Mutations in a Signal Sequence for the Thylakoid Membrane Identify Multiple Protein Transport Pathways and Nuclear Suppressors

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Abstract. The apparatus that permits protein translocation across the internal thylakoid membranes of chloroplasts is completely unknown, even though these membranes have been the subject of extensive biochemical analysis. We have used a genetic approach to characterize the translocation of *Chlamydomonas* cytochrome f, a chloroplast-encoded protein that spans the thylakoid once. Mutations in the hydrophobic core of the cytochrome f signal sequence inhibit the accumulation of cytochrome f, lead to an accumulation of precursor, and impair the ability of *Chlamydomonas* cells to grow photosynthetically. One hydrophobic core mutant also reduces the accumulation of other thylakoid membrane proteins, but not those that translocate completely across the membrane. These results suggest that the signal sequence of cytochrome f is required and is involved in one of multiple insertion pathways. Suppressors of two signal peptide mutations describe at least two nuclear genes whose products likely describe the translocation apparatus, and selected second-site chloroplast suppressors further define regions of the cytochrome f signal peptide.

Many proteins destined for the bacterial inner membrane use a translocation complex comprised of the Sec proteins (Pugsley, 1993). Proteins that are inserted or translocated through the ER interact with the signal recognition particle (SRP) that recognizes a nascent signal sequence and allows the initiation of translocation through a complex of membrane proteins (Nunnari and Walter, 1992). Neither the Sec system nor the SRP apparatus, however, are sufficient to explain the translocation or even insertion of all membrane proteins because the elimination of SRP still is permissive for ER translocation (Hann and Walter, 1991; Stirling and Hewitt, 1992), and several bacterial proteins can translocate in a Sec-independent manner (McGovern and Beckwith, 1991; Wickner, 1988). Some of these observations could be explained by the identification of both a bacterial Sec-type system in ER and an SRP-like system in *Escherichia coli* (High and Stirling, 1993; Phillips and Silhavy, 1992).

Most nuclear-encoded proteins enter the mitochondria through a proteinaceous complex that spans the inner and outer membranes (Pfanner et al., 1988), but so far this complex has little homology with either the Sec or SRP translocation machinery. The route that cytoplasmically synthesized proteins follow to insert into the inner membrane of mitochondria is less well characterized (Horst et al., 1993; Pfanner et al., 1992), as it is the insertion of mitochondrialy synthesized proteins into the inner membrane. Cytochrome c oxidase subunit II (COX2), which is synthesized in the mitochondria, is inserted cotranslationally (Sevarino and Poynton, 1980), but the insertion mechanism has not been described. It is apparent, therefore, that many distinct mechanisms for protein insertion and translocation exist, and different mechanisms often exist within one organism or organelle.

The outer and inner envelope of the chloroplast surrounds an aqueous stroma that contains thylakoid vesicles. Nuclear-encoded proteins are targeted to the chloroplast by an NH2-terminal transit peptide that is removed in the stroma. In vitro, the nuclear-encoded thylakoid membrane protein LHCP (light-harvesting chlorophyll a/b binding protein) requires ATP and several proteinaceous stromal factors (Fulson and Cline, 1988), as well as all three of its membrane-spanning regions to insert (Auchincloss et al., 1992). Cytoplasmically synthesized proteins destined for the lumen of the thylakoid, such as plastocyanin (PC) and the members of the oxygen-evolving complex (OEC33,23,17), have bipartite transit peptides where the NH2-terminal domain is similar to chloroplast transit peptides, while the COOH-terminal domain is homologous to bacterial signal sequences (Smeekens et al., 1986). The NH2-terminal transit peptide domain of the signal sequence of plastocyanin, OECs 33, 23, 17, and cytochrome c, is likely cleaved in the stroma, and the second, bacterial-like domain is cleaved upon the translocation of these proteins into the thylakoid lumen (Ko and
precursor of pea cytochrome f can be cleaved by E. coli leader peptidase in vitro (Anderson and Gray, 1991a). In addition, gene fusions made from the 5' end of the petA gene and lacZ are targeted to the inner membrane in E. coli in a SecA-dependent manner, suggesting that the cytochrome f signal sequence can function in bacteria (Rothstein et al., 1985). This does not demonstrate that chloroplasts use a Sec-like system, however, because ER signal peptides also engage the Sec Y/E translocase. A chloroplast homologue to the 54-kD subunit of SRP has been reported (Franklin and Hoffman, 1993), although its function is not known, and no bacterial Sec protein homologues have been identified in chloroplasts of land plants and green algae. There has been no direct experimental evidence that the signal sequence of cytochrome f is involved in protein translocation into thylakoids in vivo.

In vivo analysis of the cytochrome f signal sequence became possible with the development of a homologous transformation system into the chloroplast genome of Chlamydomonas Reinhardtii (Boynton and Gillham, 1993). Because petA is a single-copy gene, site-directed mutations in the signal sequence of cytochrome f that are created in vitro can replace the wild-type gene in Chlamydomonas, and the effects of these mutations can be studied. Signal sequence mutations that block the accumulation of cytochrome f will block photosynthesis and therefore autotrophic growth; because Chlamydomonas cells can grow both autotrophically and heterotrophically, maintenance of nonphotosynthetic strains is feasible. It is possible to select suppressors of such mutations by isolating cells that can grow photosynthetically. Many of these suppressors should describe proteins that interact with the signal sequence. This paper describes the characterization of seven signal sequence mutations of cytochrome f and the isolation of suppressors of two of these mutations.

