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Cloning and characterization of the nuclear AC115 gene of *Chlamydomonas reinhardtii*

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Abstract

The nuclear *ac115* mutant of *Chlamydomonas reinhardtii* is specifically blocked in the synthesis of the chloroplast encoded D2 protein of the photosystem II reaction center at a point after translation initiation. Here, we report the identification of the AC115 gene through complementation rescue of the *ac115* mutant strain, using an indexed cosmid library of *Chlamydomonas* genomic DNA. AC115 is a small, novel, intronless nuclear gene which encodes a protein of 113 amino acids. The amino terminal end of the Ac115 protein is rich in basic amino acids and has features which resemble a chloroplast transit sequence. A hydrophobic stretch of amino acids at the protein's carboxyl terminus is sufficiently large to be a membrane spanning or a protein/protein interaction domain. Various models are discussed to account for the mechanism by which Ac115p works in D2 synthesis. The *ac115* mutant allele was sequenced and determined to be an A-to-T transversion at the first position of the fourth codon of the coding sequence. This mutation changes an AAG codon to a TAG nonsense codon and results in a null phenotype.

Introduction

The biogenesis of the chloroplast requires products of genes of the organellar chromosome which are synthesized within the organelle, together with products of genes encoded by the nucleus, synthesized in the cytoplasm, and transported to their correct location within the organelle (see for review [13]). The chloroplast genome encodes about 100 of its own proteins. The synthesis of these gene products requires the products of nuclear genes, some of which act generally at all major stages of gene expression, others of which behave in a gene-specific fashion (see for review [13]). This latter class functions with a surprisingly high degree of specificity. A single chloroplast gene product may require the products of many nuclear genes for its expression. An extreme example is the chloroplast encoded photosystem I protein gene *psaA* of *Chlamydomonas reinhardtii*. The processing of this transcript is unusually complex in that the mature mRNA is built of three exons which are spliced together in *trans* [4]. This splicing requires the products of a total of at least 14 nuclear genes and one chloroplast gene [11, 12].

We are interested in the expression of the chloroplast gene *psbD* which encodes the photosystem II (PSII) reaction center protein D2 [8]. The D2 protein of *Chlamydomonas* is a *trans*-thylakoid membrane protein which has five membrane-spanning α helices [9]. Specific amino acids of the D2 protein have been shown to bind molecules essential for PS II reaction center function including chlorophyll, pheophytin, plastoquinone A, and a non-heme iron [32]. D2, together with a related protein D1, the apoproteins of cytochrome b_{559}, and several associated cofactors, are responsible for the initial photosynthetic electron transfer reactions and the generation of a charge separation across the thylakoid membrane [34]. Photosystem II is also comprised of a light-harvesting complex, an oxygen-evolving complex which provides a source of electrons from water, reaction center 'core antenna'

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AF045466 (AC115 genomic sequence) and AF045467 (AC115 cDNA sequence).

proteins CP45 and CP47, and a host of low-molecular-weight (<10 kDa) proteins, most of whose functions are unknown [1, 7].

The D2 protein is synthesized by chloroplast ribosomes which are bound to thylakoid membranes, and is believed to be co-translationally inserted into the membrane [18]. The machinery responsible for this co-translational insertion has not yet been identified. The synthesis of the D2 protein requires the products of at least three nuclear genes (NAC1, AC115, and NAC2). The NAC2 gene encodes a product which is required for the stabilization of *psbD* mRNA [27]. Mutation of the NAC2 gene results in the complete absence of *psbD* mRNA [27]. To confer stability, the product of NAC2 is believed to bind to sequences within the 5′-untranslated region of the *psbD* transcript [35]. The NAC1 and AC115 gene products are required for the synthesis of the D2 protein at a stage after translation initiation [44]. Mutations in these nuclear genes result in the complete absence of D2, despite the fact that *psbD* mRNA is present and is associated with polyribosomes in mutant cell chloroplasts (Helen Wu, unpublished results). The absence of the D2 protein in all of these mutant strains results in a non-photosynthetic, acetate-requiring phenotype.

