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# Expression of the nuclear gene encoding oxygen-evolving enhancer protein 2 is required for high levels of photosynthetic oxygen evolution in *Chlamydomonas reinhardtii*

**(photosystem II/thylakoid membrane/transit peptide)**

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**ABSTRACT** **We have cloned a cDNA encoding a 20-kDa polypeptide, oxygen-evolving enhancer protein 2 (OEE2), in *Chlamydomonas reinhardtii*. This polypeptide has been implicated in photosynthetic oxygen evolution, and it is associated with the photosystem II complex, the site of oxygen evolution in all higher plants and algae. The sequence of OEE2 cDNA, the deduced amino acid sequence of the preprotein, the N-terminal protein sequence of mature OEE2 protein, and the coding regions of the single OEE2 gene are presented. The protein is synthesized with a 57-amino acid N-terminal transit peptide that directs the transport of this polypeptide across three cellular membranes. A nuclear mutant of *C. reinhardtii*, deficient in oxygen-evolving activity, is shown to be specifically missing OEE2 polypeptides. This mutation, which results in the complete absence of all OEE2 mRNA and protein, does not affect the accumulation of other photosystem II polypeptides or their mRNAs.**

All aerobic organisms are dependent upon the evolution of oxygen from the photolysis of water during photosynthesis for the oxygen required in respiration. Photosynthesis, the conversion of light energy to chemical energy in higher plants and algae, requires the cooperation of two photosystem reaction centers and an electron transport chain that connects them. The photochemical reaction of photosystem II (PS II) induces a charge separation across the thylakoid membrane, thereby providing the oxidizing power necessary for the formation of molecular oxygen and electrons from water. The electrons derived from this reaction are passed along the electron transport chain to photosystem I and are ultimately used to reduce NADP. Considering the importance of photosynthetic oxygen evolution to the biosphere, very little is known about either the enzymatic mechanism or the enzyme(s) involved in this fundamental reaction.

The photochemical reaction center of PS II and the oxygen-evolving complex are biochemically closely linked and consist of at least five chloroplast-encoded polypeptides, which are members of the core PS II particle, and three nucleus-encoded extrinsic polypeptides, which bind to the lumen side of PS II (for review see ref. 1). With the exception of the proteins responsible for chlorophyll binding, it has proven difficult to assign particular functions to specific polypeptides within the PS II complex. Recently several groups have isolated photosynthetic membranes or submembrane particles with high specific activities of oxygen evolution *in vitro* (reports compiled in ref. 2). These particles generally contain the five core polypeptides associated with the reaction center of PS II as well as the three extrinsic polypeptides. The extrinsic polypeptides can be removed from the oxygen-evolving particles by salt washes. The removal of these polypeptides results in the loss of oxygen-evolving activities of the particle. Restoration of the activity can be accomplished by the readdition of these polypeptides (for review see ref. 3) or to a limited degree by the addition of nonphysiological levels of calcium or chloride ions (4).

An *in vivo* approach to the question of what polypeptides might be involved in oxygen evolution can be taken by analyzing mutants of *Chlamydomonas reinhardtii*, a unicellular green alga utilizing the same photosynthetic scheme as higher plants, that are deficient in oxygen evolution. These mutants can be separated into two classes; those that lack all PS II activities, including oxygen evolution, and those that are deficient only for oxygen-evolving activities. The former mutants lack polypeptides associated with the reaction center of PS II (5, 6), while the latter mutants retain at least some of the core PS II polypeptides (7).

Here we report that a *C. reinhardtii* nuclear mutant deficient in oxygen-evolving activity (7), but containing other PS II activities, specifically lacks both a 20-kDa extrinsic PS II polypeptide (oxygen-evolving enhancer protein 2, OEE2) and the mRNA encoding it. All other PS II polypeptides and their mRNAs accumulate to normal levels in these cells. We also report the cloning and nucleotide sequence of cDNA and genomic fragments encoding the OEE2 polypeptide, the predicted amino acid sequence of the entire preprotein, the coding regions of the single OEE2 gene, and the N-terminal amino acid sequence of purified mature OEE2 protein.