Materials and Methods
Construction of Mutants
The petA gene was subcloned into pUC19ΔH as a 2.3-kb KpnI fragment from pUC8Bam7 (obtained from the Chlamydomonas Genetics Center at Duke University) to create pUC19ΔHpetA. pUC19ΔH was constructed by digesting pUC19 (Vieira and Messing, 1987) with HindIII and filling in with Klenow fragment (Promega Corp., Madison, WI). Mutations in the signal sequence of cytochrome f were made using pUC19ΔHpetA and the oligonucleotide-directed in vitro mutagenesis kit from Amersham Corp. (Arlington Heights, IL). The single amino acid mutations were made by creating the following nucleotide changes in the cytochrome f signal sequence (nucleotide 1 is the site of translation initiation [Buschien et al., 1991]): A12E, nucleotides 33–36 TAG; A15E, nucleotides 42–45 GGG; A15*, nucleotides 43–45 TAG; V16D, nucleotides 45–47 AGA; M20K, nucleotides 59–63 AGGGA; M20E, nucleotides 58–63 GAGCT. The hydrophobic core frame shift (HCFS) mutation was made by adding a T after nucleotide 29 and removing nucleotide 45, both in vitro mutagenesis. Each of the mutant genes was identified in E. coli using colony lift hybridization to mutant oligonucleotides (Sambrook et al., 1989) and sequenced using Sequenase (U.S. Biochemical Corp., Cleveland, OH) to verify the existence of the mutation.

The hydrophobic core deletion (HCA) mutation was made by creating two EcoRI sites through sequential in vitro mutagenesis of nucleotides 39–43 and nucleotides 61–66, and the KpnI fragment was then subcloned into pUC19ΔHHR, a plasmid that lacked both HindIII and EcoRI sites. This construct was digested with EcoRI to remove the hydrophobic core, and was then ligated together (ligase; Promega Corp.). HCA mutant was verified by sequencing using Sequenase (U.S. Biochemical Corp., Cleveland, OH).

The signal sequence deletion (SSΔ) mutation was made by creating a new HindIII site at nucleotides 11–18 and using this and an endogenous HindIII site at nucleotides 89–94 to remove the signal sequence coding region. The in-frame deletion was verified by sequencing.

Transformation of Chlamydomonas
CC-125, a wild-type mt strain (Harris, 1989), was obtained from the Chlamydomonas Genetics Center, Duke University. DNA from each mutant gene was transformed into CC-125 using the biolistic particle gun (PDS-1000/He; Bio-Rad Laboratories, Richmond, CA) and standard transformation protocol (Boynton and Gillham, 1993), creating strains A12E, A15E, A15*, V16D, and M20K, M20E, and HCFS. 2.5 μg DNA, prepared using CsCl gradients (Sambrook et al., 1989), was cotransformed with 2.5 μg pF183 DNA, a plasmid conferring spectinomycin, streptomycin, and erythromycin resistance (Newman et al., 1990). After transformation, cells were plated in low light (15 μmol photons/m2-s) on plates containing aceta, 100 μg/ml spectinomycin, 100 μg/ml streptomycin, and 50 μg/ml ampicillin (TAP-stamp plates). Ampicillin reduces bacterial contamination but has no effect on the growth of Chlamydomonas (Harris, 1989). The strain designated throughout this paper as wild type was CC-125 transformed with 5 μg pF183 and was selected on antibiotic plates as described.

Identification of Mutant Transformants by Southern Analysis
Spectinomycin/streptomycin-resistant colonies were selected on plates as described above. To determine if these colonies also contained mutant genes for cytochrome f, DNA was extracted using the rapid whole-cell DNA isolation procedure (Newman et al., 1990), digested with KpnI (Promega Corp.), and separated on a 1% agarose gel. The DNA was transferred to Nytran (Amerham Corp.) using a modification of standard protocol (Southern, 1975). The blot was hybridized for >8 h at 37°C in 1 M NaCl,
1% BSA, 1 mM EDTA, and 0.5 M NaPO₄, pH 7.2, 7% SDS. Oligonucleotides were labeled using T4 polynucleotide kinase and [γ-³²P]ATP (Amer- sham Corp.) following standard protocols (Sambrook et al., 1989). Blots were washed 2×10 min at 25°C in 1% SDS, 1 mM EDTA, 40 mM NaPO₄, pH 7.2, and 1 M NaCl, and were exposed to film (X-OMAT-AR; Eastman Kodak Co., Rochester, NY) to ensure hybridization occurred as expected. To detect hybridization to mutant DNA sequences while excluding hybridization to wild-type DNA sequences, blots were then washed at higher temperatures. This occurred at 80°C for A12E, A15E, A15* V16D, and HCFPS DNA and 83°C for M20K and M20E DNA.

