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The 5′ Leader of a Chloroplast mRNA Mediates the Translational Requirements for Two Nucleus-Encoded Functions in *Chlamydomonas reinhardtii*

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In the green alga *Chlamydomonas reinhardtii*, the nuclear mutations *F34* and *F64* have been previously shown to abolish the synthesis of the photosystem II core polypeptide subunit P6, which is encoded by the chloroplast *psbC* gene. In this report the functions encoded by *F34* and *F64* are shown to be required for translation of the *psbC* mRNA, on the basis of the finding that the expression of a heterologous reporter gene fused to the *psbC* 5′ nontranslated leader sequence requires wild-type *F34* and *F64* alleles in vivo. Moreover, a point mutation in the *psbC* 5′ nontranslated leader sequence suppresses this requirement for wild-type *F34* function. In vitro RNA-protein cross-linking studies reveal that chloroplast protein extracts from strains carrying the *F64* mutation contain an approximately 46-kDa RNA-binding protein. The absence of the RNA-binding activity of this protein in chloroplast extracts of wild-type strains suggests that it is related to the role of the *F64*-encoded function for *psbC* mRNA translation. The binding specificity of this protein appears to be for an AU-rich RNA sequence motif.

The genes encoding the photosynthetic apparatus of plants and green algae are partitioned between the nuclear and chloroplast genomes. The nucleus-encoded protein subunits comprising the multiprotein complexes of this apparatus (photosystem II [PSII], cytochrome *b*6/f, photosystem I, ATP synthase, and ribulose bisphosphate carboxylase-oxygenase) are synthesized on cytoplasmic 80S ribosomes and, subsequently, targeted to the appropriate compartment of the chloroplast. Proteins encoded by the chloroplast genome are synthesized within the chloroplast stroma on 70S ribosomes. Expression of the correct stochiometric ratios of these polypeptides is thought to be governed by genetic circuitry between the genomes of these two compartments (reviewed in references 16 and 30). The chloroplast transcription and translation systems are similar to those of eubacteria, reflecting the procaryotic ancestry of chloroplasts. However, there are differences between the two systems. With only a few exceptions, the translation initiation sites in *Escherichia coli* contain Shine-Dalgarno sequences within the 15 nucleotides 5′ to the initiation codon (38). Of the initiation codons of chloroplast genes, however, only 40% have a potential Shine-Dalgarno sequence in this region (2). Also, five chloroplast ribosomal proteins have no known counterparts in eubacteria (40). Chloroplast gene expression requires many nuclear genes, most of which encode unknown functions (30). Thus, the expression and regulation of chloroplast genes probably differ in many respects from the expression and regulation of procaryotic genes or the nuclear genes of eucaryotes.

The unicellular green alga *Chlamydomonas reinhardtii* is an excellent experimental system for the study of chloroplast genetics, primarily because of the viability of photosynthesis-deficient mutants on media containing a reduced carbon source. Also, *C. reinhardtii* can be manipulated with microbial techniques that allow the screening of large numbers of mutagenized cells. Many mutations of the nuclear and chloroplast genes encoding subunits of the photosynthetic apparatus have been isolated. Other mutations, mostly of nuclear genes, affect a specific posttranscriptional step in the expression of a specific chloroplast gene (30), e.g., *trans* splicing (14), mRNA stability (23, 26, 36), or protein synthesis (10, 12, 21, 24, 31). The functions encoded by many of the genes identified by this class of mutation are expected to be factors involved in chloroplast gene expression or regulation. The ability to transform the chloroplast genome with exogenous DNA (4) has permitted the use of chimeric reporter genes for the identification of determinants within chloroplast mRNAs mediating the requirements for such *trans*-acting functions in *C. reinhardtii* (33). Chimeric reporter genes have also recently been used in in vivo studies of the regulation of genes of higher plant plastids and yeast mitochondria (27, 37).

The experiments described here investigate the functions encoded by two nuclear genes of *C. reinhardtii* which are required for the expression of the chloroplast *psbC* gene. It has been shown previously that two recessive nuclear mutants, *F34* and *F64*, deficient in photosynthesis, fail to accumulate any of the polypeptide subunits of PSII (5, 8, 31). *F34* and *F64* are unlinked and nonallelic and, therefore, affect different genes (1, 12). During 15-min pulse-labelling experiments (31), most chloroplast proteins appeared to be synthesized at wild-type levels in strains carrying either of these two mutations. However, synthesis of the 43-kDa chlorophyll *a*-binding PSII core subunit, P6 (the homolog of CP43 in higher plants), was not detected. In the absence of this PSII subunit, the others are degraded (31). The results of these experiments suggest that the *psbC* mRNA is not translated in strains carrying *F34* or *F64*. However, the alternative possibility that P6 is rapidly degraded could not be ruled out. Conflicting results from similar pulse-labelling experiments (21) showing that the synthesis of the D1 polypeptide of PSII is eliminated by the *F34*

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mutation were later found to result from two additional nuclear mutations in the strain used in this previous study: a suppressor of the *F34* mutation, which restores P6 synthesis, and a mutation which eliminates D1 synthesis (12). A role of wild-type *F34* function in the translation of the *psbC* mRNA was supported by a chloroplast suppressor mutant of the *F34* mutation, F34suI, which carries a single base pair change within a divergently repeated 25-bp sequence located within the *psbC* 5′ untranslated leader sequence (5′ UTR). This repeated sequence within the *psbC* 5′ UTR has the potential to form a stem-loop secondary structure (31). Further support for a role of this stem-loop structure in *psbC* translation was provided by a second chloroplast mutant, FuD34, which specifically lacks P6 synthesis and has three altered positions within the 3′ copy of the inverted repeat (31). This hairpin loop is predicted to be stabilized in FuD34 and destabilized in F34suI, suggesting that its melting is required for the translation of the *psbC* mRNA and that wild-type *F34* activity is required for this process. In order to learn more about the expression and regulation of chloroplast genes and the roles of nucleus-encoded functions in these processes, we have extended the analysis of the functions encoded by the *F34* and *F64* genes with in vivo expression experiments and in vitro RNA-protein binding studies.

MATERIALS AND METHODS

Strains and media. *F34* and *F64* strains have been described previously (1, 5, 8, 31). FuD50 (41) was grown for transformation in Tris-acetate-phosphate (TAP) medium (32). Media in petri plates were solidified with 2% agar (Difco). For genetic crosses (19), the agar was washed several times with distilled water and dried before use. For phenotypic analyses of the cross progeny, 2-ml cultures of the strains derived from each tetrad member were grown in TAP medium under dim light. Each culture (10 μl) was aliquoted onto petri plates containing various media: high-salt minimal medium (HSM) (32) (incubated in bright light), which selects for photosynthetic function; nonselective TAP medium (incubated in dim light); and TAP medium containing various concentrations of spectinomycin (50 to 600 μg/ml) (also incubated in dim light).

Nucleic acids manipulations. Standard techniques were used to manipulate and analyze nucleic acids (34). DNA was sequenced from double-stranded template with the Sequenase system (U.S. Biochemical Corp.). For Northern (RNA) blot experiments, 5-μg samples of total RNA were electrophoresed through a 1% agarose gel containing formaldehyde and MOPS (morpholine propanesulfonic acid) buffer (34), transferred to a Hybond C+ membrane (Amersham), and probed with a double-stranded DNA probe derived from a 0.81-kb *Nco*I-*Pst*I cloned DNA fragment corresponding to the *aadA* structural gene (13) which had been random-primer labelled with [α-^{32}P]dATP. Hybridization signals were revealed by autoradiography. The relative amounts of the RNA in the samples were standardized by probing the blot with a 1.9-kb *Eco*RI-*Sca*I DNA fragment corresponding to sequences located 3′ to the chloroplast *psbB* gene (26) to detect the 0.5-kb *psbH* RNA (22).