## MATERIALS AND METHODS

**Cell Culture.** Wild-type *Chlamydomonas reinhardtii* strain 137 and low chlorophyll fluorescence nuclear mutant BF25 (7) were grown in liquid Tris/acetate/phosphate medium, pH 7.0 (8), under fluorescent lighting to a density of 2–3 × $10^6$ cells per ml. The cells were harvested by centrifugation at 8000 × *g* for 10 min, resuspended in 1/20 vol fresh medium, and pelleted again at 8000 × *g* for 10 min.

**Protein Isolation, Polyacrylamide Gel Electrophoresis, Electroblotting, and Antibody Hybridization.** For protein isolation the pelleted cells were resuspended in 0.8 M Tris·HCl, pH 8.3/0.4 M sucrose/1% 2-mercaptoethanol, quickly frozen in a dry ice/ethanol bath, allowed to thaw on ice, and briefly sonicated (three 10-sec treatments) with a microtip to break the cells. The lysed cells were pelleted at 15,000 × *g* for 15 min, the supernatant (soluble fraction) was removed, and the pellet (membrane fraction) was resuspended in the above buffer. Under these conditions the three extrinsic polypeptides associated with PS II are released into the soluble

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Abbreviations: OEE1, OEE2, and OEE3, oxygen-evolving enhancer proteins 1, 2, and 3; PS I and II, photosystems I and II.

faction and can thus easily be separated from other PS II polypeptides. Polyacrylamide gel electrophoresis, protein blotting, antibody hybridization, and autoradiography were as previously described (9).

Mature OEE2 polypeptides were isolated from *C. reinhardtii* mutant F54-14 (10) which lacks both the PS I and the chloroplast ATPase complexes. Use of this mutant facilitated the isolation of PS II particles free of contaminating polypeptides. PS II particles were prepared according to Diner and Wollman (11). The PS II particles were removed from the sucrose gradient, adjusted to 5% trifluoroacetic acid, and loaded onto a HPLC reverse-phase $C_8$ column (Whatman, 300-Å pore size). The proteins were eluted from the matrix with a 0–100% gradient of acetonitrile. The samples were lyophilized to remove acetonitrile and the amino acid sequence was determined by using an Applied Biosystems model 470A protein sequenator according to standard procedures.

**Isolation of cDNAs and Genomic Clones Encoding OEE2.** A *C. reinhardtii* cDNA library was constructed in λgt10 (14). The λgt10 DNA was digested with *Eco*RI, and the cDNA inserts were purified by agarose gel electrophoresis and inserted into the unique *Eco*RI site of λgt11 (Promega Biotec, Madison, WI). Immunoscreening of the λgt11 recombinant phages was as described by Young and Davis (12), using the same antisera as used for the protein blot hybridizations. A single positive plaque was detected with antisera specific to OEE2, and the cDNA insert was subcloned in the *Eco*RI site in plasmid pUC19. The resultant plasmid, pPII-19.1, contained a 1.4-kilobase (kb) cDNA insert. To isolate genomic clones encoding OEE2 the cDNA insert of pPII-19.1 was nick-translated and hybridized by the method of Benton and Davis (13) to a *C. reinhardtii* genomic library (14) cloned in λ EMBL3. Six overlapping clones were isolated and a restriction map was compiled (Fig. 1). One of the clones, E19-2, contained the entire coding region of OEE2 and was used for subcloning in plasmid pUC19.