To enrich for homoplasmicity, cells identified as having mutant DNA by the method described above were single-cell cloned on metronidazole. 1×1-cm patches of cells on TAP/pattem plates were scraped into 1-mL TAP liquid media (Harris, 1989), vortexed, and 100 μL was plated on TAP plates. The plates were left in low light (15 μmol photons/m²-s) for 2 d. We then added 500 μL TAP liquid, gently lifted the cells from the plate with a glass rod, and transferred the liquid to 20 mM metronidazole/TAP plates (Schmidt et al., 1977). Cells were left on metronidazole for 24 h in the high light (200 μmol photons/m²-s) before being transferred back to TAP plates in 500 μL TAP liquid as just described and placed in low light. Colonies that grew up (after ~5 d) were then subjected to the Southern analysis described above. Positive isolates of each mutant strain were maintained on TAP plates in low light.

**Determination of Homoplasmicity**

Homoplasmic strains (i.e., mutant cytochrome f genes in all cells) were identified by either Southern analysis of restricted total DNA or PCR products, or by a modification of Southern analysis: DNA was loaded without restriction enzyme digestion onto a dot blot manifold (sohleicher & Schuell, Inc., Keene, NH), a vacuum was applied, and the DNA was transferred to a nylon membrane for hybridization. Neutralizing solutions (Sambrook et al., 1989). Blots were probed with oligonucleotides that recognized either the wild-type or mutant sequence of each introduced mutation. Mutant oligonucleotides are described above; a wild-type oligonucleotide spanning nucleotides 118-153 was used for strains A12E, A15* V16D, and HCFPS. A wild-type oligonucleotide from nucleotides 133-168 was used for strains M20K and M20E. Labeling of oligonucleotides, hybridization, and washing of blots were done as described above.

**Analysis of Growth**

All media used to analyze growth were made according to the Chlamydo- monas Sourcebook (Harris, 1989). Cells of each genotype were patched from a master plate (TAP, low light, 15 μmol photons/m²-s) directly onto TAP or HS (Harris, 1989), or onto 20 mM metronidazole/TAP. TAP and HS plates were then placed in low light (15 μmol photons/m²-s) or high light (200 μmol photons/m²-s) for 4 d. When plated onto metronidazole, the following procedure was used. Thin slabs of TAP media were prepared by placing 8 ml of TAP agar into patti plates and adding sterile 7-cm circular filters (Whatman Chemical Separation Inc., Clifton, NJ). The slabs were transferred to TAP plates of normal thickness and cells of each genotype were patched onto the slabs. These plates were placed in low light (15 μmol photons/m²-s) for 2 d, after which the slabs were transferred to 20 mM metronidazole/TAP plates of normal thickness and placed in high light (200 μmol photons/m²-s) for 24 h. The slabs were then transferred to fresh TAP plates of normal thickness and returned to low light (15 μmol photons/m²-s) for 24 h.

**Northern Analysis**

RNA was extracted as described (Rochaix et al., 1988). 5 μg of RNA was run on a denaturing agarose gel and transferred to Nylon N+ (Amer sham Corp.). The blots were hybridized with the cytochrome f coding region, labeled with [α-³²P]dCTP (Amer sham Corp.) using the PrimeIt kit (Stratagene, La Jolla, CA), for ≥2 h at 65°C in 1% BSA, 1 mM EDTA, 0.5 M NaPO₄, pH 7.2, and 7% SDS. Blots were washed 2×10 min at 25°C in 1% SDS, 1 mM EDTA, and 40 mM NaPO₄, pH 7.2, and exposed to film (X-OMAT-AR; Eastman Kodak Corp.). The cytochrome f coding region was excised with EcoRI/KpnI from a newly constructed plasmid, MIR1. This plasmid was constructed by introducing an EcoRI site from nucleotides 90-95 (Buschlein et al., 1991) using site-directed mutagenesis. The mutant gene was detected in E. coli by digesting plasmid DNA with EcoRI, and the existence of the mutation was confirmed by sequencing using Sequence (U.S. Biochemical Corp.).

**Western Analysis**

Western samples were prepared by resuspending cells grown on TAP plates in 1 ml TE (10 mM Tris, pH 7.5, 1 mM EDTA). Cells were spun 3,000 g for 5 min and resuspended in 20 μl TE. Protein concentrations were determined by OD280, and the equivalent of 5 μg chlorophyll (Porra et al., 1989) was added to sample dye (4% SDS, 20% glycerol, 0.2 M DTT, 0.5 M Tris, pH 6.8, and bromophenol blue) and boiled for 5 min.

Protein samples were separated on a 15% acrylamide gel and transferred to nitrocellulose (Schleicher & Schuell Inc. [Towbin et al., 1979]). Immunoblotting was done in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100 (TBS). The filters were blocked in 5% evaporated milk in TBS for ≥30 min. Blots were incubated at the following dilutions for ≥1 h: α-cytochrome f (gift from Sabeena Merchant, University of California at Los Angeles), 1:2,000; α-DI (gift from John Boynton and Nick Gillham, Duke University), 1:2,000; α-LHCP, 1:5,000; α-OEC17 (gift from J-D. Rochaix, University of Geneva, Geneva, Switzerland), 1:10,000; α-OEC23 (gift from J-D. Rochaix), 1:1,000; α-OEC33 (gift from Steve Mayfield, The Scripps Research Institute, La Jolla, CA), 1:2,000; α-plastocyanin (gift from Sabeena Merchant), 1:1,000. Blots were then washed in TBS and incubated for ≥1 h with anti–horseradish peroxidase–conjugated secondary antiserum (1:8,000; Amersham Corp.), which was then detected by the enhanced chemiluminescence reagent system (Amersham Corp.).

