Multiple Translational Control Sequences in the 5′ Leader of the Chloroplast *psbC* mRNA Interact With Nuclear Gene Products in *Chlamydomonas reinhardtii*

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ABSTRACT

Translation of the chloroplast *psbC* mRNA in the unicellular eukaryotic alga *Chlamydomonas reinhardtii* is controlled by interactions between its 547-base 5′ untranslated region and the products of the nuclear loci *TBC1*, *TBC2*, and possibly *TBC3*. In this study, a series of site-directed mutations in this region was generated and the ability of these constructs to drive expression of a reporter gene was assayed in chloroplast transformants that are wild type or mutant at these nuclear loci. Two regions located in the middle of the 5′ leader and near the initiation codon are important for translation. Other deletions still allow for partial expression of the reporter gene in the wild-type background. Regions with target sites for *TBC1* and *TBC2* were identified by estimating the residual translation activity in the respective mutant backgrounds. *TBC1* targets include mostly the central part of the leader and the translation initiation region whereas the only detected *TBC2* targets are in the 3′ part. The 5′-most 93 nt of the leader are required for wild-type levels of transcription and/or mRNA stabilization. The results indicate that *TBC1* and *TBC2* function independently and further support the possibility that *TBC1* acts together with *TBC3*.

PLASTIDS of plants and eukaryotic algae maintain and express the genomes they inherited from their endosymbiont bacterial ancestor(s). The translational machinery of plastids has many components in common with eubacterial translational systems, *e.g.*, 5S, 16S, 23S rRNAs, tRNAs, initiation factors, elongation factor EF-1A (EF-Tu), and ribosomal proteins (Harris *et al.* 1994; Sugiura *et al.* 1998). In most cases examined in *Chlamydomonas reinhardtii*, the Shine-Dalgarno (SD) sequence, which recruits the small ribosomal subunit to the initiation codon of bacterial mRNAs by base pairing with the 3′ end of the 16S rRNA, is not required for translation in plastids (Stern *et al.* 1997; Fargo *et al.* 1998; Zerges 2000; see discussion). Translation of plastid mRNAs occurs in the absence of the 5′ met ^{7}G cap structures and 3′ poly(A) tails used by cytoplasmic mRNAs to direct the small ribosomal subunit to their AUG initiation codons. Moreover, some similarities between translation in plastids and eukaryotic nuclear cytosolic systems have been found, including stimulatory effects of 3′ mRNA termini (Rott *et al.* 1998) and a possible role of a poly (A)-binding protein (reviewed by Danon 1997; Zerges 2000).

The importance of translational control sequences within the 5′ leaders of many chloroplast mRNAs has been revealed by fusing them to reporter genes and by their ability to confer translational regulation of the reporter by environmental factors such as light (Staub and Maliga 1993) or *trans*-acting factors (Nickelsen *et al.* 1994; Zerges and Rochaix 1994; Stampacchia *et al.* 1997; Zerges *et al.* 1997). For a few plastid mRNAs of *C. reinhardtii*, *cis*-acting translational control sequences have been identified within 5′ leaders by site-directed and random mutations (reviewed by Danon 1997; Stern *et al.* 1997; Bruick and Mayfield 1999; Nickelsen *et al.* 1999; Zerges 2000). Some appear to function as unstructured RNA, while others have secondary and tertiary structural features, which appear to be important (Rochaix *et al.* 1989; Mayfield *et al.* 1994; Higgs *et al.* 1999). Many *cis*-acting sequences could interact with protein factors, as determined by *in vitro* RNA-protein-binding assays (Zerges and Rochaix 1994), genetic experiments (Yohn *et al.* 1996, 1998), and *in vivo* footprinting with dimethyl sulfate (Higgs *et al.* 1999). Unlike most known eubacterial translational control elements, which are repressive and situated close to the translation initiation codon (Gold 1988), the characterized chloroplast elements have positive roles in translation and many are distant from the translation initiation