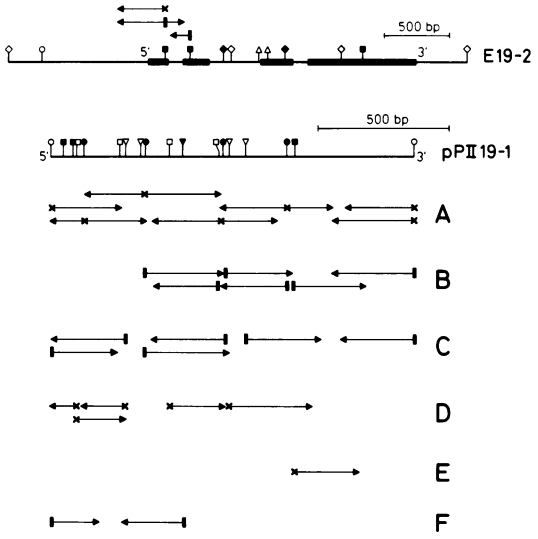


FIG. 1. Restriction map and sequencing scheme of cDNA clone pPII-19.1 and genomic clone E19-2. bp, Base pairs. The restriction sites for *Eco*RI (○), *Pst* I (◇), *Sal* I (◆), *Ava* I (■), and *Pvu* I (△) are shown for genomic clone E19-2, and the restriction sites for *Eco*RI (○), *Hin*fI (●), *Bst*NI (▽), *Taq* I (□), *Sau*96I (▼), and *Ava* I (■) are shown for cDNA clone pPII-19.1. The heavy line on genomic clone E19-2 indicates the coding regions of the single OEE2 gene. The three *Ava* I (■) sites within the genomic clone are also found in the cDNA. For sequencing the 1.4-kb cDNA clone was digested with *Hin*fI (A, B), *Bst*NI (C), *Taq* I (D), *Ava* I (E), and *Sau*96I (F). Genomic clone E19-2 was digested with *Ava* I. Fragments were labeled at either their 5′ (▌) or their 3′ (×) ends and the sequence was determined for the length indicated by the arrow.

**Sequencing of pPII-19.1 and Genomic Clone E19-2.** The 1.4-kb cDNA insert of plasmid pPII-19.1 was digested with restriction endonucleases and labeled either 5′ or 3′ as diagrammed in Fig. 1. Genomic fragments from clone E19-2 were subcloned in plasmid pUC19, digested with restriction endonuclease *Ava* I, and labeled at the 5′ and 3′ ends as diagrammed in Fig. 1. The labeled fragments were denatured, separated on strand-separating acrylamide gels, eluted, and then chemically cleaved, and the sequences were determined as described by Maxam and Gilbert (15).

**RNA and DNA Isolation, Electrophoresis, and Blotting.** RNA was isolated with guanidinium hydrochloride as described (16). Total RNA, 10 μg per sample lane, was separated on denaturing formaldehyde/agarose gels and then electroblotted to nylon membrane (GeneScreen; New England Nuclear) in 25 mM sodium phosphate, pH 6.5. After electroblotting the filters were baked in a vacuum oven at 80°C for 1 hr and stained with methylene blue (17) to check for even loading and transfer of RNA. The filters were then prehybridized and hybridized as described (18).

Isolation of *C. reinhardtii* DNA, digestion with restriction endonucleases, electrophoresis, and blotting to nitrocellulose membranes were as previously described (19).

## RESULTS

**Cloning of OEE2 cDNA and Characterization of the Corresponding Single Nuclear Gene in *C. reinhardtii*.** Indirect evidence suggested that all three of the extrinsic polypeptides associated with oxygen evolution are encoded by the nucleus in *C. reinhardtii* (6) as they are in higher plants (20). We therefore constructed a λgt11 expression vector library containing *C. reinhardtii* cDNA inserts and identified the expressed fusion protein products by reaction with antisera specific for each of the three polypeptides. cDNA clones were obtained for each of the three nucleus-encoded polypeptides. A single plaque containing a phage with a 1.4-kb cDNA insert was obtained by using antisera specific for OEE2. This cDNA clone, which hybridized to a 1.5-kb mRNA, was subcloned in plasmid pUC19 and sequenced by the method of Maxam and Gilbert (15). A restriction map and the sequencing scheme of cDNA clone pPII-19.1 are shown in Fig. 1.