**In Vivo Labeling and Immunoprecipitation of Cytochrome f in Chlamydomonas**

Cells of each genotype were grown in reduced sulfate TAP media and labeled with 750 μCi H2SO₄ (ICN Biomedicals, Inc., Costa Mesa, CA) as described (Howe and Merchant, 1992), with the following modifications. Acetone precipitated proteins were collected by centrifugation and dissolved in 0.2 ml sample buffer (2% SDS, 60 mM Tris-HCl, pH 8, 60 mM DTT, 1 mM EDTA, and 1 mM PMSF). One-tenth milliliter of this was diluted 10-fold with TBS (see above) and boiled for 5 min. 100 μL of formalin-fixed Staphylococcus aureus cells (GBCO BRL, Gaithersburg, MD) was added to TBS was added and incubated with constant mixing for 1 h at room temperature. Staphylococcus aureus cells were removed by centrifugation (16,000 g for 3 min). 10 μl of antiserum was added to each supernatant to precipitate α-cytochrome f-reactive polypeptides, and the mixture was reprecipitated for 1 h at 4°C. A-Sepharose CL-4B (Sigma Immunochemicals, St. Louis, MO) was added, and the mixture was incubated for 45 min at room temperature and centrifuged at 3,000 g for 5 min to collect the antibody–antigen complex. The precipitate was washed three times in 1 ml TBS. The final pellet was denatured in 30 μl sample buffer (see above) at 100°C for 5 min. Undissolved material was removed by centrifugation (16,000 g for 1 min), and the supernatant was analyzed by gel electrophoresis.

Cells were broken open immediately after labeling by resuspending in 200 μl of 5 mM NaPo4EDA, 10 mM Tris, pH 7.2, 2 mM PMSF, 2 μg/ml leupeptin, and 2 μg/ml E-64 (Sigma Immunochemicals) and sonicating for 2 min in a bath type sonicator (Ultraloe III Sonic Cleaner; Buehler Ltd.). Intact cells were removed by centrifugation (1,000 g for 1 min), and the supernatant was then spun at 100,000 g for 10 min at 4°C to create a soluble supernatant and a membrane pellet. Where indicated, NaHCO₃ was added to 2% wt/vol for 10 min at 4°C after sample sonication and before centrifugation.

**Generation of Suppressors**

Suppressors to A15E and V16D cells were generated with 0.27 M EMS as described (Harris, 1989). Mutagenized cells (5 × 10⁶) were plated on TAP under low light (15 μmol photons/m²-s) and on HS plates under high light (200 μmol photons/m²-s).

**Segregation Analysis**

Segregation analysis was done to determine if the mutant genes characterized by the suppressors are nuclear or chloroplast. All suppressors were isolated in a mt⁺ background. A15E mt⁺ was constructed by crossing A15Emt to cc124mt⁺. Mating type was determined with a pellicle test (Harris, 1989); the presence of the A15E mutation was determined both by the ability to grow on TAP/20 mM metronidazole and the presence of the A15E sequence by Southern analysis (see above). A15E suppressors were crossed to A15Emt. Tetrads were isolated and examined for their

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Cytochrome f Signal Sequence Mutations

C. reinhardtii cytochrome f is synthesized with 31-amino acid NH$_2$-terminal signal sequence (Bertsch and Malkin, 1991; Buschlen et al., 1991; Matsumoto et al., 1991), and it is then processed to a mature 32-kD protein. Amino acids A11 (alanine at amino acid position 11) to G19 of the cytochrome f signal sequence have the potential to form a hydrophobic core (Fig. 1). Using in vitro mutagenesis, we introduced six individual single point mutations, a deletion of most of the hydrophobic core, a deletion of most of the signal sequence, and two frame shift mutations that alter the coding sequence of only the hydrophobic core (Fig. 1). A12E (a change of alanine to glutamate at position 12), A15E (alanine to glutamate at position 15), V16D (valine to aspartate at position 16), M20K (methionine to lysine at position 20), and M20E (methionine to glutamate at position 20) were made because each of these mutations introduced a charge into or near the hydrophobic core and, by analogy to the function of the hydrophobic core in bacterial signal sequences (Biekler et al., 1990), thus may disrupt signal sequence function. A15* introduced a stop codon at position 15 in the signal sequence of cytochrome f and should, therefore, result in a cytochrome f null mutant. Each of these mutations created at least a 3-basepair change to reduce the frequency of reversions. The deletions of the hydrophobic core (hcA, deleting amino acids 14–20) and the signal sequence (ssA, deleting amino acids 5–31) were made to determine if these two structures are required. HCFS was made to destroy the hydrophobic nature of amino acids 11–19 without deleting them as it was theoretically possible that a deletion could destabilize the petA mRNA. In this mutant, the amino acids AATLAVIL were changed to RSNISCYF. The individual mutant petA genes were sequenced and transformed into wild-type Chlamydomonas.

