbD* DNA sequence including the 3' end of the gene, the DNA indicated in the boxed-in region is duplicated in FuD47. As seen in detail in Figure 5, this DNA normally codes for amino acid residues 141–155. However, the 46 bp duplication results in a frameshift which adds 30 codons to the reading frame before the first termination codon occurs (Figure 5, bottom). The peptide translated from the mutant D2 gene is predicted to contain 186 amino acid residues.

Analysis of the 5' and 3' ends of *psbD* message

The 3' end of the *psbD* transcript was investigated by S1 mapping (see Materials and methods) of the 3' end-labeled *Eco*RI–

*Ava*I fragment shown in Figure 3. DNA and RNA from both *cw15* and FuD47 were analysed in this fashion, and in all cases the results were identical. As shown in the autoradiogram in Figure 6, several labeled DNA fragments are protected from S1 digestion after hybridization with *C. reinhardtii* RNA. The 5' end of *psbD* from FuD47 was S1 mapped from the 5' labeled *Hinf*I site indicated in Figure 3. Results of this mapping were identical to those found for the wild-type *psbD* (Rochaix *et al.*, 1984). The location of all 3' and 5' S1 sites are marked in the DNA sequence in Figure 4.

Analysis of DNA from phenotypic revertants of FuD47

The results with Northern blots (Figure 2) suggested that the RNA of the phenotypic revertants of FuD47 was of normal size. To determine whether this change in size between the mutant and revertants was at the level of RNA splicing or DNA deletion, DNA from the two phenotypic revertants FuD47 PR-1 and FuD47 PR-4, from FuD47 and from *cw15* was digested with *Fnu*4HI and electrophoresed on a 1.5% agarose gel. The DNA was transferred to a membrane filter as previously described for similar analysis of genomic DNA from a herbicide-resistant *psbA* mutant (Erickson *et al.*, 1985a). Wild-type cells contain a *Fnu*4HI fragment of 300 bp (starred fragment, Figure 3) compared to one of 346 bp in the FuD47 mutant. Hybridization of the filter with the radiolabeled 300 bp *Fnu*4HI fragment showed that both phenotypic revertants contained only the smaller *Fnu*4HI fragment, indistinguishable in size from that found in wild-type cells (data not shown).

Discussion

The function of two chloroplast-encoded PSII proteins, D1 and D2, is of current interest because of their suspected role in the PSII reaction center. Analysis of *C. reinhardtii* mutants with point mutations in *psbA*, which codes for the D1 protein, has helped define the role of D1 in herbicide resistance and electron transport (Erickson *et al.*, 1984a, 1985a). A role for D1 in stabilizing the PSII particle was suggested by results of analysis of *psbA* dele-

Fig. 6. This autoradiogram shows the results of DNA sequencing and S1 mapping of the 3' end of *psbD*, using the 3' labeled 400 bp *EcoRI*–*Avall* fragment (Figure 3). The Maxam–Gilbert sequence reactions performed are listed above the lanes on this 8% acrylamide gel containing 6 M urea. DNA sequences corresponding to three major S1 cleavage sites are shown at the side of the gel, with the 5' end of one strand identified. The four strong S1 sites labeled S1a to S1d are marked in the *psbD* sequence in Figure 4. Several faint S1-protected bands and one moderately strong band are seen towards the top of the gel. The sequence of these latter sites was verified on other gels (data not shown).

tion mutants, which account for almost 90% of all mutants examined that lack PSII activity (Bennoun *et al.*, 1986). We have now extended our analysis of chloroplast mutations affecting photosynthesis to include D2 mutants.

psbA in FuD47 is normal

FuD47 falls into a general class of both nuclear and chloroplast mutants which are missing the D2 protein. However, the D1 protein is also missing or greatly reduced in these mutants. Genetic analysis of FuD47 indicates that the D1 gene is present and correct: when FuD47 cells were crossed with another mutant FuD7, lacking both copies of *psbA* which codes for D1, complementation in young zygotes resulted in a normal fluorescence pattern. Both parental strains in the cross are PSII null and show high fluorescence. Since FuD7 lacked *psbA* (Bennoun *et al.*, 1986), it is assumed that the functional D1 protein in the zygote is contributed by the FuD47 parent, while the functional D2 protein comes from FuD7. Molecular analysis of *psbA* DNA (data not shown) and RNA (Figure 2) from FuD47 confirms the presence of the D1 gene and the appropriate size and amount of *psbA* transcript. Thus, the apparent absence of D1 in FuD47 (Figure 1) is not due to a structural defect in the D1 gene or a block in *psbA* transcription.