Construction of *cg20* and *cg21*. PCR was used to amplify DNA fragments corresponding to the *psbC* promoter and 5′ UTR (from nucleotide [nt] −67 to +549 relative to the 5′ end) from cloned 780-bp chloroplast *Taq*I DNA fragments of a wild-type strain and F34suI (31). The PCR-amplified DNA fragments were cloned into the *Eco*RV site of pBluescript KS(−) (Stratagene) and then excised by digestion with *Cla*I and *Nco*I, which cleave at sites engineered into the 5′ and 3′ extremities of these fragments, respectively. The *Nco*I site was positioned immediately 3′ to the initiation codon of *psbC*. These fragments were ligated into *Cla*I- and *Nco*I-digested pUC-atpX-AAD (13), resulting in the translational fusion of the *psbC* 5′ UTR to the *aadA* structural gene. The 3′ end of the *rbcL* gene was previously positioned 3′ to *aadA* to ensure the correct termination of transcription and stability of the chimeric mRNA (13). The chimeric genes, *cg20* (*psbC*^{wt} *aadA rbcL*, where wt is wild type) and *cg21* (*psbC*^{F34su1} *aadA rbcL*), were excised from these plasmids with *Eco*RV and *Bam*HI, which cleave 5′ and 3′ to the chimeric gene, respectively, and ligated into the *Bam*HI-*Xba*I (blunted) sites of the chloroplast transformation vector atpB-INT (14) to create patpB-INT-cg20 and patpB-INT-cg21. To prevent the gene conversion of *cg20* to the other copy of the inverted repeat of the chloroplast genome and, thereby, expedite the process of obtaining homoplasmic transformants, the inverted repeat sequences to the right of *cg20* (as shown in Fig. 1) were removed by the deletion of a 1.15-kb *Kpn*I fragment (41) and the replacement of the 3′ end of *atpB* with that of *psbD*. (This 3′-end substitution is not indicated in Fig. 1 for simplicity.) patpB-INT-cg20 and patpB-INT-cg21 were transformed into the chloroplast genome of FuD50, a strain carrying a deletion of the 3′ end of the *atpB* gene and some downstream sequences (41), with a microprojectile gun as described previously (42). For genomic Southern blot analyses, total DNA was prepared as described previously (32), digested with *Bam*HI, fractionated on a 0.8% agarose gel, and analyzed as described previously (34). Double-stranded DNA probes were random-primer labelled with [α-^{32}P]dATP. The *aadA* probe was derived from a 0.81-kb *Nco*I-*Pst*I cloned DNA fragment corresponding to the structural gene (13). The second probe was derived from a 1.25-kb *Bam*HI-*Kpn*I chloroplast DNA fragment (shown in Fig. 1). In digests of DNA from the two aberrant spectinomycin-sensitive progeny of cross C, no hybridization signal was detected with the *aadA* probe (data not shown). A *Bam*HI fragment of the size of the wild-type Bam5 fragment (29) was detected with the 1.25-kb *Bam*HI-*Kpn*I probe (data not shown). Therefore, the aberrant tetrad from cross C was biparental; two members inherited the wild-type chloroplast Bam5 fragment from the *mt*$^-$ parent.

Western blot (immunoblot) analysis. The AAD polypeptide was overexpressed in *E. coli* from the 1.26-kb *Nco*I-*Sma*I fragment containing the *aadA* coding sequence and the 3′ end of *rbcL* (13) cloned into the *Nco*I-*Eco*RV sites of the pET3d plasmid (39). Aminoglycoside adenyltransferase (AAD) was extracted as described previously (18), resolved from other bacterial proteins by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE), and then visualized by soaking the gel in 0.2 M KCl at 4°C (which precipitates the SDS bound to protein). AAD was electroeluted from a gel slice with a Biotrap apparatus (Schleicher & Schuell) in 0.19 M glycine–27 mM Tris and dialyzed against either phosphate-buffered saline solution (PBS) (18) for immunizations or 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 8.0]) for coupling to Affi-Gel-15 (Bio-Rad). Approximately 200 μg of AAD in PBS was mixed with an equal volume of Freund's adjuvant (Gibco) and used for each immunization of a rabbit as described previously (18). Anti-AAD antibodies were affinity purified from the serum on a column of AAD which had been linked to Affi-Gel-15 by incubating 0.5 ml of the affinity support with approximately 5 mg of AAD in 1.0 ml of 0.1 M HEPES (pH 8.0) for 4 h at 4°C (Bio-Rad). After passage of 25 ml of the serum over this column two times, the column was washed with 10 mM phosphate (pH 8.0). Antibodies retained on the column were

eluted first with 100 mM glycine (pH 2.5) and then with 100 mM triethylamine (pH 11.5) as described previously (18) and then dialyzed against PBS.

The tested strains were grown in 12 ml of TAP medium in dim light to approximately 2×10^6 cells per ml. These cells were pelleted at 3,000 rpm for 5 min, resuspended in 1.0 ml of TAP medium, pelleted again, resuspended in 200 μl of protein loading buffer (10% glycerol, 1% SDS, 100 mM dithiothreitol, 30 mM Tris-HCl [pH 6.8], 0.01% bromophenol blue), incubated for 1.0 min at 100°C, and centrifuged in a microcentrifuge for 5 min. The supernatant was carefully transferred to a clean tube. Approximately 10 μg of total soluble protein from this supernatant (estimated relative to molecular weight standards of known concentrations by SDS-PAGE) was resolved by electrophoresis on a 12% polyacrylamide gel, electrotransferred to Hybond-C Extra (Amersham), and blocked with Blotto-Tween (5% [wt/vol] nonfat dry milk, 0.2% [vol/vol] Tween 20 in PBS) as described previously (18). The filter was then probed with the affinity-purified anti-AAD antibodies. Secondary labelling was done with ^{125}I-protein A as described previously (34). Ribulose bisphosphate carboxylase, detected with an antiserum raised against the holoenzyme, was used to standardize the amount of protein in each lane.

In vitro RNA-protein binding experiments. Protein extracts were prepared from strains carrying the *cw-15* mutation, which affects the cell wall (7), to allow breakage of cells, but not chloroplasts, by equilibration with nitrogen at high pressure in a Yeda press (Weizmann Institute of Science, Rehovot, Israel) as described previously (15). Cultures (1.5 liters) were grown in TAP medium exposed to indirect light to 1.5×10^6 cells per ml. A chloroplast-enriched fraction was obtained by centrifuging broken cells to 3,000 × *g*, immediately decelerating, and resuspending the pellet in a hypotonic lysis buffer (17). To purify chloroplasts, this pellet of enriched chloroplasts was gently resuspended in 1× breaking buffer (0.3 M sorbitol, 50 mM HEPES-KOH [pH 7.2], 2 mM EDTA, 1 mM $MgCl_2$) and then fractionated on a 25-ml linear 10 to 80% Percoll gradient in 1× breaking buffer containing 5 mM β-mercaptoethanol. Soluble protein extracts from chloroplast-enriched fractions or from pure chloroplasts were prepared as described previously (17). Each extract was standardized for protein concentration by the Bradford assay (4).

RNA probes were synthesized in 20 μl of reaction mixtures containing 1 μg of linear DNA template; 40 mM Tris-HCl (pH 7.5); 6 mM $MgCl_2$; 2 mM spermidine; 10 mM dithiothreitol; 50 U of porcine RNase inhibitor (Biofinex); 60 μCi of [α-^{32}P]UTP (800 mCi/mmol; Amersham); 12 μM nonradiolabelled UTP; 0.3 mM (each) ATP, CTP, and GTP; and 15 U of T7 RNA polymerase (Promega) for 45 min at 37°C. RNase-free DNase (1 U) was added, and the reaction mixture was incubated for an additional 10 min at 37°C. The reaction mixtures were extracted once with phenol-chloroform. The RNA probes were separated from the nonincorporated nucleotide triphosphates on G-50 spin columns (34). The *psbC* 5′-UTR RNA probe was derived from pDH245, a subcloned 245-bp *Dde*I-*Hin*dIII fragment (see Fig. 5) in pBluescript KS(−), cleaved by *Sal*I adjacent to the insert. The *psbD* 5′-UTR RNA probe was derived from ppsbD(−79), a 79-bp fragment corresponding to the *psbD* 5′ UTR cloned into pBluescript KS(−) and cleaved with *Eco*RI. The *psaA* probe was derived from a DNA fragment corresponding to the 3′ end of the first intron of *psaA* (from −109 bp to +9 relative to the splice acceptor site) cloned in pBluescript KS(−). The 248-nt control RNA probe was derived from pBluescript KS(−) cleaved with *Bst*NI. Each probe had the expected length as determined by denaturing PAGE (data not shown). The conditions of the binding reactions were described previously (28). Binding reaction mixtures were incubated for 5 min at room temperature (22 to 25°C) before being irradiated. Each reaction contained 5 μg of protein extract and approximately 2×10^5 cpm of RNA probe. Cross-linking reactions were performed with either a 254-nm UV irradiation of 1.0 J/cm^2 and approximately 7 min duration using a Stratalinker (Stratagene) UV cross-linker or a 266-nm laser pulse of 79 mJ/cm^2 and 5-ns duration with a pulsed YAG laser (Quanta-Ray GCR). After irradiation, samples were treated with 10 μg of RNase A (Sigma) for 10 min at 37°C, boiled for 1.0 min in protein loading buffer (10% glycerol, 1% SDS, 100 mM dithiothreitol, 30 mM Tris-HCl [pH 6.8], 0.01% bromophenol blue), quickly cooled on ice, and analyzed by SDS-PAGE (12 to 15% acrylamide gradient). Control samples containing *F64* extract were incubated in the presence of 1 μg of proteinase K (Boehringer Mannheim) at room temperature (22 to 24°C), or at 65°C for 5 min, before the addition of the RNA probe.