Chlamydomonas is well known as an excellent model organism in which to study the genetics of photosynthesis. Recent advances in the field include the development of straightforward means of introducing DNA into both the nuclear and chloroplast genomes [3, 24, 25], and the availability of genomic, cDNA, and YAC libraries of *Chamydomonas* DNA [20, 38, 46]. Zhang *et al.* [46] have constructed an indexed cosmid library of *Chlamydomonas* genomic DNA. This library consists of 11 280 individually maintained clones stored in the wells of 120 microtiter plates. An ordered series of mutant cell transformations can be performed using pools of DNA isolated from the clones of each plate of the library, followed by pools of DNA from the clones stored in the rows and columns of complementation-positive plates, to identify specific cosmids which carry genes that rescue mutant phenotypes [46]. Here, we report the isolation of the AC115 gene of *Chlamydomonas* from the indexed cosmid library. The gene encodes a 113 amino acid polypeptide which has a putative chloroplast transit sequence at its amino terminal end. The carboxyl terminus of the protein is rich in hydrophobic amino acids and may be a transmembrane domain or a region involved in protein/protein interaction. Ac115p may function within the thylakoid membrane to stabilize the D2 protein and/or its translation intermediates, or it may serve to localize the *psbD* message and/or the nascent D2 protein to the thylakoids.

Materials and methods

Chlamydomonas reinhardtii *cell culture conditions*

Chlamydomonas reinhardtii cells were grown under constant light in tris acetate phosphate (TAP) medium [15]. HSM minimal medium was used for selection of photoautotrophic transformants [16]. For DNA extraction and transformation experiments, cells were harvested for experimentation at a cell concentration of ca. $1–2 \times 10^6$ cells/ml.

Isolation of cosmid DNA from the indexed library of Chlamydomonas reinhardtii *DNA*

Bacterial clones from each microtiter plate were grown overnight at 37 °C in a Bioblock device (a device with 96 2-ml wells arranged in an array identical in spacing to the wells of a 96-well microtiter plate). After 18 h of growth, cosmid DNA from the pooled cultures was extracted with pZ523 spin columns ($5' \rightarrow 3'$ Inc.). The average amount of cosmid DNA isolated by this method was ca. 500 μg.

For 'cross-hair' transformation experiments, bacterial clones of a microtiter plate were inoculated into 6 Bioblock devices and grown overnight at 37 °C. Bacterial cells were pooled from each of the eight rows (A–H) and from each of the twelve columns (1–12) of the microtiter plates. Cosmid DNA from each pool was isolated by using pZ523 spin columns ($5' \rightarrow 3'$ Inc.)

Transformation experiments

Ac115;cw15 mutant cells were grown in TAP medium to 2×10^6 cells/ml. The *cw15* mutation produces cells with an abnormal cell wall [19]. This phenotype is required for the introduction of DNA into cells by the transformation method described below [24]. Mutant cells were concentrated to 2×10^8 cells/ml in HSM minimal medium [16] and were shaken under continuous light at room temperature for 1–2 h before transformation.

For the transformation of *Chlamydomonas* cells with cosmid DNA pools, the procedure employed was that described by Zhang *et al.* [46] with some modification. Transformations were performed in 50 ml

conical centrifuge tubes. For each transformation, 20 of pooled cosmid DNA and 0.6 g of acid-washed glass beads were added to a tube containing 1.2 ml of 2×10^8 mutant cells. The cells were vortexed at high speed for 15 s. After vortexing, 30 ml of minimal medium was added, the cells were spun down by centrifugation (2500 rpm for 5 min), resuspended in 1 ml of minimal medium, and plated onto the surface of 2% agar minimal medium containing plates. The plates were incubated under continuous light at 25 °C. Prototrophic colonies appeared in two to three weeks. For the transformation of *Chlamydomonas* cells with a single cosmid clone or with plasmid DNA, the procedure employed was that described by Kindle [24]. To identify a subclone of a cosmid insert which could rescue the *ac115;cw15* mutant, cosmid DNA was digested with various restriction enzymes and 2 μg of each of the restricted DNAs were used for transformation of the *ac115;cw15* mutant.

Table 1. Number of transformants from transformations of the *ac115* mutant.