psbD 5' and 3' flanking regions

S1 mapping of the 5' end of *psbD* from FuD47 shows two S1-sensitive sites as previously reported for *cw15* (Rochaix *et al.*, 1984). This suggests that the 5' end of the *psbD* message in FuD47 is not significantly different from that of *psbD* message in wild-type cells. The presence of two S1-protected bands is a common feature of other chloroplast genes (Dron *et al.*, 1982; Erickson *et al.*, 1984b) and may represent alternate transcription initiation sites or a processing site. Interestingly, a comparison of both primary and secondary structure at the 5' end of the D1 and D2 genes shows that 25 short sequences are conserved in order (Figure 4) and that secondary structures can be drawn which are remarkably similar for the two genes (Figure 7). This includes the locations of the S1 sites and the putative Pribnow transcription initiation sequences. Any functional significance associated with these homologies and/or secondary structures will not be clear until factors regulating the transcription of these two chloroplast genes can be studied in detail. DNA homology extends into the coding region of the two genes, and it has been noted that the genes for these chloroplast-encoded core proteins may have arisen by a process involving the duplication of DNA sequences (Alt *et al.*, 1984).

S1 mapping at the 3' end of *psbD* gave the same pattern for *cw15* as for FuD47 cells. We conclude that the 3' end of the message is not significantly different between these two strains. However, both strains show an unusual pattern with multiple S1-sensitive sites (Figure 6). The first site, which lies within three bases of the end of the coding region and is in a stretch of five consecutive A residues, is presumed to be an artifact. The second two sites are found in a perfect inverted repeat extending through 40 bases, which can form a large stem–loop structure (see Figure 4). Similar structures are associated with S1-sensitive sites at the 3' end of other chloroplast genes (Dron *et al.*, 1982; Erickson *et al.*, 1984b). With one exception, the remaining S1-sensitive sites seen on the autoradiogram in Figure 6 and indicated on the sequence in Figure 4 are all found in inverted repeats that can form stem–loop structures. It is not clear if these sites represent heterogeneity in the termination of the transcription, in processing, or whether they are artifacts of the mapping

Fig. 7. A comparison of possible secondary structures in the 5' flanking regions of *psbA* (upper line, D1) and *psbD* (lower line, D2). S1 cleavage sites (S1) fall in the stems of the two conserved structures closest to the coding region of each gene. A conserved ACG triplet is found 3' to one of these stems, while a conserved ATG triplet is found following the other. These residues and other conserved residues, including the Pribnow box in the largest loop, are outlined in the diagram. The 6 bp inverted repeats, found in both *psbA* and *psbD* within the ~ 30 bp between the two pairs of stem-loop structures, are marked with arrows below the sequence. The potential ribosome binding sites are indicated by dashed boxes. The first ATG triplet in the *psbD* open reading frame is underlined, while MET shows the location of the second methionine residue in the open reading frame, as indicated in the text and the Figure 4 legend. Twenty-one base pairs of *psbA* have been omitted at the point indicated.

procedure. Unlike the higher plant D2 genes sequenced to date (Rasmussen *et al.*, 1984; Alt *et al.*, 1984; Holschuh *et al.*, 1984), in which the last 50 bp of *psbD* overlap with the first 50 bp of *psbC* which codes for the 43–47 kD core protein of PSII, there is no obvious overlapping reading frame in the *C. reinhardtii psbD*. Moreover, a single mRNA of $\sim 1.2 \pm 0.1$ kb hybridizes with the cloned D2 gene (Figure 2).

Molecular basis of the FuD47 mutation

Comparison of the nucleotide sequence of *psbD* from FuD47 and *cw15* showed that the only difference between these two strains was a 46 bp direct duplication of DNA in the coding region, resulting in a frameshift and early termination in FuD47 after 186 amino acid residues (Figure 5). Southern and Northern analysis of FuD47 DNA and RNA, respectively, confirmed that the duplication exists in the FuD47 chloroplast genome and in the *psbD* mRNA, and that it is not simply an artifact of the lambda cloning procedure. The reason for the lower amount of *psbD* message in FuD47 cells (Figure 2) is not known. It may be caused by a lower rate of transcription initiation or elongation, or an increase in the turnover of this message. The normal amounts of *psbA* and *rbcL* messages in FuD47 suggest that whatever is affecting the message level in FuD47 is specific for the D2 gene.