For RNase T_1 protection gel mobility shift (T_1-GMS) assays (25), 30 μl of the binding reaction mixture containing RNA probe (approximately 5×10^4 cpm) was incubated for 5 min, treated with 15 U of RNase T_1 (Boehringer Mannheim) for 5 min, and then electrophoresed on a 5% native acrylamide gel (50:1 acrylamide-bisacrylamide ratio) at 2 to 3 W in 0.19 M glycine–27 mM Tris (ultrapure reagents from Schwarz/Mann Biotech). All steps were done at room temperature (22 to 24°C). Gels were dried and visualized by autoradiography.

RESULTS

Expression of a heterologous reporter gene from the *psbC* promoter and 5′ UTR requires the wild-type *F34* and *F64* functions in vivo. To test the hypothesis that translation of the *psbC* mRNA, rather than the stability of the polypeptide product of *psbC*, requires the wild-type functions encoded by the *F34* and *F64* genes, a chimeric reporter gene was employed to determine whether the *psbC* 5′ UTR can confer *F34* and *F64* dependence on the expression of a heterologous reporter gene in vivo. This chimeric gene, called *cg20* (Fig. 1), consists of the *psbC* promoter and 5′ UTR fused to the coding region of the *E. coli aadA* gene encoding AAD. Expression of *aadA* in chloroplasts confers spectinomycin and streptomycin resistance (13). A transformation construct containing this chimeric gene, patpB-INT-cg20, was transformed into the chloroplast genome of FuD50, a strain carrying a deletion of the 3′ end of the *atpB* gene and some downstream sequences (41). Integration of the *atpB* selectable marker gene by homologous recombination rescues the photosynthetic defect of this strain (Fig. 1); therefore, transformants were selected for growth on HSM medium in bright light. *cg20* is located in a site removed by the *FuD50* deletion and therefore was integrated with the selectable marker. The following genomic Southern blot analyses were used to identify chloroplast transformants and to confirm that *cg20* was transmitted to all four members of a representative tetrad from each of the crosses described below. The results of the analyses of the four members of one tetrad are shown in Fig. 1. The *aadA* probe (see Materials and Methods) hybridized only to a 3.1-kb *Bam*HI fragment (Fig. 1B) in DNA digests of the transformants. This probe did not hybridize to any fragment in digests of FuD50 DNA. When these DNA samples were digested with *Eco*RI, which cuts at sites on either side of the *FuD50* deletion, the *aadA* probe hybridized to a fragment with an approximate size of 7.4 kb (data not shown), the expected size for the integration of one copy of the chimeric gene and the 3′ end of the *atpB* gene, as indicated in Fig. 1. A probe derived from the 1.25-kb *Bam*HI-

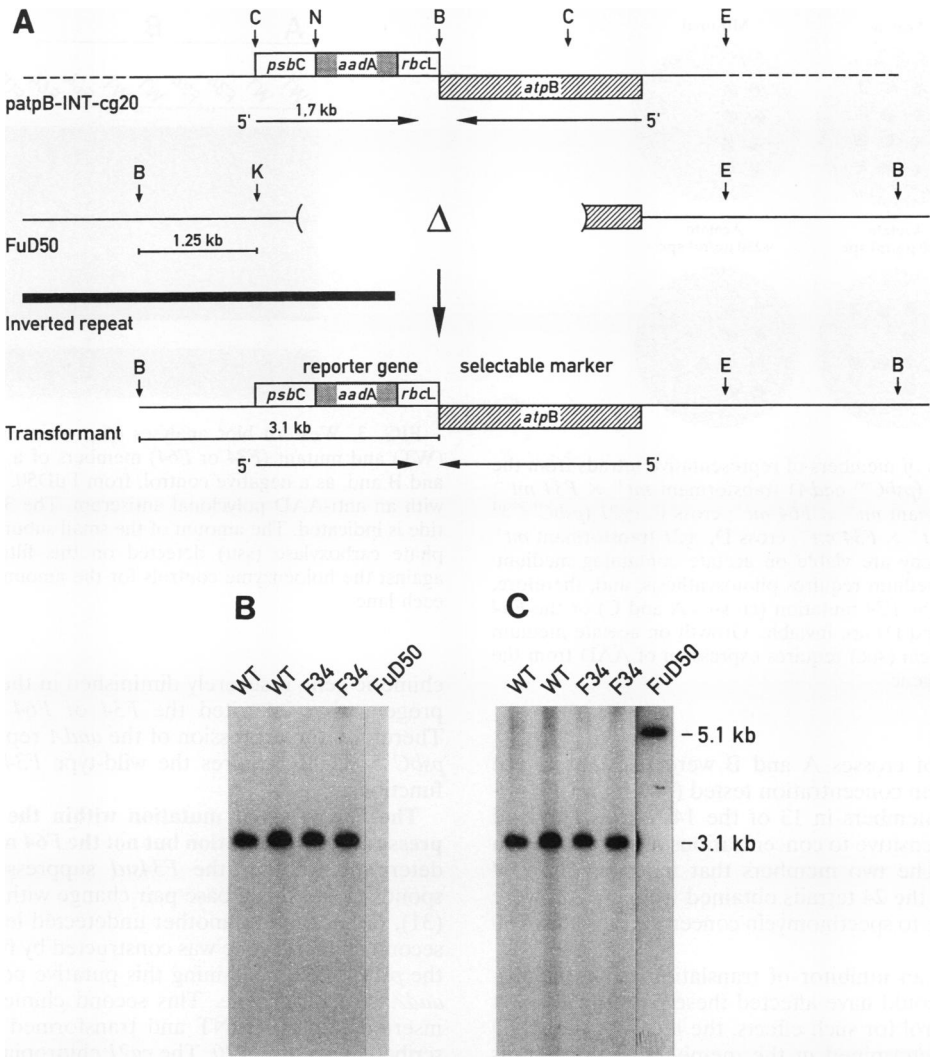

FIG. 1. (A) The transformation construct patpB-INT-cg20 is shown above the Bam5 chloroplast DNA fragment of the deletion mutant FuD50. The solid bar indicates the location of the end of the inverted repeat region. Photosynthetic transformants result from the integration by homologous recombination of a functional copy of the *atpB* gene, which rescues the photosynthetic deficiency of Fud50. The 3′ end of *atpB* in this construct had been previously replaced with that of the *psbD* gene. The chimeric reporter genes (*cg20* and *cg21*) are cointegrated with this selectable marker because they are inserted into a site within the *FuD50* deletion. The Southern blot contains *Bam*HI-digested total DNA from each member of one tetrad from cross A. E, *Eco*RI; B, *Bam*HI; C, *Cla*I; K, *Kpn*I; N, *Nco*I. (B) Only the 3.1-kb fragment, which is diagnostic of correct integration of the chimeric gene, hybridized to a probe derived from the *aadA* gene in digests of DNA from the four members of the tetrad but not to any fragment in a control digest of DNA from FuD50. (C) The 1.25-kb *Bam*HI-*Kpn*I fragment indicated on the map (A) also detected this 3.1-kb fragment in the tetrad members and not the 5.1-kb Bam5 fragment of FuD50. WT, wild type.