The 1.4-kb cDNA clone pPII-19.1 was nick-translated and hybridized to genomic DNA blots. As shown in Fig. 2, the OEE2 cDNA hybridized to a single or small set of DNA fragments, depending on the restriction enzyme used. Genomic clones encoding OEE2 were isolated from a *C. reinhardtii* library. Several overlapping clones were obtained

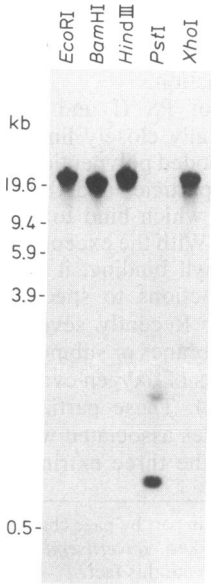


FIG. 2. Southern analysis of wild-type *C. reinhardtii* genomic DNA. DNA was digested with a restriction endonuclease as indicated on the figure, separated by agarose gel electrophoresis, and blotted to nitrocellulose. The blot was hybridized with a nick-translated cDNA insert from plasmid pPII-19.1.

and a restriction map of the chromosomal segment was compiled by using common restriction endonucleases (Fig. 1). Note that in the *Pst* I digest of genomic DNA shown in Fig. 2 there are only two hybridization signals and that the restriction map of the cloned genomic segment (Fig. 1) predicts 2 fragments of approximately 900 bp (strong band) and 1 fragment of 1.7 kb (weak band). Comparison of other restriction fragments from the genomic clones with Southern blot analysis of genomic DNA confirmed that OEE2 is a single-copy gene (data not shown). Genomic clones containing the entire OEE2 gene were subcloned in plasmid pUC19, digested with the appropriate restriction endonuclease, and labeled at either their 5′ or 3′ ends. The labeled fragments were then hybridized with an excess of *C. reinhardtii* RNA and the coding regions were mapped by using S1 exonuclease (21). The four coding regions and the 5′ and 3′ boundaries of the single OEE2 gene are shown in the upper map of Fig. 1.

**OEE2 Is Encoded as a 245-Amino Acid Precursor Polypeptide That Is Cleaved to a Mature Protein of 188 Amino Acids.** To verify that pPII-19.1 encoded OEE2 and to determine the amino acid sequence of the OEE2 protein, the OEE2 cDNA was sequenced. OEE2 cDNA was digested and labeled at either the 5′ or 3′ end as diagrammed in Fig. 1. The labeled fragments were then sequenced by the chemical cleavage method of Maxam and Gilbert (15). The cDNA pPII-19.1 contained the 3′ poly(A) tail but was truncated at the 5′ end. To obtain the 5′ coding sequence of OEE2, subclones of genomic fragments containing the 5′ end of the gene were digested with *Ava* I, labeled at both the 5′ and 3′ ends, and sequenced as before (see map in Fig. 1). The composite