Whole-cell DNA was isolated from transformed colonies obtained from single-cell cloning and Southern blots were performed using the mutagenic or wild-type oligonucleotides as probes (Fig. 2). We chose seven mutant strains where the probe specific for the introduced mutation hybridized only to the mutant Chlamydomonas DNA, while the probe specific for wild-type sequences hybridized only to wild-type DNA and did not hybridize to mutant Chlamydomonas DNA (Fig. 2). This demonstrated that there were no copies of wild-type Chlamydomonas DNA in each of the seven mutant strains. Cells containing only the cytochrome f mutants A12E, A15*, A15E, V16D, M20K, M20E, and HCFS were isolated in this way. A strain containing hcA was isolated only after transformants were selected on acetate and spectinomycin/streptomycin in the dark, and as these cells grow very slowly we have been unable to characterize them extensively. No transformants were recovered that contained ssA. Each of the recovered mutations was maintained through a series of genetic crosses (see below), providing further evidence that the genome contained only mutant copies of cytochrome f.
Signal Sequence Mutations Reduce Accumulation of Cytochrome f

Cytochrome f is a member of the photosynthetic electron transport chain, and mutations that block the synthesis or accumulation of cytochrome f therefore inhibit the ability of those cells to grow autotrophically. To examine this, wild-type strain cc125 transformed with the genes conferring spst* (referred to as wild type throughout the paper) and the seven strains carrying mutant petA genes were plated under low light (15 μmol photons/m²-s) on media containing (TAP) or lacking (HS) acetate as a carbon source (Fig. 3 A). All strains grew on acetate. Wild type, A12E, M20K, and M20E grew well on HS, while A15*, V17D, and HCFS did not grow at all. A15E grew well on acetate but had severely reduced growth on HS. These cells were also examined for their ability to grow on metronidazole, which is an electron acceptor downstream of cytochrome f, and the reduced form is toxic. Growth under low light on this drug would indicate a direct effect on electron transport. As expected, wild type, M20K, and M20E did not grow at all on metronidazole, while A15*, V16D, and HCFS grew well. A15E had intermediate growth on metronidazole. This indicated that the inability of A15E, A15*, V16D, and HCFS to grow on HS was caused by an alteration of electron transport, which is consistent with a block in the accumulation of cytochrome f.

When the steady-state level of cytochrome f was examined in all seven mutant strains, reduced amounts of cytochrome f were seen in only those strains that grew on metronidazole but not on HS (Fig. 3 B). Equal amounts of whole-cell protein from cultures grown on TAP in the low light were run on an acrylamide gel, transferred to nitrocellulose, and cytochrome f was detected with polyclonal antisera. As predicted from the growth characteristics, the null mutation A15* and V16D and HCFS lacked detectable cytochrome f. A15E had ~10% cytochrome f as compared to wild type, consistent with its low level of growth under autotrophic conditions and intermediate growth on metronidazole. A12E, M20K, and M20E had reduced but near normal levels of cytochrome f.

To determine if the reduction in cytochrome f levels was caused by changes in mRNA abundance, total RNA from each strain, grown under low light in TAP, was isolated and equal amounts were examined by Northern blot hybridization to the petA coding region (Fig. 3 B). Near-normal levels of message were seen in the mutant strains, indicating that the cytochrome f mutations did not affect mRNA accumulation. A15* had a reduced mRNA level; however, in this case, a full-length protein was not expected (higher molecular weight hybridization was caused by contaminating DNA).

Pleiotropic Effect of a Signal Sequence Mutation

We wondered whether the cytochrome f mutations had an influence on the accumulation of other proteins, and therefore, we examined by immunoblotting the steady-state levels of both chloroplast- and nuclear-encoded proteins found in the thylakoid membrane and the lumen. Fig. 4 A demonstrates that the A15* and V16D mutations do not appear to affect the accumulation of proteins other than cytochrome f. In A15E, the levels of both LHCP and D1, two proteins integral to the thylakoid, were reduced although the lumenal oxygen evolving complex protein OEC33 and PC were not affected. The changes in thylakoid proteins were not caused by a destabilizing effect of reduced cytochrome f because A15* and V16D lacked cytochrome f completely but had...
normal levels of LHCP and D1. These experiments were carried out both with independent isolates of the original A15E mutant strain and with the progeny of crosses between A15E strains (mt) and wild type (mt; data not shown), thus making it more likely that this effect was not caused by an additional mutation in our original A15E strain. It appeared, therefore, that A15E, which had reduced accumulation of cytochrome f, had a pleiotropic effect on both nuclear-(LHCP) and chloroplast-(D1) encoded integral membrane proteins but not those of the thylakoid lumen (PC and OEC33). The thylakoid proteins cytochrome f and LHCP in M20K accumulated to slightly lower levels than in wild type, and D1 accumulation was increased.

Alterations in levels of thylakoid proteins can lead to ultrastructural changes in the chloroplast (Somerville, 1986). We examined ultra-thin sections of each of the cytochrome f mutant strains by electron microscopy, and we were unable to detect any gross morphological alterations (data not shown).

The observed reduction of other integral membrane proteins in the A15E strain could have several causes. One possibility is that a low level of cytochrome f, rather than no cytochrome f, caused an increased turnover in D1. Increased D1 turnover is usually associated with photoinhibition (Greenberg et al., 1989; Kyle et al., 1984). *Chlamydomonas* strain FI8 carries an unidentified nuclear mutation (Lemaire et al., 1986) that results in a reduction in the steady-state level of cytochrome f similar to that found in A15E. The immunoblot shown in Fig. 4 B demonstrates, however, that D1 accumulated to normal levels in FI8 but not A15E. A second interpretation is that the cytochrome f signal sequence mutation is interfering with the translocation of integral thylakoid proteins, but not those of the lumen. To test this hypothesis, we needed to determine that cytochrome f translocation itself was affected.