*Kpn*I chloroplast DNA fragment (Fig. 1) also detected the 3.1-kb *Bam*HI fragment in the lanes containing transformant DNA and did not detect the nontransformed 5.1-kb Bam5 fragment. The latter fragment was detected in the lane containing DNA of FuD50 (40). (The wild-type Bam 5 fragment is 7.6 kb.) Ectopic integration would not generate this 3.1-kb *Bam*HI fragment (the left-hand *Bam*HI site is absent in patpB-INT-cg20) or eliminate the Bam5 fragment of FuD50.

Expression of AAD from the *cg20* chimeric gene in the chloroplast transformants confers resistance to the highest concentration of spectinomycin tested (600 μg/ml). In order to determine whether expression of the *aadA* reporter gene from the *psbC* 5′ UTR requires wild-type *F34* and *F64* alleles, the transformed *cg20* chimeric gene was crossed into nuclear backgrounds containing either the *F34* mutation or the *F64* mutation as follows. A mating-type-plus (mt^+) *cg20* transformant strain was crossed to mating-type-minus (mt^-) strains carrying either the *F34* mutation (cross A) or the *F64* mutation (cross B). *cg20* was transmitted to all of the progeny because the chloroplast genome in *C. reinhardtii* is transmitted uniparentally from the mt^+ strain (11). The results of the phenotypic analyses of a representative tetrad from each cross are shown in Fig. 2. All of the progeny were viable on a medium containing acetate, which permits heterotrophic growth of the photosynthesis-deficient strains. The *F34* and *F64* nuclear mutations segregated 2:2, as indicated by the inviability of two members of each tetrad on a minimal medium which selects for photosynthetic growth. The members of each tetrad were tested for growth on acetate medium containing various concentrations of spectinomycin (from 50 to 600 μg/ml). The

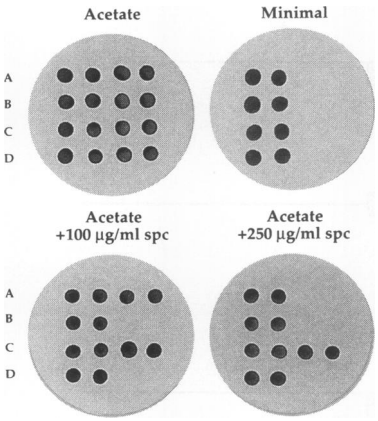

FIG. 2. Phenotypes of members of representative tetrads from the crosses. cross A, *cg20* (*psbC*^{wt} *aadA*) transformant *mt*$^+$ × *F34 mt*$^-$; cross B, *cg20* transformant *mt*$^+$ × *F64 mt*$^-$; cross C, *cg21* (*psbC*^{F34suI} *aadA*) transformant *mt*$^+$ × *F34 mt*$^-$; cross D, *cg21* transformant *mt*$^+$ × *F64 mt*$^-$. All progeny are viable on acetate containing medium. Growth on minimal medium requires photosynthesis, and, therefore, the progeny carrying the *F34* mutation (crosses A and C) or the *F64* mutation (crosses B and D) are inviable. Growth on acetate medium containing spectinomycin (spc) requires expression of AAD from the transformed reporter gene.

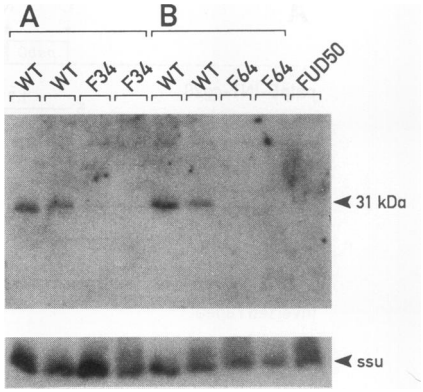

FIG. 3. Western blot analyses of protein extracts from wild-type (WT) and mutant (*F34* or *F64*) members of a tetrad from crosses A and B and, as a negative control, from FuD50. This filter was probed with an anti-AAD polyclonal antiserum. The 31-kDa AAD polypeptide is indicated. The amount of the small subunit of ribulose bisphosphate carboxylase (ssu) detected on this filter with an antiserum against the holoenzyme controls for the amount of protein extract in each lane.

wild-type progeny of crosses A and B were resistant to the highest spectinomycin concentration tested (600 μg/ml). However, the two *F34* members in 13 of the 14 tetrads obtained from cross A were sensitive to concentrations of spectinomycin above 250 μg/ml. The two members that inherited the *F64* mutation in each of the 24 tetrads obtained from cross B were found to be sensitive to spectinomycin concentrations above 50 μg/ml.

Spectinomycin is an inhibitor of translation in the chloroplast and, as such, could have affected these patterns of *aadA* expression. To control for such effects, the levels of the AAD polypeptide were determined in the members of the tetrads from crosses A and B shown in Fig. 2 grown in the absence of the drug. On the Western blot in Fig. 3, a polyclonal antiserum raised against AAD detected an approximately 32-kDa polypeptide in soluble protein extracts of the wild-type progeny (lanes 1, 2, 5, and 6) but not in extracts from either the *F34* members of the tetrad from cross A (lanes 3 and 4) or the *F34* members of the tetrad from cross B (lanes 7 and 8). This protein is ADD and not a cross-reacting species, because it has the correct apparent molecular mass (31.6 kDa) (20) and was not detected in protein extracts from FuD50, the recipient strain used for transformation. The inability to detect AAD in the *F34* progeny of cross A by Western blot, even though these strains show a low level of spectinomycin resistance, is probably due to the low level of AAD (less than 0.01% of total protein; data not shown) in *cg20* chloroplast transformants having a wild-type nuclear genome. Thus, the Western blot analysis is done at the limit of detection. Despite this low level of AAD expression, the chimeric gene transformants are resistant to spectinomycin concentrations of above 1.5 mg/ml. Therefore, an exceedingly low level of AAD expression in the *F34* progeny of cross A, which was undetectable on the Western blot, could account for the low relative level of spectinomycin resistance of these strains. This possibility is supported by a previous report of trace amounts of the *psbC*-encoded polypeptide in the *F34* mutant (9).

These results show that expression of AAD from the *cg20* chimeric gene is severely diminished in the chloroplasts of the progeny that inherited the *F34* or *F64* nuclear mutations. Therefore, the expression of the *aadA* reporter gene from the *psbC* 5′ UTR requires the wild-type *F34*- and *F64*-encoded functions.

The *F34suI* point mutation within the *psbC* 5′ UTR suppresses the *F34* mutation but not the *F64* mutation. In order to determine whether the *F34suI* suppressor mutation corresponds to the single base pair change within the *psbC* 5′ UTR (31), rather than to another undetected lesion in this strain, a second chimeric gene was constructed by fusing the 5′ UTR of the *psbC* allele containing this putative point mutation to the *aadA* structural gene. This second chimeric gene, *cg21*, was inserted into atpB-INT and transformed into FuD50 as described above for *cg20*. The *cg21* chloroplast transformant was resistant to spectinomycin, showing that AAD is expressed from this chimeric gene. To determine whether *aadA* expression from the *psbC*^{F34suI} 5′ UTR suppresses the requirement for wild-type *F34* or *F64* alleles, the *mt*$^+$ *cg21* transformant was crossed to *mt*$^-$ strains carrying either the *F34* mutation (cross C) or the *F64* mutation (cross D), and the progeny were tested for growth on different media as described above (Fig. 2). The spectinomycin sensitivity phenotype produced by the *F64* mutation was not suppressed by the *F34suI* suppressor mutation in *cg21*; the two members that inherited the *F64* mutation in each of the 12 tetrads obtained from cross D were sensitive to spectinomycin concentrations above 25 μg/ml, while the two wild-type members were resistant to the highest concentration tested (600 μg/ml). In contrast to the *F34* progeny of cross A, however, the *F34* members of 9 of the 10 tetrads obtained from cross C were resistant to the highest spectinomycin concentration tested (600 μg/ml). The one tetrad in which all of the progeny were not spectinomycin resistant was found to be biparental; two members inherited the nontransformed chloroplast genome from the *mt*$^-$ parent (see Materials and Methods). Thus, similar to the *psbC*^{F34suI} allele, expression of *aadA* from the *psbC*^{F34suI} 5′ UTR does not require the wild-type *F34*-encoded function but does require the wild-type *F64*-encoded function. These results confirm that the single base pair change in the *psbC* 5′ UTR of the F34suI strain (31), and not another undetected mutation, is responsible for the