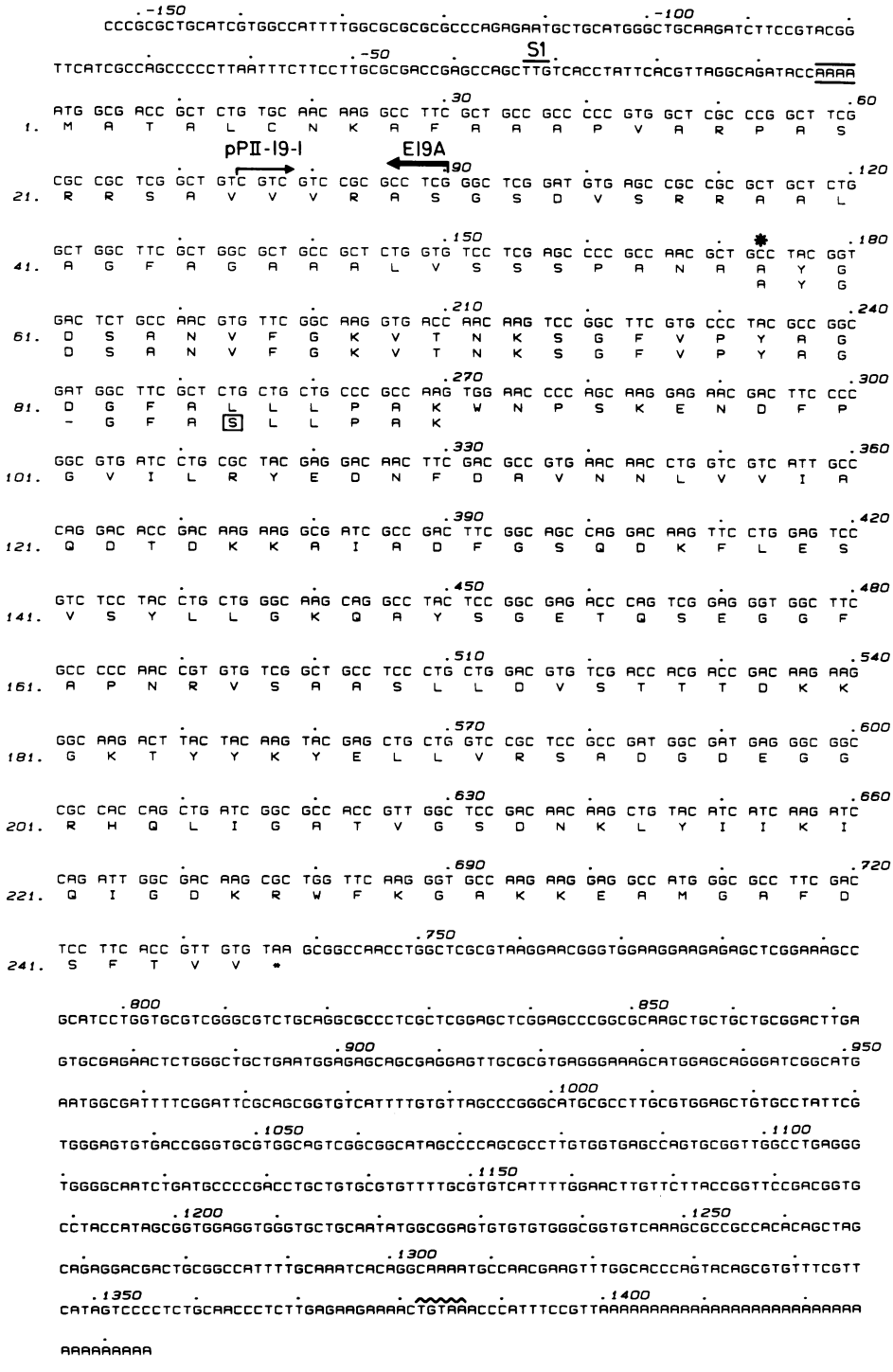


FIG. 3. Nucleotide sequence of cDNA pPII-19.1 (arrow pointing right) and genomic fragment E19-2 (arrow pointing left), predicted amino acid sequence of OEE2, and N-terminal sequence of mature OEE2 protein. Amino acid residues are represented by the standard one-letter code. The 5′ terminus (S1) of the mRNA is 32 bases from the methionine initiation codon ATG (residue 1). Note that A is never used in the third position of any codon and that the methionine initiation codon is preceded by four A residues (⎓); this renders all three reading frames null just prior to the start codon. The N terminus of the mature OEE2 protein is 58 amino acids (*) from the N terminus of the deduced preprotein, and differs at one point (S), which is probably due to a misreading of the mature protein sequence. A possible polyadenylylation site (wavy overline) has been found in all *C. reinhardtii* nuclear genes sequenced to date (refs. 6 and 22; S.P.M. unpublished data). Also note that the 3′ untranslated portion of the sequence is almost as long as that of the translated portion; the reason for this long 3′ extension is not clear.

sequence derived from both the cDNA and genomic fragments is presented in Fig. 3.