DNA	Number of transformants[a]
cosmid DNA (plate 40)	32 ± 3
cosmid DNA (row E of plate 40)	62 ± 9
cosmid DNA (column 1 of plate 40)	124 ± 11
cosmid DNA (clone 40E1)	50 ± 9
*Ava*I-digested #40E1 cosmid DNA	1 ± 1
*Eco*RI-digested #40E1 cosmid DNA	0
*Pst*I-digested #40E1 cosmid DNA	84 ± 8
*Pvu*II-digested #40E1 cosmid DNA	51 ± 8
0.9 kb *Pst*I fragment of clone 40E1 in pGEM-4Z	97 ± 8
4 kb *Pst*I fragment of clone 40E1 in pGEM-4Z	0
cDNA clone #C5	67 ± 7
negative control[b]	< 1[c]

[a]Number of *ac115;cw15*-derived colonies growing on minimal medium containing plates after transformation. Values are averages ±SD. [b]Mock transformations of the *ac115;cw15* mutant with no DNA. [c]Value calculated from negative controls of all transformation experiments.

DNA extraction, electrophoresis and Southern analysis

Genomic DNA was extracted from log-phase *C. reinhardtii* cells by the SDS/proteinase K miniprep protocol [39]. For Southerns, equal amounts of *Bam*HI-digested DNA were electrophoretically size-fractionated on 1% agarose gels and transferred onto nylon membranes (Duralon, Stratagene) by capillary blotting. DNA was fixed to the nylon membrane by UV light crosslinking using a Stratagene 1800 Stratalinker. Prehybridization, hybridization, and autoradiography procedures were done as described in Kuchka *et al.* [26].

Chlamydomonas reinhardtii *cDNA library screening*

The *C. reinhardtii* cDNA library in λ NM1149 was kindly provided by Dr M. Goldschmidt-Clermont. The *Escherichia coli* host used for the transfection was strain BHB2600. The library was screened with a ^{32}P-labeled probe by standard methods [31]. The probe used in this experiment was the 0.9 kb *Pst*I fragment of the cosmid clone of *Chlamydomonas* genomic DNA that rescues the *ac115;cw15* mutant. cDNA clones were purified by standard methods [31].

Cloning of the ac115 *mutant allele*

The *ac115* mutant allele was cloned by polymerase chain reaction amplification of *ac115* mutant cell DNA using as primers 21-mer sequences which correspond to positions 50 nucleotides upstream of the start of the cDNA sequence and 350 nucleotides downstream of the putative translation termination signal (see Figure 1). Thermal cycling conditions for amplification were: 1 cycle (4 min at 94 °C); 35 cycles (45 s at 94 °C, 1 min at 55 °C, and 2 min at 72 °C); 1 cycle (10 min at 72 °C).

DNA sequencing

The genomic and cDNA clones were sequenced by Dr Jikang Fang, Veterinary School, University of Pennsylvania, Philadelphia (ABI373A Automatic DNA Sequencer, Dye-terminator cycle sequencing).

Results

Identification of cosmid clones of an indexed library of Chlamydomonas reinhardtii *genomic DNA that complement the* ac115 *mutant*

To identify a clone of the indexed library which rescues the *ac115* mutant, 'glass bead' transformations of the *ac115;cw15* mutant were conducted with pools of cosmid DNA isolated from the bacterial clones stored in each of the 120 microtiter plates. Putative transformants were selected for photoautotrophic growth on minimal medium containing plates. Transformations