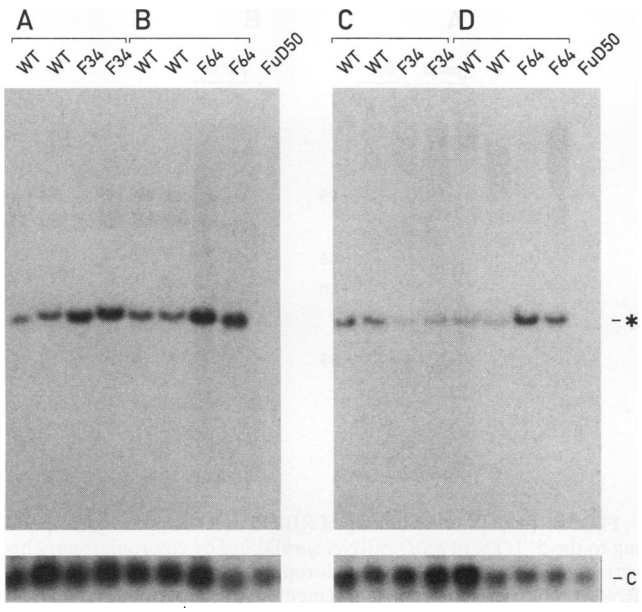

FIG. 4. Northern blot analyses of the *cg20* and *cg21* mRNAs expressed in representative tetrads from the crosses and with RNA isolated from the transformation recipient, FuD50, are shown. The 1.7-kb transcript (*) was detected with a probe derived from the *aadA* structural gene sequences. The level of the 0.5-kb *psbH* transcript (C) controls for the amount of RNA in each lane. WT, wild type.

suppression of *F34*. Moreover, the spectinomycin resistance of the progeny of cross C that inherited the *F34* mutation excludes the possibility that spectinomycin resistance conferred by AAD requires photosynthesis since these strains are both spectinomycin resistant and deficient for photosynthesis (because their endogenous *psbC* gene does not carry the suppressor mutation).

The *F34* and *F64* mutations do not reduce the expression of the chimeric genes at the level of the accumulation of their mRNAs. To determine whether the effects of the *F34*, *F64*, and *F34sul* mutations on the expression of the chimeric genes result from either qualitative or quantitative alterations of the chimeric mRNAs, the following Northern blot analyses were performed on samples of total RNA preparations from the representative tetrad from each cross (Fig. 4). The level of a 0.5-kb mRNA encoded by the chloroplast *psbH* gene (22) was used to normalize the amount of RNA in each lane. A probe derived from the *aadA* structural gene hybridized to a 1.7-kb mRNA species in the lanes containing RNA from the *cg20* and *cg21* transformants. This mRNA species is expressed from the chimeric genes because it has the expected size and is absent in RNA from FuD50, the recipient strain used for transformation. The levels of the chimeric mRNAs in RNA preparations from the progeny which are deficient in *aadA* expression (the progeny of crosses A and B carrying the *F34* and *F64* mutations, respectively, and the progeny of cross D carrying the *F64* mutation) were found to be slightly higher than the levels in preparations from their respective wild-type sibling strains. Therefore, the requirements for functional *F34* and *F64* alleles for AAD expression from the chimeric genes are not manifested at the level of the chimeric mRNAs.

The elevated levels of the chimeric mRNAs in the progeny strains carrying *F34* or *F64* are probably irrelevant to any effects of these mutations on the endogenous *psbC* mRNA because RNase protection assays revealed equivalent levels of the latter in the samples of total RNA from the tetrads from crosses A and B (data not shown).

In vitro cross-linking analysis of the RNA-binding activities in chloroplast extracts from wild-type and mutant strains. To identify any *trans*-acting factors which mediate the *F34* and *F64* dependence of *psbC* mRNA translation, UV cross-linking experiments were carried out to identify proteins capable of binding to the *psbC* 5′ UTR in vitro. Protein extracts were prepared either from chloroplast-enriched fractions or from purified chloroplasts of the wild-type and mutant strains (see Materials and Methods). A 245-nt RNA probe corresponding to the central region of the *psbC* 5′ UTR (Fig. 5) was incubated with protein extracts from chloroplast-enriched fractions of a wild-type strain and strains carrying either the *F34* mutation or the *F64* mutation. The binding reactions were then irradiated with UV light as a cross-linking agent.

In extracts of chloroplast-enriched fractions of the strain carrying the *F64* mutation, and from purified chloroplasts of this strain, the *psbC* 5′-UTR RNA probe was cross-linked to an approximately 46-kDa protein (Fig. 5). This protein was not detected in chloroplast extracts of the wild-type strain or the strain carrying the *F34* mutation (Fig. 5A). This difference was reproduced in numerous binding reactions with five independent extract preparations from the three strains (Fig. 5 and data not shown). This protein is hereafter referred to as RBP46 for RNA-binding protein with a mass of 46 kDa. RBP46 was also cross-linked to the *psbC* 5′ UTR RNA probe during a 5-ns laser pulse (Fig. 5B) and was, therefore, probably not labelled during the 7-min UV irradiation by virtue of a weak or transient association with this probe. No signals were detected in control samples which lacked extract (Fig. 5D) or were not irradiated (data not shown). Moreover, this binding activity, and the four others described below, correspond to protein species because they were eliminated by 5-min incubations either with proteinase K or at 65°C before the addition of the probe (Fig. 5C).

In chloroplast-enriched extracts from each of the three strains, protein species with masses of 95, 65, 40 and 33 kDa (as estimated by SDS-PAGE) were also labelled in this way (Fig. 5A). The 33-kDa species was not detected in protein extracts of purified chloroplasts (shown for *F64* in Fig. 5B) and therefore is probably not involved in *psbC* translation. Although the 95-, 65-, and 40-kDa protein species were detected in the extract of purified chloroplasts, they were not reproducibly detected even when the same extract preparation was used. Moreover, the cross-linking of these proteins to the *psbC* 5′ UTR did not differ reproducibly when chloroplast extracts from the wild-type and mutant strains were used. Thus, there is presently no evidence that these proteins are related to the roles of the wild-type *F34*- and *F64*-encoded functions in the translation of the *psbC* mRNA. No additional proteins were detected with a probe corresponding to the entire *psbC* 5′ UTR (data not shown), which detected the same proteins as the probe shown in Fig. 1.

In order to determine whether the *F64* mutation might eliminate a factor which inhibits or regulates RBP46 RNA-binding activity, rather than activate RBP46 directly, a sample of a chloroplast-enriched protein extract from a strain carrying *F64* was mixed with the same amount of chloroplast-enriched extract from a wild-type strain. This sample and samples of each extract alone were incubated for 5 min at room temperature (22 to 24°C) before the addition of the RNA probe and subsequent UV irradiation. Comparison of the proteins detected in the sample containing *F64* extract alone with those detected in the mixture of wild-type and *F64* extracts revealed