To confirm the identity of pPII-19.1 as the cDNA encoding OEE2, we isolated mature OEE2 protein from *C. reinhardtii* cells and determined the N-terminal amino acid sequence. As shown in Fig. 3, the N terminus of the mature OEE2 protein does not correspond to the N terminus of the deduced preprotein, but rather corresponds to a section of the polypeptide that is 57 amino acids from the methionine initiation amino acid. The 25-amino acid sequence derived from the mature protein differs at only one point from the deduced amino acid sequence (Fig. 3, amino acid 67). We take this as proof that pPII-19.1 and E19-2 encode OEE2.

**A Nuclear Mutant of *C. reinhardtii* Deficient in Oxygen Evolution Specifically Lacks OEE2 Polypeptides.** A nuclear mutant of *C. reinhardtii* (BF25) that has only 5% of the oxygen-evolving activity of wild-type cells yet contains normal levels of other activities associated with PS II (7) is lacking a 20-kDa polypeptide associated with PS II particles (Fig. 4*A*). Proteins were isolated from wild-type and BF25 cells, separated into soluble and membrane protein fractions, and electrophoretically separated on denaturing polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate. After electrophoresis either the protein gels were stained with Coomassie blue or the proteins were transferred to CNBr-activated paper for reaction with specific antisera. Examination of the stained gels reveals that a 20-kDa protein is missing from the soluble protein fraction of mutant BF25, while most other polypeptides appear to be present in equal quantities in both membrane and soluble protein fractions in both wild-type and mutant cells (Fig. 4 *A* and *C*). Reaction with antisera specific to polypeptides associated with PS II shows that only the 20-kDa extrinsic polypeptide (OEE2) is missing from the mutant (Fig. 4*B*). The 26-kDa (OEE1, referred to as the 33-kDa protein in spinach) and 17-kDa (OEE3) extrinsic polypeptides and all of the core PS II proteins accumulate to similar levels in both wild-type and BF25 cells (Fig. 4 *B* and *D*).

**Mutant BF25 Lacks Only the mRNA Encoding OEE2.** To determine if the loss of OEE2 protein was directly due to a mutation in the gene encoding this polypeptide and not to a pleiotropic effect of a mutation at another site, we used the OEE2 cDNA to probe RNA and DNA blots of wild-type and BF25 cells. Equal amounts of total RNA from wild-type and BF25 cells were fractionated on denaturing agarose gels and electroblotted onto nylon membranes. The blots were then hybridized with nick-translated cDNA probes encoding the 26- and 17-kDa polypeptides associated with oxygen-evolving activity (OEE1 and OEE3; cloning and identification to be reported elsewhere) and with nick-translated pPII-19.1. RNA blots were also probed with nick-translated cloned chloroplastic genomic fragments specific for mRNAs encoding the core PS II polypeptides D1, D2, P5, and P6 (23). As shown in Fig. 5, all of the chloroplast mRNAs encoding core PS II polypeptides accumulate to similar levels in both wild-type and BF25 cells. The two nuclear mRNAs encoding OEE1 and OEE3 also accumulate to wild-type levels in BF25 cells. The only mRNA encoding a PS II protein that failed to accumulate in BF25 cells was OEE2 mRNA. Southern blot analysis of genomic DNA from wild-type and BF25 cells failed to reveal any differences between the two (data not shown).