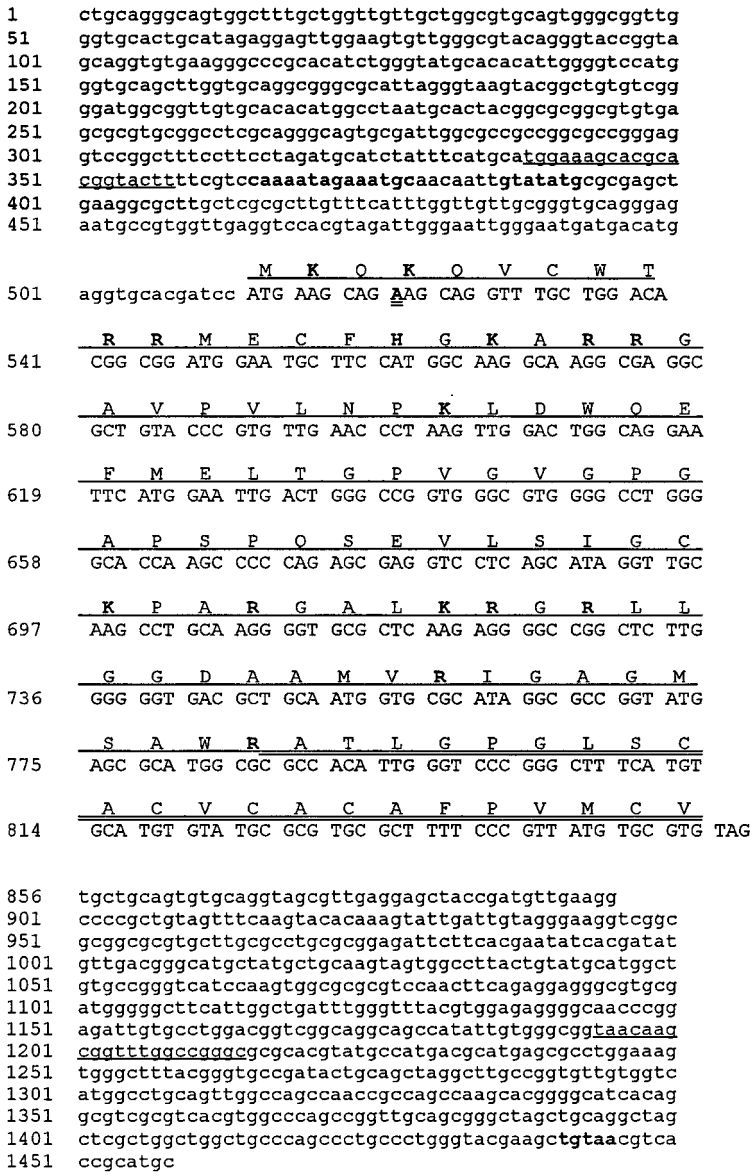

Figure 1. The nucleotide sequence of the *Chlamydomonas reinhardtii* AC115 gene and flanking sequences. Genomic DNA sequenced extends from nucleotide position 1 to 1214. The 0.9 kb *Pst*I fragment of cosmid clone #40E1 extends from nucleotide position 1 to 863. The cDNA sequence includes nucleotides 411 through 1458. Lower-case letters refer to genomic sequences flanking AC115. The CAAT and TATA sequences (positions 365–377 and 387–392, respectively) and the putative polyadenylation signal (positions 1441–1445) are bold-faced. Sequences corresponding to those used as primers to amplify genomic DNA from the *ac115* mutant are underlined (positions 338–358 and 1194–1214). The AC115 open reading frame is shown in upper-case letters. The single letter abbreviations for amino acids is used. 'Z' denotes a stop codon. Basic amino acids are shown in bold-face type. A hydrophobic stretch of amino acids at the carboxyl end of the protein is double-underlined. The cysteine repeat extends from amino acid 100 to 107. The site of the *ac115* mutation (nucleotide position 523) is bold-faced and underlined.

of *ac115;cw15* mutant cells with cosmid DNA pools from plates #40, #68, #77, and #81 produced numbers of prototrophic colonies appreciably above that of the negative control (a mock transformation of the *ac115;cw15* mutant with no cosmid DNA) (Table 1). The reversion frequency of mutant *ac115* is very low ($<10^{-8}$). Negative control plates typically had one or no colonies growing on them. To target a specific cosmid clone which rescues the *ac115* mutant, 'cross-hair' transformation experiments were performed with DNA pools isolated from the clones stored in the rows and columns of one plate (#40). The results showed that cosmid DNA pools from the clones in row E and in column 1 of microtiter plate #40 were able to complement the *ac115* mutant. Cosmid DNA from the 12 clones in row E produced an average of 62 ± 9 colonies on minimal plates, while the cosmid DNA from the 8 clones in column 1 produced an average of 124 ± 11 acetate-independent colonies. Therefore, the cosmid clone in well E1 of microtiter plate #40 was expected to contain a gene that complements the acetate requiring phenotype of the *ac115* mutant. To confirm that the DNA in well E1 of microtiter plate #40 complements the *ac115* mutant, cosmid DNA was isolated from this single bacterial clone and used for transformations. In a total of five experiments, transformations of *ac115;cw15* with #40E1 DNA yielded an average of 50 ± 9 colonies on selective medium.