psbD deduced amino acid sequence

Analysis of the *psbD* nucleotide sequence shows that this gene codes for a protein that has 92% overall homology with the D2 protein in higher plants (see Erickson *et al.*, 1985b, for a com-

parison of D2 in various species). In contrast to a previous report (Rochaix *et al.*, 1984) we now find that this homology extends through the entire protein, including the COOH terminal end. The *C. reinhardtii psbD* codes for two methionine residues in the first 11 amino acids of the same open reading frame. The second of these is preceded by the consensus ribosomal binding site indicated in Figure 4. Translation from this second methionine gives a protein of 352 amino acid residues which is similar in size to the 353 amino acids found in the higher plant D2 protein. Thus, the first methionine in the higher plant open reading frame, also preceded by a strong putative ribosomal binding site, corresponds to the second methionine in the *C. reinhardtii* reading frame. Because of this comparison, we have numbered the amino acid residues in the algal D2 protein starting with the second methionine in the open reading frame. There is no concrete evidence as to where translation actually begins.

True or phenotypic revertants?

The frequency of phenotypic reversion of FuD47 is about 10^{-4} , which is of the same order of magnitude as that seen for deletions of small, direct duplications in λ phage (Calos *et al.*, 1978). Southern and Northern analysis of the DNA and RNA, respectively, from two FuD47 phenotypic revertants suggested that the duplicated DNA was removed, resulting in a true genetic reversion. However, sequence analysis of this region in the revertants was not carried out, and it is therefore not known whether these are exact or inexact reversions. It is clear, however, that the reading frame is restored and that the subsequent D2 product

functions in a fashion not detectably different from that of the wild-type cells.

Effect of the psbD mutation on FuD47 proteins

A major consequence of the *psbD* mutation in FuD47 is the general destabilization of PSII proteins and the subsequent lack of PSII particles. The stained gel in Figure 1 shows that both chloroplast and nuclear-encoded PSII proteins are missing or greatly reduced in the mutant. Western analysis of total cell proteins, using rabbit antibodies against proteins 5, 6 and 12, as well as D1 and D2, confirm the lack of accumulation of all these proteins in the FuD47 mutant (S.Mayfield, M.Kuchka, J.-D.Rochaix and P.Bennoun, in preparation). However, all chloroplast-encoded PSII proteins except D1 and D2 are synthesized, as shown by pulse-labeling in the presence of cycloheximide (Figure 1), and are found in the membrane fraction. A similar result is seen in several *psbA* mutants of *Chlamydomonas*, typified by FuD7. These mutants entirely lack the D1 protein, fail to accumulate all PSII proteins, but synthesize the PSII chloroplast-encoded proteins other than D1 which are found firmly anchored in the membrane, resistant to removal by washing with 6 M guanidine (Bennoun *et al.*, 1986). On the basis of these results, we attributed to D1 an important role in stabilizing the PSII particle. Although our results with FuD47 suggest that D2 may have a similar effect on the stability of the PSII complex, we cannot rule out the possibility that the instability of PSII in FuD47 is due to the absence of D1 rather than the absence of D2. Two other mutants have been analysed, with results that are relevant to this question. One is a *C. reinhardtii* chloroplast mutant which does not synthesize the 43–47 kD core PSII protein but does synthesize all other chloroplast-encoded core proteins, including D1, although none of these proteins accumulate (M.Kuchka, J.Girard-Bascou, J.-D.Rochaix, P.Bennoun, unpublished). The other is a nuclear maize mutant which fails to accumulate both nuclear and chloroplast-encoded PSII proteins (Metz and Miles, 1982). Although the identity of the nuclear gene containing the mutation is not known, the maize mutant does synthesize at least two of the chloroplast-encoded core components, D1 and the 43–47 kD protein, which are found in the stromal lamellae but are rapidly degraded (Leto *et al.*, 1985). The 47–52 kD core protein was never seen by pulse-labeling in this nuclear mutant. These results, while suggesting that D1 may not be more important to PSII stability than are any of the other core proteins, do emphasize the fact that the apparent absence of D1 synthesis in FuD47 reflects a peculiar relationship between D2 and D1, and is not simply a direct consequence of any general instability of PSII.