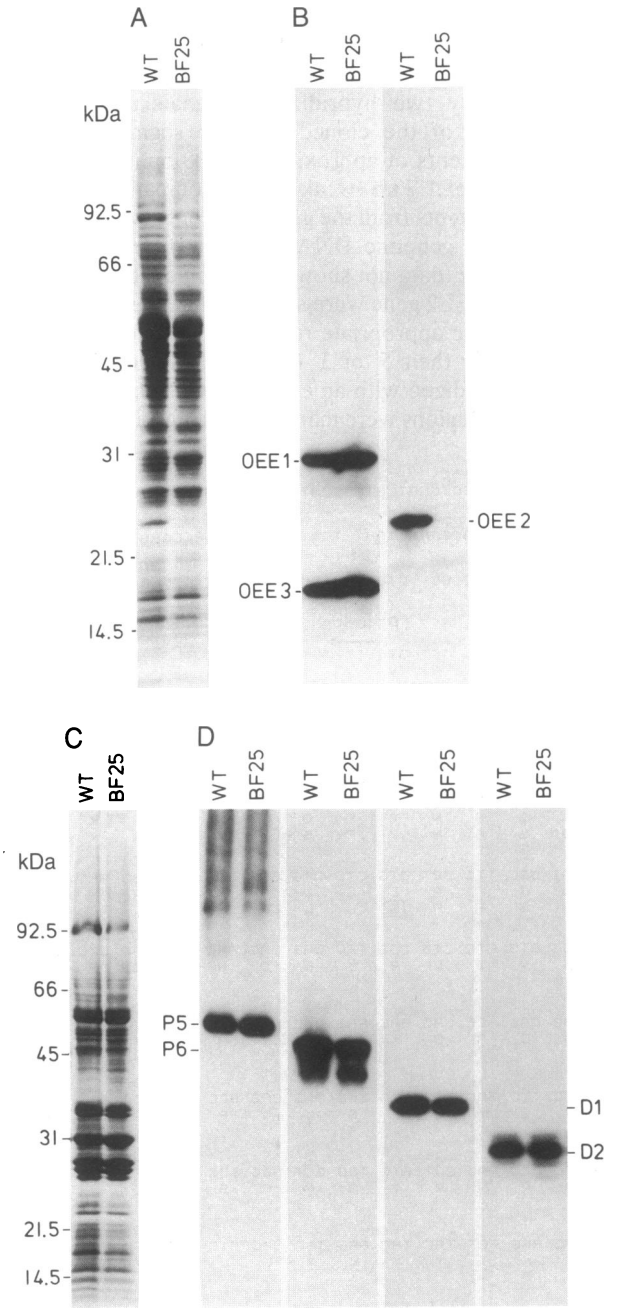


FIG. 4. Polyacrylamide gel electrophoresis of water-soluble (*A* and *B*) and membrane (*C* and *D*) proteins isolated from wild-type (WT) and mutant BF25 cells. After electrophoresis the proteins were either stained with Coomassie blue (*A* and *C*) or transferred to CNBr-activated paper (*B* and *D*). The protein blots in *B* were allowed to react with antisera specific for three extrinsic polypeptides associated with PS II: OEE1, OEE2, and OEE3. The protein blots in *D* were allowed to react with antisera specific for the PS II core proteins D1, D2, P5, and P6. The proteins were visualized by autoradiography after labeling of the antigen–antibody complexes with $^{125}$I-labeled *Staphylococcus aureus* protein A.

## DISCUSSION

We have shown that a nuclear mutant of *C. reinhardtii* that has only 5% of the oxygen-evolving activity of wild-type cells lacks a single 20-kDa water-soluble protein (OEE2) associated with PS II. The loss of OEE2 polypeptide is due to the complete failure of OEE2 mRNA accumulation. Thus, the mutation of BF25 is most likely within the single OEE2 gene, but it may also be in another gene that somehow affects the stable accumulation of OEE2 mRNA. The loss of OEE2 polypeptide does not affect the accumulation of other PS II polypeptides or their mRNAs. Sequence analysis of mature OEE2 protein and of cloned OEE2 cDNA and genomic fragments showed that the polypeptide is synthesized as a 245-amino acid precursor polypeptide, which is cleaved to a 188-amino acid mature protein by the time it reaches its final destination within the chloroplast.

Several groups have isolated chloroplast membrane particles that are capable of high rates of oxygen evolution *in vitro*.