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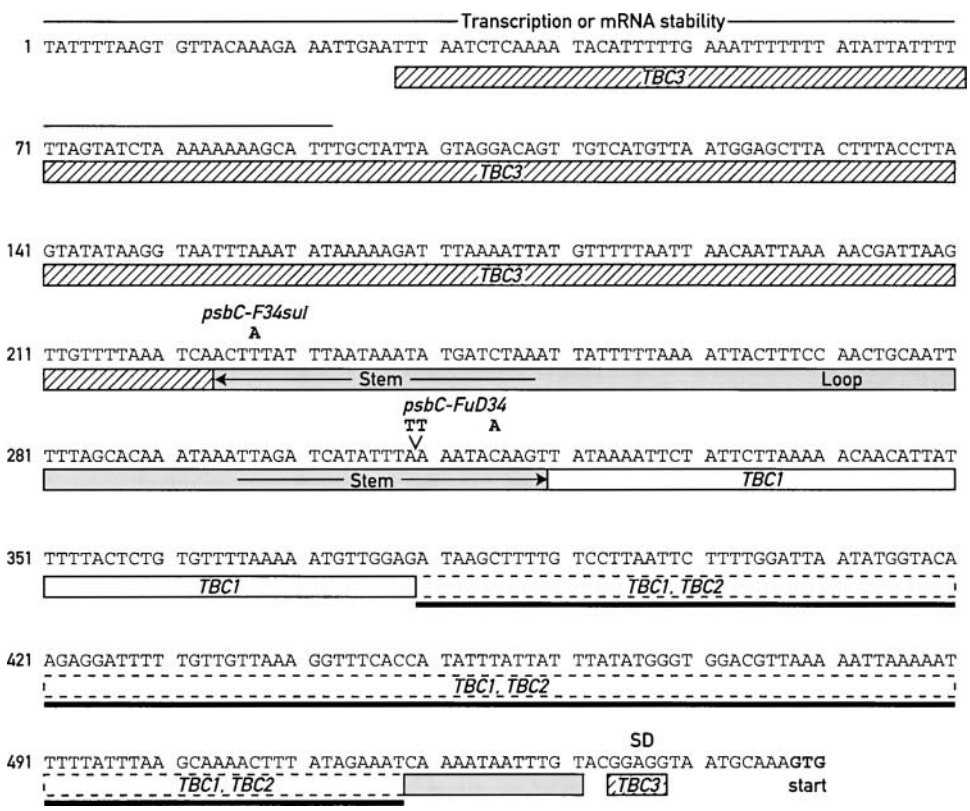


FIGURE 1.—Identification of functional regions within the *psbC* 5′ leader. The sequence of the 547-nt *psbC* 5′ leader (ROCHAIX *et al.* 1989) is shown with the relevant regions indicated as follows. Horizontal arrows indicate the complementary sequences that result from two inverted repeats in the DNA from which the 5′ leader is transcribed. Regions that are partially required for translation are indicated with striped boxes (27–222) and open boxes (321–378). The regions that are important for translation are indicated with a shaded box: 223–320 and 519–532. "*TBC1*" and "*TBC3*" indicate the target regions of the factors that are encoded or controlled by these loci. The region that is dispensable for translation and partially required for the interactions with *TBC1* and *TBC2* is indicated by a box with a dashed border and is underlined. The GUG initiation codon is in boldface type. The SD-like sequence (GGAGG) is indicated as "SD." The mutations *psbC-FuD34* and *psbC-F34suI* are indicated.

codon in the 5′ leader (MAYFIELD *et al.* 1994; SAKAMOTO *et al.* 1994; ZERGES *et al.* 1997; reviewed by DANON 1997; STERN *et al.* 1997; BRUICK and MAYFIELD 1999; ZERGES 2000).