Identification of a subclone of cosmid #40E1 that complements the ac115 *mutant*

The approximate size of the #40E1 cosmid clone is 40 kb (data not shown). To identify a subclone of #40E1 which could complement *ac115*, the cosmid was digested with four restriction enzymes (*Ava*I, *Eco*RI, *Pst*I, and *Pvu*II) and the digested DNAs were used to transform the *ac115* mutant. The results showed that *Pst*I-digested and *Pvu*II-digested #40E1 DNA produced an average of 84 ± 8 and 51 ± 8 putative transformants, respectively, while *Ava*I-digested and *Eco*RI-digested #40E1 cosmid clones produced 1 or 0 colonies, respectively, on minimal plates (Table 1). These results suggest that *Pst*I and *Pvu*II cut outside the complementing sequence while *Ava*I and *Eco*RI cut,within this sequence and abolish complementation. The sequence of interest was expected to be within, for example, a *Pst*I fragment which carries an *Eco*RI site. The #40E1 cosmid clone was digested with these two enzymes individually and with both enzymes together, and the restriction digestion products were separated on a 1% agarose gel. The results showed that there were two PstI fragments (4 kb and 0.9 kb in size) that were digested by *Eco*RI (data not shown). One of these two *Pst*I fragments was expected to contain a sequence that complements the *ac115* mutant.

The 4 kb and 0.9 kb *Pst*I fragments were cloned into the vector pGEM-4Z and these clones were tested for their ability to rescue the *ac115* mutant. Only the plasmid carrying the 0.9 kb *Pst*I fragment could complement the acetate requiring phenotype of *ac115* (Table 1). Since this 0.9 kb *Pst*I fragment was expected to contain the sequence that rescues the *ac115* mutant, it was sequenced from the SP6 and T7 promoter sequences which flank it in the pGem-4Z vector (Figure 1).

Screening of a Chlamydomonas reinhardtii *cDNA library with the 0.9 kb* Pst*I restriction fragment of clone #40E1*

A *C. reinhardtii* cDNA library was screened using the 0.9 kb *Pst*I restriction fragment of cosmid clone #40E1 as a probe. About 1.5×10^6 plaques were screened and five positive clones identified. One clone (C#5) was tested for its ability to complement the *ac115* mutant. Transformation with this DNA produced an average of 67 ± 7 putative transformants. The size of the *C. reinhardtii* cDNA insert in this lambda clone is ca. 1.1 kb (data not shown). This was determined by polymerase chain reaction (PCR) amplification of the cDNA sequence, using as primers the *Eco*RI/*Not*I adapter sequences which flank the cDNA in the lambda vector. The *C. reinhardtii* cDNA was sequenced from these primer sequences (Figure 1). The cDNA sequence overlaps with the sequence of the 0.9 kb *Pst*I fragment of the genomic clone in a region of ca. 450 bp (Figure 1). Northern blots of total RNA isolated from wild-type and *ac115* mutant cells, probed with radiolabeled 0.9 kb *Pst*I subclone of cosmid #40E1 revealed a single message of ca. 1.2 kb in size (data not shown). The size of this message is consistent with the size of the cDNA sequence.

Cloning and determination of the ac115 *mutant sequence*

There are two possible ways by which the sequence within the 0.9 kb *Pst*I fragment of cosmid #40E1 could rescue the *ac115* mutant. This sequence could carry the wild-type AC115 gene or it could encode a product which suppresses the *ac115* mutant phenotype.

Therefore, it was of interest to clone from the *ac115* mutant strain the sequence corresponding to those isolated from the genomic and cDNA libraries which complement *ac115*, and to determine if there were differences between the wild-type and mutant sequences. The *ac115* mutant sequence was cloned by PCR using *ac115* genomic DNA as a template. The two primers used in this experiment flank the complementing sequences on the genomic and cDNA clones. One primer corresponds to a region of genomic sequence situated 50 nucleotides upstream of the start of the cDNA sequence, while the other is complementary to a region of the cDNA 350 nucleotides downstream of the putative translation termination signal (see Figure 1).

The product of the PCR amplification is 876 bp in size (data not shown). This DNA was sequenced using as primers the same sequences that were used for the PCR. A comparison between wild-type and mutant sequences revealed a single nucleotide substitution from A to T at the position corresponding to the first base of the fourth codon of the largest open reading frame. This point mutation changes the fourth codon from AAG to an amber (TAG) stop codon. The rest of the mutant sequence is exactly the same as the genomic and cDNA sequences (Figure 1). These results strongly suggest that the sequence within the complementing cosmid and cDNA clones is AC115.