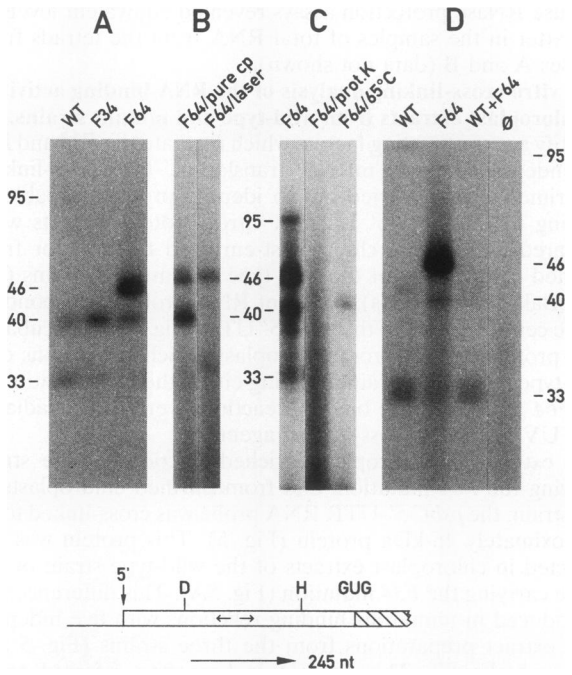

FIG. 5. UV cross-linking of proteins to ^{32}P-labelled probe derived from the 245-bp *Dde*I (D)-*Hin*dIII (H) fragment (indicated by the arrow) from *psbC* 5′ UTR and resolved by SDS-PAGE. Their apparent molecular masses (in kilodaltons) are indicated. The open and striped rectangles on the map correspond to the *psbC* 5′ UTR and 5′ end of the *psbC* coding sequence, respectively. The location of the GUG initiation codon is indicated. The small arrows indicate the inverted repeat within the *psbC* 5′ UTR. (A) A 46-kDa protein (RBP46) was detected in chloroplast-enriched protein extracts from a strain carrying the *F64* mutation (F64) but not in similarly prepared extracts from a wild-type strain (WT) or a strain carrying the *F34* mutation (F34). (B) RBP46 was detected in an extract of purified *F64* chloroplasts (F64/pure cp) and was labelled during a laser pulse (F64/laser). (C) None of the proteins detected in the chloroplast extract from the strain carrying the *F64* mutation (F64) was detected in samples of the same extract that had been previously incubated either with proteinase K (F64/prot.K) or at 65°C (F64/65°C). (D) RBP46 was not detected in extracts from the wild-type strain (WT) or from a mixture of wild-type and *F64* extracts (WT+F64) but was detected in the *F64* extract alone (F64). No signals were detected in a control sample lacking extract (Ø).

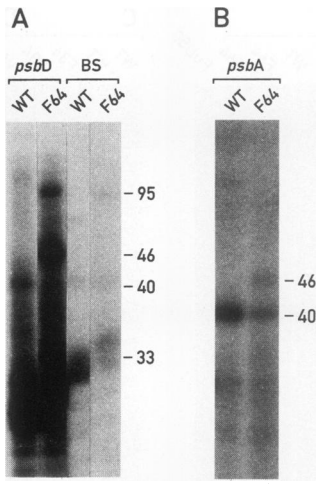

FIG. 6. (A) UV cross-linking of RBP46 to RNA probe corresponding to the 5′ UTR of *psbD* mRNA (*psb*D) in *F64* chloroplast-enriched extract (F64) but not wild-type chloroplast-enriched extract (WT). An RNA probe derived from a fragment of pBluescript (BS) did not detect RBP46 in either wild-type or *F64* chloroplast extracts. (B) An RNA probe corresponding to the 5′ UTR of the *psbA* mRNA (*psb*A) did not detect any protein in the 46- to 47-kDa molecular mass range in an extract of purified chloroplasts from a wild-type strain, although the 40-kDa species was detected. This RNA probe labelled RBP46 in extracts of purified chloroplasts from the strain carrying the *F64* mutation.

that incubation with wild-type extract results in the disappearance of the RBP46 activity in *F64* extract (Fig. 5D).

In order to determine whether RBP46 binding is specific to the *psbC* 5′ UTR, similar UV cross-linking experiments were carried out with RNA probes corresponding to portions of three other chloroplast mRNAs: the 5′ UTR of the *psbD* mRNA (Fig. 6A), the 5′ UTR of the *psbA* mRNA (panel B), and the 3′ end of the first intron of the *psaA* pre-mRNA (data not shown). Like the *psbC* 5′-UTR probe, these probes detected a 46-kDa protein species, which we presume to be RBP46, in chloroplast extracts of the strain carrying the *F64* mutation but not in similarly prepared extracts from the wild-type strain. An RNA probe derived from a 248-bp fragment of the pBluescript phagemid vector [pBluescript KS(−)] (Fig. 6A) did not detect any protein in the 46-kDa molecular mass range in *F64* chloroplast extract (or in wild-type chloroplast extract). Competition studies using nonradiolabelled RNAs corresponding to the same chloroplast mRNAs sequences and the pBluescript fragment were consistent with these results; the RNAs derived from *psbC*, *psbD*, *psbA*, and *psaA* sequences efficiently competed for the binding of RBP46 to the *psbC* RNA probe while only weak competition was observed with the RNA derived from pBluescript (data not shown).

Chloroplast RNAs have a high A+U content, while the control probe derived from the pBluescript fragment has a normal base composition and lacks any stretches of either A or U residues longer that 4 nt. The observation the RBP46 binds to the chloroplastic RNA sequences and not the RNA derived from pBluescript suggested that this protein recognizes an AU-rich sequence motif. To test this hypothesis, homopolymers of each of the four ribonucleotides were tested for the ability to compete for RBP46 binding. Samples of *F64* chloroplast-enriched extract were incubated with either 0.2 or 1.0 μg of nonlabelled homopolymers of each of the four ribonucleotides for 5 min before the addition of the 245-nt *psbC* probe and subsequent UV irradiation (Fig. 7). In the absence of competitor, all four proteins in *F64* chloroplast-enriched extract were detected. Incubation with poly(C) or poly(G) had no effect on RBP46 binding to the *psbC* RNA probe. Similar incubations with poly(A) or poly(U), however, eliminated RBP46 binding, demonstrating efficient competition by these ribohomopolymers. Therefore, RBP46 recognizes an AU-rich RNA sequence motif.

Novel RNA-protein complexes generated by *F64* protein extracts are detected by RNase T_1-GMS assays. In order to determine whether RBP46 forms a stable complex with the RNA probes derived from chloroplast mRNAs, and possibly other factors, RNase T_1-GMS assays (25) were performed. The RNA probes derived from *psbC*, *psbD*, *psbA*, and pBluescript (described above) were incubated with samples of chloroplast-enriched protein extracts (see Materials and Methods) of a wild-type strain or the mutants. These samples were treated

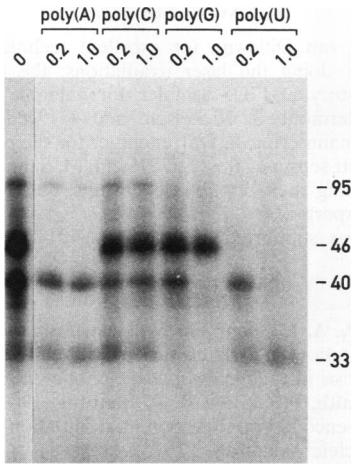

FIG. 7. SDS-PAGE analysis of proteins in *F64* chloroplast-enriched extract labelled by RNA probe derived from *psbC* 5′ UTR following incubation in the presence of 0.2 or 1.0 μg of the various homopolynucleotides indicated. Lane 0, control sample without competitor. Molecular masses (in kilodaltons) of the principal protein species are indicated on the right.

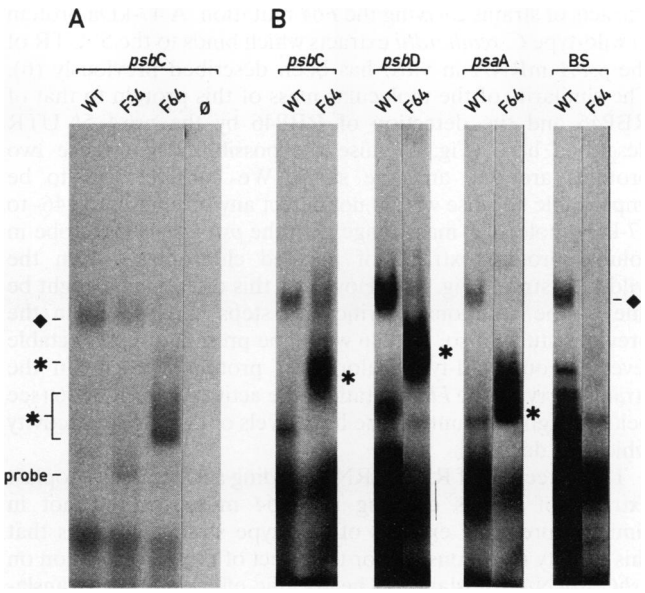

FIG. 8. RNase T_1-GMS assays. The bands indicated correspond to complexes between the RNA probe and factors in extracts from the wild-type strain (WT) or from strains carrying either the *F34* mutation or the *F64* mutation. Complexes which were detected only with chloroplast extracts from the strain carrying the *F64* mutation are indicated by asterisks. Another complex, which is discussed in the text, is indicated by ◆. (A) Results of T_1-GMS assays using the *psbC* 5′-UTR RNA probe and extracts from the wild-type and mutant strains. The position of the band on this native acrylamide gel (which is not shown) corresponding to the intact *psbC* 5′-UTR RNA probe is indicated by probe. The lane containing a control sample lacking protein extract and treated with RNase T_1 is labeled "Ø." (B) Results of T_1-GMS assays using RNA probes corresponding to the *psbC* 5′-UTR probe (*psbC*), the *psbD* 5′-UTR probe (*psbD*), the *psaA* first-intron probe (*psaA*), and the probe derived from pBluescript (BS) sequences, with chloroplast-enriched protein extracts from a wild-type strain (WT) and the strain carrying the *F64* mutation (F64).