Translation of the *psbC* mRNA in the chloroplast of the unicellular eukaryotic alga *C. reinhardtii* is controlled by interactions between its 547-nucleotide (nt) 5′ leader and the products of the nuclear loci *TBC1*, *TBC2*, and *TBC3*. (*psbC* encodes the 51-kD chlorophyll-binding PSII reaction center subunit P6, the homolog of the CP43 protein in vascular plants.) The region of the *psbC* 5′ leader between positions 222 and 320 is required for translation initiation (ZERGES *et al.* 1997). (The 5′ end of the mRNA is +1.) This central region has the potential to form a stable stem-loop secondary structure (see Figure 1; ROCHAIX *et al.* 1989). Two bulges in the stem, caused by two sites of noncomplementarity between the strands, are essential for translation because a mutation that eliminates them, *psbC-FuD34* (Figure 1), completely abolishes translation of the mRNA (ROCHAIX *et al.* 1989; ZERGES *et al.* 1997). A point mutation at position 227 within this region (Figure 1, *psbC-F34suI*) partially reverses the translation block caused by the *TBC1* mutation, but not that caused by a *TBC2* mutation. Thus, this position is critical for the *trans*-acting role of *TBC1* (ROCHAIX *et al.* 1989; ZERGES and ROCHAIX 1994).

A dominant nuclear suppressor mutation partially reverses the translational blocks caused by mutations of this central region (*psbC-FuD34* and a deletion of this region) and *tbc1-F34*. This suppressor mutation has a weak phenotype on its own and induces a twofold decrease in the rate of synthesis and in the accumulation of P6 (ZERGES *et al.* 1997). Thus, this suppressor mutation identifies a third nuclear locus, *TBC3*, which could be involved in the initiation of translation of *psbC* mRNA, possibly through other factors. Like the *cis*-acting suppressor mutation *psbC-F34suI*, the *tbc3-rb1* suppressor mutation does not reverse the translational block caused by a mutation in *TBC2*, *tbc2-F64* (ZERGES *et al.* 1997). Our current model is described in the DISCUSSION.

While *TBC1* and *TBC3* have not yet been characterized at the molecular and biochemical levels, the *TBC2* coding sequence predicts a protein of 1115 residues with nine copies of a novel degenerate 39-amino-acid repeat with a quasi-conserved PPPEW motif near its C-terminal end (AUCHINCLOSS *et al.* 2002). The central region of the protein displays partial amino acid sequence identity with Crp1, a protein in *Zea mays* that is implicated in the processing and translation of the chloroplast *petA* and *petD* mRNAs. The Tbc2 protein cofractionates with chloroplast stroma, is associated with a 400-kD protein complex, and has been proposed to play a role in the specific translation of the *psbC* mRNA.

In this study, the *psbC* 5′ leader was dissected for sequences that control translation and interact, directly or indirectly, with the *TBC1*, *TBC2*, and *TBC3* gene products. We have generated mutant and hybrid *psbC* 5′ leaders and examined their ability to mediate a re-

sponse to the products of the nuclear genes *TBC1*, *TBC2*, and possibly *TBC3*.