Southern blotting analysis of genomic DNA of putative transformants

To determine if *ac115;cw15*-derived colonies able to grow on minimal medium containing plates after transformation were true transformants, and to determine the number of integrated copies of the transformed sequence, Southern blotting analysis of genomic DNA of these cells was carried out, using the radiolabeled 0.9 kb *Pst*I restriction fragment as a probe. Ten clones which grew on minimal medium after transformation were randomly selected for this analysis (designated as T1–T10). The #40E1 cosmid clone, genomic DNA from the *ac115* mutant, and genomic DNA from these ten randomly selected clones were digested with the *Bam*HI, electrophoretically separated on a 1% agarose gel, and transferred to a nylon membrane. The membrane was probed with the radiolabeled 0.9 kb *Pst*I subclone of #40E1. The autoradiogram of this Southern blot shows a 6 kb *Bam*HI fragment in all samples, and one or two additional *Bam*HI fragments in 7 of the 10 clones (T1, T2, T4, T5, T6, T8, and T10) (Figure 2). The size of these extra *Bam*HI fragments is different in each clone, demonstrating that they are independent isolates and that they result from the nonhomologous integration into the nuclear genome of sequences within the 0.9 kb *Pst*I fragment. The other three clones may be revertants or homologous recombinants because these cells grow on minimal medium but carry only one copy of the 0.9 kb hybridizing sequence.

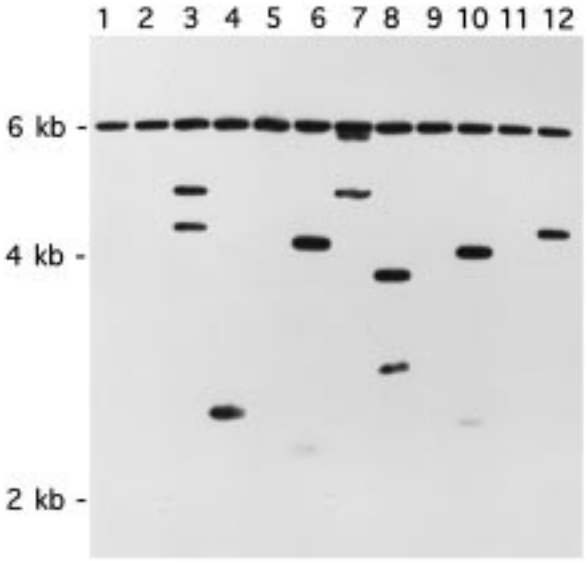

Figure 2. Southern blot analysis of putative transformants. The #40E1 cosmid DNA clone, *ac115* genomic DNA and genomic DNA from 10 putative transformants were digested with *Bam*HI size-fractionated on a 1% agarose gel, blotted onto a nylon membrane and hybridized with nicked-translated 0.9 kb *Pst*I fragment of cosmid clone #40E1. Lane 1, #40E1 cosmid DNA; lane 2, *ac115* mutant; lanes 3–12, putative transformants rescued by plasmid carrying 0.9 kb *Pst*I DNA fragment).

Discussion

The first description of the *ac115* mutant was reported in 1961 [28]. The strain was originally characterized as a nuclear mutant defective in photosynthesis and deficient in cytochrome b_{559} [14, 28, 41]. The *ac115* mutation was mapped to the right arm of chromosome I [29]. Years later, chloroplast gene expression in this strain was studied, and it was determined that the *ac115* mutation causes a specific block in the synthesis of the chloroplast-encoded D2 protein of PSII [26]. The *psbD* message encoding D2 is present in *ac115* mutant cells, but no D2 protein is made, even in very short pulse-labeling experiments [26, 44]. A pleiotropic effect of this mutation is the instability of