**Signal Sequence Mutations Affect Protein Translocation**

If the signal sequence mutations were blocking protein translocation, then we expected to detect an accumulation of the precursor because processing occurs in the lumen of the thylakoid (Gray, 1992). To detect the precursor, we performed a pulse chase experiment. Cells of wild-type and the mutant strains A15E, V16D, and M20K were grown in media containing acetate and reduced sulfate, incubated with [35S]SO4, and then washed and incubated in rich media for 5 or 60 min. The chase was terminated by the addition of acetone to the cells. Cytochrome f was immunoprecipitated from a whole-cell extract and visualized by denaturing gel electrophoresis and autoradiography (Fig. 5 A). We detected only mature cytochrome f (m) in wild-type and M20K cells; precursor (pr) was never seen. This is also true for cells that were pulsed for shorter times, but had no chase (data not shown). The 35-kD precursor, however, was detected in both A15E and V16D cells. In A15E cells, the precursor was seen after a 5-min chase, but only a background smear was detected after a 1-h chase. Because the mature cytochrome f can be detected by immunoblotting in A15E cells (Fig. 4 A), the smear likely includes mature cytochrome f. In V16D cells, the precursor was seen after the 5-min chase, but it was degraded during a 1-h long chase, and no mature sized cytochrome f was seen. The levels of [35S]-labeled protein detected are likely not quantitative as varying levels of unlabeled endogenous mature protein in the different strains can influence the efficiency of immunoprecipitation with cytochrome f antiserum, which itself may not be saturating. In addition, the various strains appear to incorporate label into total protein to different degrees (data not shown), and we cannot exclude the possibility that A15E and V16D cytochrome f mRNA are less efficiently translated than wild type. These results do indicate that cytochrome f is indeed synthesized in each of the mutant strains examined, but the rate at which the precursor is processed to the mature form is reduced in A15E and V16D.

The reduced accumulation of mature cytochrome f in A15E and V16D is most likely caused by a defect in protein translocation. Translocation was slowed in A15E, while translocation was blocked altogether in V16D. If the signal sequence mutations are indeed blocking protein translocation, then we would expect not only precursor accumulation, but also an altered localization of mutant cytochrome f. Pulse-labeled cells were therefore broken open by sonication and separated into a soluble (S) or membrane (M) fraction. Samples were also treated with Na2HCO3 after sonication and then separated into a washed soluble (SW) and washed membrane (MW) fraction. Cytochrome f was immunoprecipitated and visualized by denaturing gel electrophoresis and autoradiography. Mutant strains are indicated above each lane by the type of amino acid substitution. pr, precursor; m, mature cytochrome f.
2% NaHCO₃, a chaotropic agent used to wash peripheral but not integral proteins from membranes, did not remove most of the wild-type mature cytochrome f from the thylakoid (Fig. 5 B, lane MW) although some was washed off into the supernatant (lane SW), and this has been observed before (Auchincloss et al., 1992). NaHCO₃, however, removed much but not all of the A15E precursor from the membrane fraction (Fig. 5 B, lanes SW vs MW). Thus some A15E precursor is stromal, some is peripheral to the membrane, and some may be integral to the thylakoid.

Suppressors of Signal Sequence Mutations

The initial characterization of the signal sequence mutant strains led the way for the isolation of suppressors of these mutations. While A15E cells grew slowly in the absence of acetate in low light, they are unable to grow autotrophically (i.e., on HS, without acetate) in high light (Fig. 6 A). No revertants capable of photosynthetic growth were seen when 10⁶ nonmutagenized cells were plated on HS. We mutagenized 10⁹ A15E cells with EMS and selected cells that were now able to grow on acetate minus media, thereby generating 130 putative A15E suppressor strains. The growth on HS and TAP of three of these strains carrying suppressors of A15E is shown in Fig. 6 A. Wild type, A15E, and the suppressors all grew on acetate, while only wild type and the suppressors grew on HS. The same strategy was used to isolate suppressors to V16D (Fig. 6 A), but only two suppressors were recovered from >10⁷ EMS-mutagenized cells. Each of the A15E and V16D suppressor strains shown in Fig. 6 A were then examined by immunoblotting for their steady-state level of cytochrome f and were found to have increased levels of cytochrome f relative to the original mutant strain (Fig. 6 B). The level of restoration varied between the A15E suppressors, and it was directly related to the ability of that strain to grow autotrophically. We have observed the same results for the other 127 A15E suppressor strains (data not shown). The amount of LHCP, D1 (both reduced in A15E), and OEC33 was also examined in the three A15E suppressor strains shown in Fig. 6 A, and it was found that the suppressors had near normal levels of each of these proteins (Fig. 6 B).

To determine whether the A15E and V16D suppressors were not revertants, we performed Southern analysis using the mutagenic oligonucleotides as probes against DNA isolated from the suppressor strains. The results for three A15E and two V16D suppressors are shown in Fig. 7. At nonstringent conditions (25°C), the mutant oligonucleotides hybridized to both wild-type and the appropriate suppressor strains. However, after a stringent wash (75°C), the mutant oligonucleotides remained hybridized to either the A15E or V16D suppressor strains but not to the wild type. Thus these strains, and all 20 that were tested, still carry the original cytochrome f signal peptide mutations. Genetic crosses between five of the A15E suppressor strains (all mt+) and an A15E mt- strain have shown that at least four of the A15E suppressors are nuclear mutations while one appears to be chloroplastic. Similar analysis with the V16D suppressors shows that their phenotypes are both caused by a chloroplast mutation.