MATERIALS AND METHODS

Construction of *psbC* 5′ leader deletions: To construct the mutant 5′ leaders shown in Figure 2A, DNA fragments that flank the deletions were generated as two series. In one series fragments extend from position −68 bp [with respect to the site corresponding to the 5′ terminus of mature *psbC* mRNA (Rochaix *et al.* 1989)] to various positions within the 5′ leader (93, 167, 222, 320, 391, 467, 519, and 532; see Figures 1 and 2A). In the second series, DNA fragments extend from the GUG initiation codon in an upstream direction to various endpoints within the 5′ leader (positions 484, 475, 408, 379, 321, 223, 168, 95, 27, and 3; see Figures 1 and 2A). To generate many of these 5′ leader subfragments, two series of deletions were generated with *Exo*III. First, a fragment of the *psbC* 5′ flanking and 5′ leader sequences (between positions −68 and +550, the third base of the GUG initiation codon), which had been generated by PCR and cloned in a previous study (Zerges and Rochaix 1994), was excised by digestion with *Cla*I and *Bam*HI, rendered blunt at its ends, and cloned into the *Hinc*II site of the pBluescribe vectors pBS+ and pBS− (Stratagene, La Jolla, CA). Each recombinant plasmid was digested with *Bam*HI and *Kpn*I, which cleave adjacent to and on the same side of the insert, for the pBS+ at the distal side and for the pBS− clone at the proximal side, *i.e.*, near the GUG initiation codon. Short treatments (30–90 sec) with *Exo*III and S1 nuclease (at 30°) followed by intramolecular ligation (Guo and Wu 1983; Sambrook and Russell 2001) generated a series of plasmids with varying amounts of sequences deleted from one end of the 5′ leader or the other. Deletion endpoints were determined by DNA sequencing (Sambrook and Russell 2001). Various combinations of these partial 5′ leaders were combined by ligating *Hin*dIII (rendered blunt)-*Sac*I fragments with 5′ flanking and distal 5′ leader regions into plasmids with the proximal 5′ leader sequences digested with *Sac*I and *Hin*dIII (rendered blunt). Other endpoints were generated by PCR, cloned, sequenced to verify that no errors had occurred during PCR (Sambrook and Russell 2001), and used to generate 5′ leader deletion mutants in similar cloning steps to create the set of deletion-mutant 5′ leaders shown in Figure 2A. Mutagenesis of the SD-like sequence was performed by PCR with an oligonucleotide that hybridizes 67–81 nt upstream from the 5′ end of the transcription unit and a mutagenic oligonucleotide (5′ CCATGGACACTTTGCA TTAGGAGGGTACAAATTATTTTGATT 3′), which hybridizes to sequences from 516 to 551 (Figure 1) and introduces a CCTCC in place of the SD-like sequence, GGAGG.

The resulting fragments with the 5′ flanking region and mutant 5′ leaders were excised from their plasmid vector by digestion with *Cla*I and *Nco*I. The corresponding restriction sites were present within the 5′ and 3′ oligonucleotides. They were ligated to similarly digested cg20-atpB-INT, a chloroplast transformation vector containing the *aadA* coding sequence translationally fused within the *Nco*I site. Fused to the 3′ end of *aadA* are the 3′ untranslated region (UTR) and 3′ flanking sequence of the chloroplast *rbcL* gene, which serves to generate processed transcripts of uniform length (Goldschmidt-Clermont 1991).

To generate the hybrid *psbC-atpA* 5′ leader, PCR was used to amplify a DNA fragment with the oligonucleotide that hybridizes 67–81 nt upstream of the 5′ end and an oligonucleotide that hybridizes immediately 5′ to the SD-like element of *psbC*. This fragment was cloned into a plasmid vector and sequenced to exclude mutations introduced during PCR. This fragment was excised by digestion at the *Cla*I and *Xho*I sites introduced at its 5′ and 3′ extremities, respectively, and ligated into similarly digested p-atpB-INT, a plasmid containing a chimeric gene with the *atpA* 5′ leader translationally fused to *aadA*, containing the *rbcL* 3′ untranslated region and flanking sequences (Goldschmidt-Clermont 1991). Digestion of this plasmid with *Cla*I and *Xho*I excises most of the *atpA* 5′ flanking region and 5′ leader, but not the proximal 57 nt and ATG initiation codon (Figure 4; Dron *et al.* 1982; Leu *et al.* 1992). This resulted in a fusion of the distal 533 nt of the *psbC* 5′ leader, up to but not including the SD-like sequence, to the proximal 57 nt of the *atpA* 5′ leader, translationally fused to the *aadA* coding sequence.