with RNase T_1 to trim the free probe sequences from any complexes that had formed. Complexes between the RNA probe and factors in the protein extract were detected by native PAGE. In the absence of extract, the probe was completely degraded by the RNase T_1 treatment (Fig. 8A). The position of the intact probe on this gel is indicated.

In binding reactions with extract from the strain carrying the *F64* mutation, but not with extracts from the wild-type strain, a complex was detected with each of the three RNA probes corresponding to chloroplast mRNA sequences (Fig. 8). Other minor complexes were also generated when extracts from this mutant were used. However, when the control RNA probe derived from the pBluescript fragment and extract from the strain carrying the *F64* mutation were used this complex was not apparent. Thus, the *F64*-specific complexes were detected only with the probes that detected RBP46 in the UV cross-linking experiments. This correlation suggests that the *F64*-specific complexes result from the binding of the chloroplastic RNA probes to RBP46 and, possibly, other factors. This binding must be relatively stable since dissociation of an RNA-protein complex in these experiments would render the probe susceptible to digestion by RNase T_1 or separation from the protein components during electrophoresis.

Another complex (◆ in Fig. 8) was generated when each of the RNA probes, including the probe derived from the pBluescript fragment, was incubated with extracts from either wild-type or the mutant strains. Although this complex was often diminished when extract from the strain carrying the *F64* mutation was used (Fig. 8), in similar experiments extract from this strain generated this complex to the same extent as did wild-type extract (data not shown). Therefore, further experiments are required to determine if this complex is related to the roles of the *F34*- and *F64*-encoded functions for *psbC* translation.

DISCUSSION

In vivo expression experiments reveal that the *F34* and *F64* nucleus-encoded functions are required for the initiation of *psbC* mRNA translation. In this study, the 5′ UTR of the *psbC* mRNA is shown to be sufficient to make the expression of a heterologous reporter gene dependent on wild-type *F34* and *F64* nucleus-encoded functions in vivo. These results rule out the possibility that the *F34* and *F64* mutations cause the destabilization of P6, the polypeptide encoded by *psbC*. Moreover, the finding that the expression of the *aadA* reporter gene from the *psbC* 5′ UTR of the F34suI strain is not affected by the *F34* mutation shows that the single base pair change within this UTR, and not another undetected mutation, is responsible for the suppression of *F34*. Northern blot analyses revealed that the *F34* and *F64* mutations do not diminish expression of the reporter gene via an alteration of the transcription or stability of the chimeric mRNAs. These results demonstrate that the wild-type *F34*- and *F64*-encoded functions are required either for the initiation of *psbC* mRNA translation or to inhibit premature translation termination caused by the *psbC* 5′ UTR. We favor the first possibility, as we consider it highly improbable that the *psbC* 5′ UTR can mediate premature translation arrest or termination within both the coding sequence of the endogenous *psbC* mRNA and the *aadA* reporter gene in the absence of wild-type *F34*- or *F64*-encoded functions.

Chloroplasts of the *F64* mutant display a novel RNA-binding activity. Our in vitro RNA-protein cross-linking studies revealed a 46-kDa RNA-binding protein in chloroplast

extracts of strains carrying the *F64* mutation. A 47-kDa protein in wild-type *C. reinhardtii* extracts which binds to the 5′ UTR of the *psbA* mRNA in vitro has been described previously (6). The similarity of the molecular mass of this protein to that of RBP46 and the detection of RBP46 by the *psbA* 5′ UTR described here (Fig. 6) raise the possibility that these two proteins are one and the same. We consider this to be improbable because we did not detect any protein in the 46- to 47-kDa molecular mass range with the *psbA* 5′-UTR probe in soluble protein extracts of purified chloroplasts from the wild-type strain (Fig. 6B). However, this discrepancy might be due to the additional purification steps carried out in the previous study; if so, RBP46 would be present at undetectable levels in our wild-type chloroplast protein extracts. In the strains carrying the *F64* mutation, the activation of RBP46 (see below) might account for the high levels of this binding activity which we detected.

The detection of RBP46 RNA-binding activity in chloroplast extracts of strains carrying the *F64* mutation, but not in similarly prepared extracts of wild-type strains, suggests that this activity is responsible for the effect of the *F64* mutation on *psbC* mRNA translation. The specific effect on *psbC* translation, however, is probably determined by factors other than RBP46 since the binding specificity of this protein was found to extend to other chloroplast mRNAs. Also, RBP46 binding was inhibited by poly(A) and poly(U), suggesting that it recognizes stretches of either of these residues in RNA. Such factors conferring a specific effect of RBP46 might include the context of the binding site(s) on the *psbC* mRNA or other specific *trans*-acting factors which were absent from our extracts.

The observation that the *F34suI* point mutation suppresses the requirement for wild-type *F34* function suggests that the putative stem-loop secondary structure in the *psbC* 5′ UTR is the target site of an *F34*-dependent activity required for the translation of the *psbC* mRNA. In the *F64* mutant RBP46 probably binds to the longest stretches of A and U residues in the *psbC* 5′ UTR. Since these AU-rich stretches are located on either side of this stem-loop structure, this secondary structure probably does not mediate the requirement for wild-type *F64* function. This hypothesis is consistent with inability of the *F34suI* suppressor mutation to suppress the *F64* mutation.

A specific effect mediated by an RNA-binding protein with little sequence specificity is not unprecedented. In *Drosophila melanogaster*, the *Sex-lethal* gene encodes an RNA-binding protein involved in the determination of sexual identity via its regulation of specific alternative splicing reactions. Yet, the primary binding specificity of the protein encoded by *Sex-lethal* is for stretches of more than five U residues (35). Consequently, this protein binds to many RNAs containing stretches of U residues that are probably not regulated targets sites.

A mutation which activates a repressor of *psbC* mRNA translation would be expected to produce a dominant phenotype. The recessive nature of *F64* (1) suggests, therefore, that this mutation does not activate RBP46 directly. Moreover, the observation that RBP46 was not detected following the incubation of *F64* chloroplast extract with wild-type chloroplast extract suggests that RBP46 is not encoded by the *F64* gene. One possibility, which is consistent with our data, is that the wild-type *F64* gene encodes an activity which inactivates RBP46 RNA-binding activity. Alternatively, RBP46 may function in a complex which also contains the *F64* gene product. The absence of the latter in strains carrying the *F64* mutation might result in aberrant or unregulated binding of RBP46 to which the translation of the *psbC* mRNA is particularly sensitive.