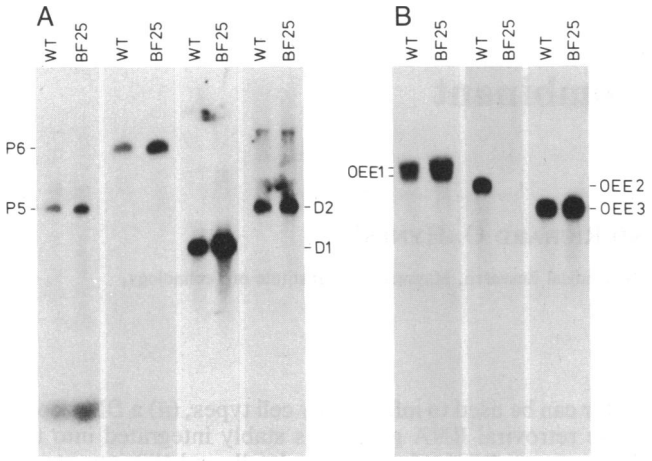


FIG. 5. Blot hybridization analysis of mRNA isolated from wild-type (WT) and mutant BF25 cells. Total RNA was separated on denaturing formaldehyde gels and electroblotted to nylon membranes. The filters were hybridized with nick-translated cloned cDNAs encoding OEE1, OEE2, or OEE3 (*B*) and with cloned chloroplast genomic fragments encoding the PS II core proteins D1, D2, P5, or P6 (*A*).

Analyses of these particles have shown them to contain the reaction center proteins of PS II and in most cases three extrinsic polypeptides, referred to here as OEE1, OEE2, and OEE3, which could be removed from the core PS II particle by salt washing. Removal of these proteins from the PS II core particle resulted in the loss of oxygen-evolving activity, which could be restored to various degrees by the readdition of the extrinsic polypeptides. The general picture emerging from this *in vitro* work is that OEE2 and OEE3 (23- and 17-kDa polypeptides of spinach) may play some role in the binding or concentrating of $Ca^{2+}$ and $Cl^-$, while OEE1 (33-kDa polypeptide of spinach) is necessary for the binding of $Mn^{2+}$ (for review see refs. 1 and 3).

There is some debate on which of the peripheral PS II polypeptides are absolutely necessary for $O_2$ evolution. Several groups have reported that the PS II core and the OEE1 protein are sufficient for $O_2$ evolution (4, 24–30), while others have reported that the PS II core and OEE2 constitute the minimal oxygen evolving complex (31–33). Here we show that, *in vivo*, OEE2 is absolutely required for high levels of oxygen evolution. However, it is important to note that mutant BF25, although deficient in $O_2$ evolution, is not completely devoid of $O_2$ evolution and in fact is capable of photosynthetic growth (approximately 5% of wild-type levels). Although OEE2 is required for $O_2$ evolution it is apparent from the protein blotting data that OEE2 is not necessary for the accumulation of the core PS II particle or for the accumulation of either OEE1 or OEE3.

The 57-amino acid transit peptide of OEE2 must direct the polypeptide across the chloroplast outer and inner envelope membranes and then across the thylakoid membrane in order for the polypeptide to reach its binding site on the lumen side of the PS II complex. Examination of the transit peptide shows it to contain mostly hydrophobic and uncharged amino acids, in particular alanine (33%), serine (14%), and valine (10%). The charged residues, mainly arginine, are distributed evenly throughout the N-terminal half of the sequence, but are missing in the C-terminal portion. A similar uncharged region has also been found in the C terminus of the transit peptide of plastocyanin, a protein that, like OEE2, is located on the lumen side of the thylakoid membrane (34). Similar features are also found in the transit sequence of the nucleus-encoded yeast mitochondrial cytochrome $c_1$, a protein that is bound to the inner mitochondrial membrane and extends into the intermembrane space. The N-terminal half of this transit sequence is able to target the protein into the mitochondrial matrix, while it appears that the C terminus of the transit peptide acts as a stop transfer signal for the inner membrane (35). It may be that a similar mechanism is used for the sorting of OEE2 within *C. reinhardtii*.

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