Indeed, the most surprising characteristic seen in FuD47 is the dramatic effect of D2 on the presence of D1: when D2 is missing in pulse-labeled cells, D1 is also absent or reduced below the detectable level (Figure 1). The reciprocal relationship does not hold, that is, in the absence of D1 in FuD7, D2 is still synthesized. One distinction between the general nature of these two mutants is that FuD47 is a duplication mutant, containing both a mutant *psbD* gene and message, whereas FuD7 is a deletion mutant completely lacking *psbA* genes and message. The importance of this distinction may not be clear until deletion mutants of *psbD* or early termination mutants of *psbA* have been characterized. Since the levels of *psbA* message in FuD47 are normal and genetic analysis shows that the gene is functional, the block in D1 expression is presumed to be post-transcriptional. It is tempting to speculate that D2 plays a role in regulating the translation of D1 by a process involving attachment of polysomes to the membranes or association of the message with ribosomes.

On the other hand, we cannot rule out the possibility that the D1 protein is synthesized normally in FuD47, but degraded so rapidly that it is never seen. It has been shown that D1 is degraded fairly rapidly in *Spirodela* (Mattoo *et al.*, 1981). The normal instability of D1 is proportional to light intensity and linked to the function of D1 in PSII electron transport (Mattoo *et al.*, 1984). A similar light-dependent degradation of D1 is seen in *Chlamydomonas* (Kyle *et al.*, 1984) and in isolated pea thylakoids (Ohad *et al.*, 1985). However, since FuD47 has no detectable PSII particles or activity, the absence of D1 in FuD47 is not easily explained by such a mechanism. The effect of different light intensities on transcription and translation of D1 and D2 in FuD47 will be reported elsewhere (P.Malnoë and P.Bennoun, in preparation). The lack of accumulation of the other PSII proteins in FuD47, including the truncated D2 protein, is most likely due to their enhanced degradation in the absence of proper PSII assembly. A similar situation has been seen for the multi-subunit enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase, in which unassembled subunits are rapidly degraded (Schmidt and Mishkind, 1983; Spreitzer *et al.*, 1985).

In summary, analysis of FuD47, a *psbD* mutant, has suggested that the D2 protein is needed for assembly of a stable PSII complex. In addition, this mutant reveals the particular effect of D2 on the regulation of D1 expression, either at the translational or post-translational level. Genetic and molecular analysis of *psbB*, *psbC* and *psbE* null mutants, as well as strains with point mutations in these genes and in *psbD*, will greatly aid in studying the role of these various proteins in PSII assembly and function.

Materials and methods

Algal and bacterial strains

C. reinhardtii strains cw15 (a cell-wall mutant which is wild-type with respect to photosynthesis, Davies and Plaskitt, 1971), wild-type strain 137c, FuD47 and the phenotypic revertants FuD47 PR-1 and FuD47 PR-4 were maintained in continuous dim light on solid Tris-acetate-phosphate (TAP) medium (Gorman and Levine, 1965). *Escherichia coli* RY1073 (Young and Davis, 1983) was used to select for recombinant phage in the λ vector NM1149 (Murray, 1983). λ NM1149 and pBR322 recombinant plasmids were propagated in the *E. coli* strain C600 (rk⁻, mk⁻, SuII), while those recombinants constructed in the pUC9 plasmid vector (Vieira and Messing, 1982) were propagated in either *E. coli* strains JM83 or JM101. *In vitro* packaging extracts were prepared from *E. coli* strains BHB2688 and BHB2690 (Arber *et al.*, 1983).

DNA preparation and Southern analysis

Total DNA was prepared from the cell-wall mutant *C. reinhardtii* cw15 strain as previously described (Rochaix, 1980). DNA from 137c and FuD47, which have wild-type cell walls, was prepared as described for *C. reinhardtii* DCMU4 (Erickson *et al.*, 1984a). Rapid preparations of CsCl-banded FuD47 PR-1 and FuD47 PR-4 from 40 ml cultures of cells in stationary phase were performed according to Weeks *et al.* (1986). Pelleted cells were resuspended in water and lysed with buffered SDS. CsCl was added directly to this lysate and DNA was banded by centrifuging the mixture in a VTi50 Beckman rotor at 45 K r.p.m. for 16 h. Rapid preparations of plasmid DNA from 1.5 ml of culture were performed by a modification of the Holmes and Quigley (1981) procedure, as previously described (Erickson *et al.*, 1984b). Rapid preparations of λ DNA from 0.4 ml lysates were performed as described (Cameron *et al.*, 1977). DNA separated by agarose gel electrophoresis was transferred to a Gene Screen Plus membrane filter (New England Nuclear) as previously described (Erickson *et al.*, 1985a).