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REFERENCES

1. **Bennoun, P., A. Masson, and M. Delosme.** 1980. A method for complementation analysis of nuclear and chloroplast mutants of photosynthesis in *Chlamydomonas*. Genetics **95**:39–47.
2. **Bonham-Smith, P. C., and D. P. Bourque.** 1989. Translation of chloroplast-encoded mRNA: potential initiation and termination signals. Nucleic Acids Res. **17**:2057–2080.
3. **Boynton, J. E., N. W. Gillham, E. H. Harris, J. P. Hosler, A. M. Johnson, A. R. Jones, B. L. Randolph-Anderson, D. Robertson, T. M. Klein, K. B. Shark, and J. C. Sanford.** 1988. Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science **240**:1534–1538.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
5. **Chua, N. H., and P. Bennoun.** 1975. Thylakoid membrane polypeptides of *Chlamydomonas reinhardtii*: wild type and mutant strains deficient in photosystem II reaction center. Proc. Natl. Acad. Sci. USA **72**:39–47.
6. **Danon, A., and S. P. Y. Mayfield.** 1991. Light regulated chloroplast RNA-binding proteins: candidates as translational activators. EMBO J. **10**:3993–4001.
7. **Davies, D. R., and A. Plaskitt.** 1971. Genetical and structural analyses of cell-wall formation in *Chlamydomonas reinhardtii*. Genet. Res. **17**:33–43.
8. **Delepelaire, P.** 1984. Partial characterization of the biosynthesis and integration of the photosystem II reaction centers in the thylakoid membranes of *Chlamydomonas reinhardtii*. EMBO J. **3**:701–706.
9. **de Vitry, C., J. Olive, D. Drapier, M. Recouvreur, and F.-A. Wollman.** 1989. Posttranslational events leading to the assembly of photosystem II protein complex: a study using photosynthesis mutants from *Chlamydomonas reinhardtii*. J. Cell Biol. **109**:991–1006.
10. **Drapier, D., J. Girard-Bascou, and F.-A. Wollman.** 1992. Evidence for a nuclear control on the expression of the *atpA* and *atpB* chloroplast genes in *Chlamydomonas reinhardtii*. Plant Cell **4**:283–295.
11. **Gillham, N. W.** 1978. Organelle heredity. Raven Press, New York.
12. **Girard-Bascou, J., Y. Pierre, and D. Drapier.** 1992. A nuclear mutation affects the synthesis of the chloroplast *psbA* gene product in *Chlamydomonas reinhardtii*. Curr. Genet. **22**:47–52.
13. **Goldschmidt-Clermont, M.** 1991. Transgenic expression of aminoglycoside adenyl transferase in the chloroplast: a selectable marker for site-directed transformation in *Chlamydomonas reinhardtii*. Nucleic Acids Res. **19**:4083–4089.
14. **Goldschmidt-Clermont, M., Y. Choquet, J. Girard-Bascou, F. Michel, M. Schirmer-Rahire, and J.-D. Rochaix.** 1991. A small chloroplast RNA may be required for *trans*-splicing in *Chlamydomonas reinhardtii*. Cell **65**:135–143.
15. **Goldschmidt-Clermont, M., P. Malnoë, and J.-D. Rochaix.** 1989. Preparation of *Chlamydomonas* chloroplasts for the *in vitro* import of polypeptide precursors. Plant Physiol. **89**:15–18.
16. **Gruissem, W.** 1989. Chloroplast gene expression: how plants turn their plastids on. Cell **56**:161–170.
17. **Gruissem, W., B. M. Greenberg, G. Zurawski, and R. B. Hallick.** 1986. Chloroplast gene expression and promoter identification in chloroplast extracts. Methods Enzymol. **118**:253–270.
18. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Harris, E. H.** 1989. The *Chlamydomonas* sourcebook: a compre-

hensive guide to biology and laboratory use. Academic Press, Inc., San Diego, Calif.
20. **Hollingshead, S., and Vapnek, D.** 1985. Nucleotide sequence analysis of a gene encoding a streptomycin/spectinomycin adenyltransferase. Plasmid **13**:17–30.
21. **Jensen, K., D. L. Herrin, F. G. Plumley, and G. W. Schmidt.** 1986. Biogenesis of photosystem II complexes: transcriptional, translational, and post-translational regulation. J. Cell Biol. **103**:1315–1325.
22. **Johnson, C. H., and G. W. Schmidt.** 1993. The *psbB* gene cluster of the *Chlamydomonas reinhardtii* chloroplast: sequence and transcriptional analysis of *psbN* and *psbH*. Plant Mol. Biol. **22**:645–658.
23. **Kuchka, M. R., M. Goldschmidt-Clermont, J. van Dillewijn, and J.-D. Rochaix.** 1989. Mutation at the *Chlamydomonas* nuclear NAC2 locus specifically affects stability of the chloroplast *psbD* transcript encoding the polypeptide D2 of photosystem II. Cell **58**:869–76.
24. **Kuchka, M. R., S. P. Mayfield, and J.-D. Rochaix.** 1988. Nuclear mutations specifically affect the synthesis and/or degradation of the chloroplast-encoded D2 polypeptide of photosystem II in *Chlamydomonas reinhardtii*. EMBO J. **7**:319–324.
25. **Leibold, E. A., and H. N. Munro.** 1988. Cytoplasmic protein binds *in vitro* to a highly conserved sequence in the 5′ untranslated region of ferritin heavy- and light-subunit mRNAs. Proc. Natl. Acad. Sci. USA **85**:2171–2175.
26. **Monod, C., M. Goldschmidt-Clermont, and J.-D. Rochaix.** 1992. Accumulation of the chloroplast *psbB* RNA requires a nuclear factor in *Chlamydomonas reinhardtii*. Mol. Gen. Genet. **231**:449–459.
27. **Mulero, J. J., and T. D. Fox.** 1993. *PET111* acts in the 5′-leader of the *Saccharomyces cerevisiae* mitochondrial *COX2* mRNA to promote its translation. Genetics **113**:509–516.
28. **Nickelsen, J., and G. Link.** 1989. Interaction of a 3′ RNA region of the mustard *trnK* gene with chloroplast proteins. Nucleic Acids Res. **17**:9637–9648.
29. **Rochaix, J.-D.** 1978. Restriction endonuclease map of the chloroplast DNA of *Chlamydomonas reinhardtii*. J. Mol. Biol. **126**:597–617.
30. **Rochaix, J.-D.** 1992. Post-transcriptional steps in the expression of chloroplast genes. Annu. Rev. Cell Biol. **8**:1–28.
31. **Rochaix, J.-D., M. Kuchka, S. Mayfield, M. Schirmer-Rahire, J. Girard-Bascou, and P. Bennoun.** 1989. Nuclear and chloroplast mutations affect the synthesis or stability of the chloroplast *psbC* gene product in *Chlamydomonas reinhardtii*. EMBO J. **8**:1013–1021.
32. **Rochaix, J.-D., S. Mayfield, M. Goldschmidt-Clermont, and J. Erickson.** 1988. Molecular biology of *Chlamydomonas*, p. 253–275. *In* C. H. Shaw (ed.), Plant molecular biology: a practical approach. IRL Press, Oxford.
33. **Salvador, M. L., U. Klein, and L. Bogorad.** 1993. 5′ sequences are important positive and negative determinants of the longevity of *Chlamydomonas* chloroplast gene transcripts. Proc. Natl. Acad. Sci. USA **90**:1556–1560.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Samuels, M. E., D. Bopp, R. A. Colvin, R. F. Roscigno, M. A. Garcia-Blanco, and P. Schedl.** 1994. RNA binding by Sxl proteins in vitro and in vivo. Mol. Cell. Biol. **14**:4975–4990.
36. **Sieburth, L. E., S. Berry-Lowe, and G. W. Schmidt.** 1991. Chloroplast RNA stability in *Chlamydomonas*: rapid degradation of *psbB* and *psbC* transcripts in two nuclear mutants. Plant Cell **3**:175–189.
37. **Staub, J. M., and P. Maliga.** 1993. Accumulation of D1 polypeptide in tobacco plastids is regulated via the untranslated region of the *psbA* mRNA. EMBO J. **12**:601–606.
38. **Stormo, G. D., T. D. Schneider, and L. Gold.** 1982. Characterization of translational initiation sites in *E. coli*. Nucleic Acids Res. **10**:2971–2996.
39. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185**:60–89.
40. **Subramanian, A. R.** 1993. Molecular genetics of chloroplast ribosomal proteins. Trends Biochem. Sci. **18**:177–181.
41. **Woessner, J. P., A. Masson, E. H. Harris, P. Bennoun, N. W. Gillham, and J. E. Boyton.** 1984. Molecular and genetic analysis of the chloroplast ATPase of *Chlamydomonas*. Plant Mol. Biol. **3**:177–190.
42. **Zumbrunn, G., M. Schneider, and J.-D. Rochaix.** 1989. A simple particle gun for DNA-mediated cell transformation. Technique **1**:204–216.