The chloroplastic A15E and V16D suppressor mutations are not revertants and are caused by second-site mutations. We have sequenced the cytochrome f signal peptide of two V16D suppressors and found a leucine residue at position 10 instead of the wild-type arginine (mutation R10L) in addition to the V16D mutation; the chloroplastic A15E suppressor has a cysteine substitution at this position, as well as the original A15E mutation (Baillet, B., and B. D. Kohorn, unpublished results). The cis mutations help further define the hydrophobic core and the role of other regions within the signal sequence.

Figure 6. Suppressors of signal peptide mutations. (A) Growth of suppressors of signal peptide mutations. Wild-type (+) and mutant strains A15E and V16D were patched to growth media containing (TAP) or lacking (HS) acetate as a carbon source, along with three A15E and two V16D suppressor strains. All were grown under high light (200 μmol photons/m²-s). (B) Cytochrome f levels are restored by suppressor mutations. Total protein extracts were isolated from wild-type (+), A15E, V16D, and suppressor strains (A15ESUP1,67,71; V16DSUP1,2). 5 μg of each was run in a denaturing acrylamide gel, transferred to nitrocellulose, and probed with different antisera. Antisera are indicated to the right of each panel.

Figure 7. Mutant oligonucleotide hybridization to DNA from suppressor strains. Chlamydomonas DNA from wild-type (+), three A15E, and two V16D suppressor strains was digested with KpnI, transferred to a nylon membrane, and hybridized to the corresponding 32P-labeled mutant oligonucleotide. The hybridized filter was washed at nonstringent conditions (25°C) and autoradiographed, and then washed at a stringent temperature (75°C) and subjected to autoradiography again.


**Discussion**

**The Hydrophobic Core of the Cytochrome f Signal Sequence Is Required for Integration of Cytochrome f into Thylakoids**

We have demonstrated that mutations within the 31-amino acid signal sequence of *Chlamydomonas* cytochrome f can reduce or eliminate protein translocation. Single-point mutations A15E and V16D that introduced charged amino acids into the predicted hydrophobic core led to an accumulation of precursor cytochrome f and reduction in mature protein. A12E, M20K, and M20E, however, had little effect on photosynthetic growth or cytochrome f accumulation and suggest, therefore, that the hydrophobic core only extends from T13 to G19.

A stably transformed *Chlamydomonas* strain that contained a deletion of L14 to M20 (hcΔ) was only recovered when cells were kept in complete darkness, further emphasizing that these amino acids are important for cytochrome f accumulation. A mutation that eliminated the majority of the signal sequence (ssΔ) could never be recovered, suggesting that it was not stable. Cytochrome f itself is not required for *Chlamydomonas* growth, as evidenced by the isolation of the A15* strain. This indicates that the inability to retrieve the ssΔ strain was not caused by the absence of cytochrome f, but the causes for the apparent instability of this mutation are unclear.

Precursor cytochrome f having the A15 mutation accumulates in the stroma and on the surface of the thylakoid, and some may be within the membrane. This precursor is labile because it is only detected when several protease inhibitors are present during the sonication and fractionation, or if intact cells are immersed in acetone. V16D precursor appears to be more labile than A15E protein, as it is only found in acetone treated cells, and likely degrades during the sonication and fractionation steps. The different stabilities of A15E and V16D may reflect their different associations with chloroplast proteins, although other explanations are possible. Nevertheless, these mutations do appear to disrupt the correct translocation of cytochrome f. While the levels of mutant cytochrome f mRNA are near normal, we have not measured the rates of protein synthesis. However, possible reductions in the translation rates of mutant cytochrome f mRNA would be unlikely to result in the accumulation of precursors, especially ones that are no longer integral. Further evidence for a block in translocation comes from the observation that the signal sequence is removed on the luminal side of the thylakoid membrane and cytochrome f must therefore be translocated before it can be processed (Gray, 1992). It is possible that if the signal sequence was not removed and some protein was inserted, this cytochrome f would be labile. This is unlikely, however, because membrane-associated precursor cytochrome f in a plastome mutant in *Oenothera* accumulated to similar levels as mature cytochrome f in wild-type chloroplasts (Johnson et al., 1991). It is also unlikely that the signal sequence mutations we have introduced affect heme binding by the mature protein; even if they do, they would not affect cytochrome f accumulation as studies have shown that heme addition is not a prerequisite for maturation (Anderson and Gray, 1991b; Davies et al., 1990).

The signal sequence mutation A15E not only inhibits the insertion of cytochrome f, but it also decreases the accumulation of integral thylakoid membrane proteins LHC and D1 (Fig. 4A). The accumulation of PC and OEC17, 23, and 33, which are found in the lumen, are not affected (Fig. 4A). The pleiotropic effect seen in A15E on D1 and LHC is not caused by a reduced level of cytochrome f because both the total absence of the protein in A15* and its 80% reduction in F18 does not cause a corresponding reduction in D1 (Fig. 4B). Thus, it is likely that the signal sequence mutation is influencing the accumulation of D1 and LHC. If the reduction of D1 in A15E cells was also caused by a translocation defect, then we would expect to see an accumulation of the D1 precursor. This experiment is problematic in A15E cells because labeling and D1 antisera are inefficient. Preliminary experiments indicate that precursor D1 in A15E cells remains for <1 h in a pulse chase, while in wild-type cells precursor D1 is matured within 10 min (data not shown). Thus, the cytochrome f A15E mutation may interfere directly with the translocation of D1.