the entire PSII core. Because D2 is not present, all other major proteins of the PSII core are absent or greatly reduced [26]. Proteins of the oxygen evolving and PSII light-harvesting complexes, however, are unaffected in their accumulation and are present at normal levels in *ac115* cells [26]. It has been determined that despite the complete lack of D2 protein in *ac115*, the message encoding this protein is present on polyribosomes (Helen Wu, unpublished results). For this reason, it is expected that the AC115 gene product functions in D2 synthesis after translation initiation, and it is hypothesized that this nuclear gene product may work to localize the *psbD* message and/or the nascent D2 protein to thylakoids, or to stabilize D2 and/or its translation intermediates within these membranes. To help determine the function of the AC115 gene product, the gene was cloned by complementation and sequenced. A cosmid clone of *Chlamydomonas* genomic DNA and a *Chlamydomonas* cDNA clone have been isolated, both of which rescue the acetate-requiring phenotype of *ac115*. The 0.9 kb subclone of the genomic clone and the cDNA clone overlap in an area of about 450 bp. An open reading frame within this area of overlap encodes a small protein of 113 amino acids. This open reading frame sequence has within it restriction sites for *Eco*RI (Figure 1, position 517) and *Ava*I (Figure 1, position 799), both of which have been shown to abolish the ability of the genomic clone to rescue *ac115*. The open reading frame is flanked by the expected regulatory sequences, including TATA and CAAT boxes at its 5′ end, and a polyadenylation site at its 3′ end. The TATA box (TTGTATAT), is positioned ca. 20 nucleotides upstream of the start of the cDNA sequence. A CAAT box sequence (CAAAATAGAAA) is found 12 nucleotides upstream of the TATA sequence. This CAAT box is very similar to the CAAT box consensus sequence (CAAAATNGAAAA) found in plant genes [17]. A long 3′-untranslated region (635 nucleotides, including a 32 nucleotide long poly(A) tail) is present on the cDNA clone, downstream of the translation stop site. A conventional polyadenylation signal (TGTAA), is located on the cDNA sequence 13 nucleotides upstream of the polyA tail (positions 1441-1445). This same polyadenylation sequence is found in several other *Chlamydomonas* genes [10, 33, 40, 45]. Several additional small open reading frames are present on the cDNA sequence, downstream of AC115. The largest encodes a 78 amino acid polypeptide with no homology to any known protein. Since this sequence is not within the 0.9 kb *Pst*I fragment of the genomic clone which rescues *ac115*, and since this sequence is unchanged in the *ac115* mutant, we do not believe that it is AC115. The 1.2 kb message which hybridizes with AC115 on northern blots is large enough to include this sequence. However, at present it is not clear if it is at all relevant to this system.

The *ac115* mutant sequence was isolated by PCR amplification of mutant cell DNA. The PCR product is 876 base pairs in size and includes sequences 50 nucleotides upstream of the first nucleotide of the cDNA and 350 nucleotides downstream of the AC115 translation termination codon. Within this 876 bp region, the single difference between the *ac115* mutant sequence and the wild-type sequences of the cDNA and genomic clones is an A-to-T transversion of the tenth nucleotide of the open reading frame. This corresponds to the first position of the fourth codon, and changes the sequence of this codon from AAG to TAG (amber). This nonsense mutation would block AC115 expression and result in a null phenotype.

The exact correspondence between the genomic and cDNA sequences demonstrates that AC115 is an intronless nuclear gene. To our knowledge, this is the first report of an intronless nuclear gene in *Chlamydomonas*. All other *Chlamydomonas* nuclear genes which encode chloroplast proteins have one or more introns with conventional splice sites [16]. Some nuclear genes which encode organellar proteins of higher plants are hypothesized to be derived from organellar genes which were transferred to the nucleus through evolution [37, 43]. Some of these nuclear gene sequences presently resemble cDNA copies of processed and edited organellar RNAs [6, 36]. It is possible that the AC115 gene evolved in such a manner. The AC115 gene of *Chlamydomonas* may be a cDNA copy of a former chloroplast-encoded RNA, or a single exon of a chloroplast mRNA, which was transferred from the chloroplast to the nucleus early in evolution. Integration of this sequence into the nuclear genome downstream of the appropriate control elements, and adjacent to a sequence which encodes a chloroplast transit signal, would be sufficient for expression and localization of the gene product to the organelle.