RNA preparation and Northern analysis

Conditions for RNA extraction, formaldehyde gels, transfer of RNA to nitrocellulose filters and hybridization of the filters with radio-labeled DNA probes are as previously described (Goldschmidt-Clermont, 1986). RNA was prepared from TAP liquid cultures of wild-type strain 137c, FuD47, FuD47PR-1 and FuD47PR-4, all grown in continuous dim light to a density of $1-2 \times 10^6$ cells/ml. After hybridization, nick-translated DNA probes were removed from the filter

by rinsing twice in 0.1 N NaOH at room temperature for 2 min each, followed by two rinses in distilled water, and two 10-min rinses in $2 \times \text{SSC}$ ($1 \times \text{SSC}$; 0.15 M sodium chloride, 0.015 M sodium citrate).

Cloning

The λ vector NM1149 (Murray, 1983) was used to clone *Hind*III fragments from total DNA of *C. reinhardtii* mutant FuD47. Ligated DNA was packaged *in vitro* (Arber *et al.*, 1983) and the resulting phage were infected into *E. coli* strain RY1073 which prevents growth of parental phage. Recombinant plaques were screened by the method of Benton and Davis (1977), using the cloned chloroplast *Eco*RI fragment R3 from *C. reinhardtii* *cw15* as a probe. Hybridizing phage were plaque-purified several times on RY1073 before DNA mini-preparations were made from recombinant phage propagated in *E. coli* strain C600. The DNA was digested with *Hind*III alone and both *Hind*III and *Eco*RI in order to verify the presence of the desired 7.5 kb chloroplast *Hind*III fragment. One recombinant phage which contained the appropriate *Hind*III fragment was called λ FuD47-H3 and used for further studies.

To facilitate sequence analysis, the 7.5 kb *Hind*III chloroplast fragment contained in λ FuD47-H3 and in λ CW-H3 (Rochaix *et al.*, 1984) was cloned into the plasmid vector pUC9. Digestion of this 7.5 kb *Hind*III fragment produces two *Eco*RI chloroplast fragments; the 2.85 kb R3 fragment and the 1.05 kb R06 fragment. R3 and R06 from FuD47 and from *cw15* were each subcloned into pUC9 resulting in the recombinant plasmids pFuD47-R3, pCW-R3, pFuD47-R06, and pCW-R06. Conditions for ligations, transformations, nick-translation, hybridization and autoradiography are as previously described (Erickson *et al.*, 1981).

DNA sequence analysis

DNA fragments, electro-eluted from polyacrylamide gels and digested with the desired restriction enzymes, were labeled at either the 3' or 5' ends as previously described (Erickson *et al.*, 1984b), strand-separated by electrophoresis on an acrylamide gel, and sequenced by the chemical cleavage technique of Maxam and Gilbert (1980). Reaction products were separated by electrophoresis on 8 and 20% acrylamide gels containing 6 M urea, and visualized by autoradiography with Kodak X-ray film XR5. A Hewlett Packard computer model 9845 and a Victor Sirius computer were used to analyse DNA sequences.

S1 mapping

S1 mapping was performed according to Berk and Sharp (1977). DNA fragments marked S1 in Figure 3 were labeled at the 5' or 3' end as described above and strand separated before hybridization with 20 μ g of total *C. reinhardtii* RNA. S1 mapping of the FuD47 *psbD* was performed using both FuD47 and *cw15* RNA. We identified the location of the S1 cleavage sites by size comparison of S1-protected fragments with the Maxam–Gilbert sequence reactions for the same labeled fragment, taking into account the fact that a DNA fragment cleaved by S1 nuclease migrates approximately 1½ bp more slowly than its chemically cleaved counterpart (Sollner-Webb and Reeder, 1979).

Analysis of proteins

The preparation of whole cell proteins, thylakoid membrane proteins and pulse-labeled proteins, and the conditions for gel-electrophoresis and autoradiography are as previously described (Bennoun *et al.*, 1986), with the modification noted in the legend to Figure 1.

Enzymes, isotopes

Restriction endonucleases, the large fragment of DNA polymerase I (Klenow), and T4 polynucleotide kinase were purchased from Genofit (Geneva), Biofinex (Fribourg), New England Biolabs, Bethesda Research Laboratories Inc., Boehringer Mannheim or Amersham, and used as recommended by the supplier. Calf intestine alkaline phosphatase and S1 nuclease were purchased from Boehringer Mannheim. Radioisotopes were purchased from Amersham.

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