One would also expect that a mutant cytochrome f protein would interfere with the translocation of wild-type cytochrome f. Indeed, strains containing both wild-type and A15E mutant cytochrome f signal sequences on different copies of the genome, a heteroplasmic strain, show a 90% reduction in cytochrome f (data not shown), indicating that the A15E mutation is also dominant on wild-type cytochrome f.

A15E has a pleiotropic affect on the accumulation of D1 and LHC, but not PC and OEC33, and one possible explanation is that integral thylakoid proteins and lumenal proteins use distinct, perhaps overlapping, translocation apparatus. Indeed, there is recent in vitro evidence that supports this conclusion (Cline et al., 1993). A15E precursor is found both in the stroma and peripherally associated with the thylakoid, and it is unclear at which step the mutation is interfering with the translocation apparatus. Identification of the genes described by the suppressor mutations should clarify the components and mechanism of the translocation machinery.

Some variability in the reduction of D1 and LHC in the cytochrome f mutant strains is seen. D1 and LHC are always reduced in strain A15E, but the level of reduction is variable between experiments. This may be caused by changing levels of D1, LHC, and cytochrome f synthesis, which fluctuate during the cell cycle and therefore differentially compete for the translocation apparatus. This model can be tested once the apparatus is identified. At present, we have no explanation for the increased levels of D1 seen in strain M20K (Fig. 4A), which is also variable, but we do note that M20K has slightly reduced cytochrome f levels as compared to wild type (Fig. 4A).

Our results suggest that cytochrome f, which has an NH2-terminal signal sequence, and D1, which does not, share a common insertion mechanism. Although the majority of chloroplast-encoded proteins reside in the thylakoid (Jagendorf and Michaelis, 1990; Simpson and Von Wettstein, 1989), only three have potential NH2-terminal signal sequences (cry f; atpI [Cozens et al., 1986]; psbK [Murata et al., 1988]). It is unclear, therefore, why some thylakoid proteins have NH2-terminal signal sequences and most do not. It is possible that the signal sequence is required for the
translocation of any large hydrophilic lumenal domain across thylakoids, but the fact that mutations in cytochrome f do not inhibit PC or OEC33 (lumenal proteins that have hydrophobic NH2-terminal signal sequences similar to that of cytochrome f) suggests that this is not a requirement for one general pathway. Alternatively, the NH2-terminal signal sequence may be required for translocation across the membrane for both cytochrome f and PC, but there is separate machinery for lumenal and thylakoid proteins. It is possible to test this experimentally by making mutations within the hydrophobic core of PC and OEC33 signal sequences and determining if these mutants are affected by A15E suppressor strains.

Suppressor Mutations

Suppressors to A15E and V16D restore steady state cytochrome f to wild-type levels (Fig. 6 B). Four A15E suppressors have been identified as mutations in nuclear genes and one has been identified in a chloroplast gene. Both V16D suppressors are chloroplastic. It is possible that the nuclear suppressors may be mutations that increase petA RNA synthesis to compensate for low levels of protein translocation. An increase in cytochrome f synthesis in the presence of a translocation defect might result in the accumulation of precursor detectable by immunoblots. Of the 10 A15E suppressor strains examined, all have near normal levels of cytochrome f mRNA (Baillet, B., and B. D. Kohorn, unpublished results). Immunoblots do detect a precursor-sized cytochrome f in A15E suppressor 67 (Fig. 6 B), suggesting that this mutant might have increased cytochrome f synthesis. Precursor-sized cytochrome f is not detected in the other suppressors strains, and these suppressors are likely to lie in genes whose products directly influence protein translocation. This must be verified by pulse-chase analysis and the eventual isolation of the suppressor genes. The suppressors are also more likely to reside in the translocation apparatus and not in regulation of expression as one might expect increases in the levels of all other chloroplast-encoded mRNA translation in addition to cytochrome f mRNA. Although we see an increase in DI accumulation relative to that strain A15E (Fig. 6 B), all other chloroplast-encoded proteins appear to accumulate to wild-type levels (data not shown). It is likely, therefore, that some of the nuclear suppressors are mutations within components of the thylakoid insertion machinery. The nuclear suppressors are being mapped, and the genes can be cloned by complementation using cosmid libraries made from the A15E suppressor strains.

In conclusion, we have shown that the NH2-terminal sequence is required for the correct insertion of cytochrome f into thylakoids. A signal sequence mutation that blocks the stable insertion of cytochrome f also inhibits the accumulation of two other integral thylakoid proteins, but does not inhibit the accumulation of lumenal proteins, suggesting that there are at least two pathways for translocation across the thylakoid. Lastly, we have identified nuclear suppressors to a cytochrome f signal sequence mutation that restore the accumulation of cytochrome f to normal levels, and it is hoped that by characterizing these nuclear genes, we will begin to identify the components and mechanisms of thylakoid protein insertion machinery.

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