The longest open reading frame of the cDNA clone, the only open reading frame present in the area of overlap between the cDNA and genomic clones, and the open reading frame which carries a mutation in the *ac115* mutant, encodes a protein of 113 amino acids. The amino terminal portion of the protein is rich in basic amino acids residues (9 out of the first 30 amino acids) which is characteristic of

chloroplast transit signals [5]. The N-terminus of this protein also contains a loosely conserved sequence for transport to the thylakoid lumen compartment (amino acids 20–30). This conserved sequence consists of a stretch of hydrophobic amino acids flanked by two arginines at the N-terminal end and a basic amino acid at the C-terminal end [5]. The carboxy-terminal end of the polypeptide is rich in hydrophobic amino acids (amino acids 92–113). This region is large enough to be a membrane spanning domain. The Ac115 protein may be a single-pass *trans*-thylakoid membrane protein which is oriented with its amino terminal end in the thylakoid lumen and its carboxy terminal 21 amino acids inserted into the membrane. Its small size and its potential association with the thylakoid membrane are reminiscent of the low-molecular-weight PSII proteins [7, 21, 22, 30]. Many such low-molecular-weight PSII proteins have been identified and characterized (*psb0*–*psbX*). Several of these have been shown to be single pass *trans*-thylakoid membrane proteins [2, 7, 21, 30] and at least one is known to interact physically with D1 and D2 [21]. Alternatively, the hydrophobic region of Ac115p may be a site of protein/protein interaction. Within this hydrophobic region, at the extreme carboxy terminal end of the protein, is a motif of repeated cysteine residues (C_{100}-A-C-V-C-A-C-A_{108}). This motif shares a low degree of homology with cysteine rich domains of the heavy metal binding proteins metallothioneins (BLAST – Basic Alignment Search Tool). It is tempting to speculate that this region has functional significance in Ac115p. It is possible that it is a site for heavy-metal binding. The hydrophobic domain and adjacent cysteine residues may be a region of the Ac115 protein which is used for homodimerization or for interaction with other proteins. These interactions may be required for Ac115p function in D2 synthesis.

The AC115 gene product is presumed to function in D2 protein synthesis at a point after translation initiation. In mutant cell chloroplasts, the *psbD* message is present in normal quantities and is loaded onto ribosomes, yet no D2 protein is detectable. Immunoprecipitations of pulse-labeled *ac115* protein reveal trace amounts of full-sized D2, as well as a 15 kDa protein which may be a translation intermediate [44]. There are a number of ways we envision the AC115 gene product working in D2 synthesis. The protein may associate with the D2 protein within the thylakoid membrane, while it is being synthesized, or after its synthesis is complete. This association might be essential for the correct co-translational folding and stabilization of D2 into the thylakoid membrane. Alternatively, the AC115 protein may assist in the co-translational binding of a D2-specific ligand such as chlorophyll, plastoquinone, or the non-heme iron. Such roles in D1 protein synthesis have been proposed for the VIR115 nuclear gene product in barley [23]. The absence of such binding may lead to improper folding of D2 and protein destabilization. A third possibility is that the AC115 gene product is a constituent of the PSII complex where it serves to stabilize D2. Although we consider it unlikely, it is possible that Ac115p is a heretofore unidentified PSII component. Finally, it is possible that Ac115p is a stromal protein which directs the psbD message and/or the nascent D2 protein to the thylakoid membrane as the protein is being made. To do this, Ac115p may need to interact with other proteins, e.g., Nac1p and/or Nac2p, through its hydrophobic and cysteine rich domain. In the absence of a functional AC115 gene product, the D2 protein may be synthesized in an improper location, it may not be properly folded into the thylakoid membrane, and as a consequence it may be exceptionally labile. This would account for the lack of detectable D2 synthesis in *ac115* mutant cells despite the association of the psbD message with polysomes. The role of the AC115 gene product will be clarified once the protein is localized within the organelle and its interactions with neighboring proteins is assayed.

Of the ten putative transformants assayed by Southern analysis, three carry a single copy of the AC115 gene. Although it is possible that these strains are revertants, the reversion frequency of *ac115* is extremely low ($<10^{-8}$). Therefore, it is possible that these transformants are ones in which the complementing sequence has recombined homologously into the genome. While it is most common for DNA introduced into *Chlamydomonas* cells by transformation to integrate nonhomologously into the genome, homologous recombination has been detected [42]. It is somewhat surprising that the AC115 cDNA clone successfully complements the *ac115* mutant. To do so, this sequence in transformants must either be integrated at nonhomologous sites adjacent to a promoter, or it could have recombined homologously into the genome.

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