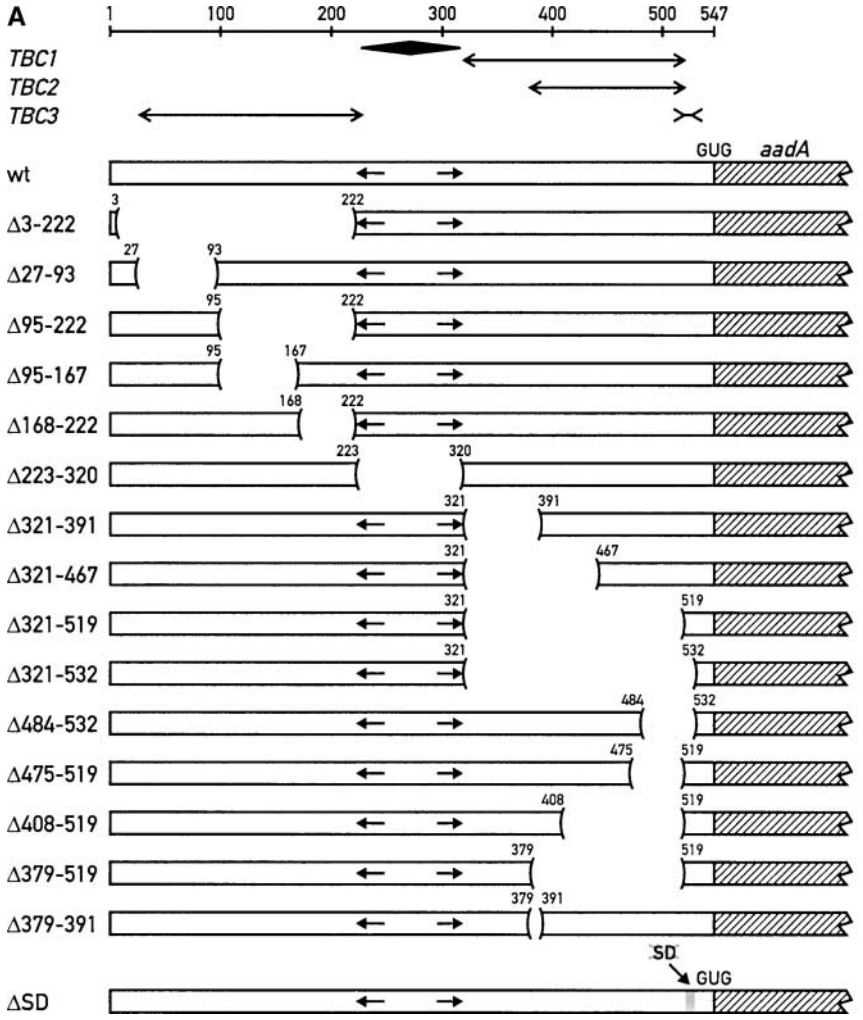
These chloroplast transformation vectors have the *atpB* gene as a marker to select for rescue of the photosynthesis deficiency of *FuD50*, a mutant with a deletion of the 3′ portion of this chloroplast gene (Woessner *et al.* 1986; Goldschmidt-Clermont 1991). Thus, following delivery of the DNA into the chloroplast by a biolistic particle gun (Zumbrunn *et al.* 1989), stable insertions of the reporter and marker genes by homologous recombination can be selected on "minimal" medium, which requires photosynthesis for growth. Homoplasmic transformants (strains with all copies of the multicopy chloroplast genome transformed) were selected on minimal medium and identified by genomic Southern blot analysis as described previously (Zerges and Rochaix 1994).

Culture conditions and genetic analyses: Strains were grown in Tris/acetate/phosphate medium (Gorman and Levine 1965). Cultures were grown under a light intensity of 100–150 $\mu E/m^2/sec$. Induction of gametes, crosses, maturation of zygotes, and tetrad dissections were carried out as described previously (Goldschmidt-Clermont *et al.* 1990). Mutant strains carried *tbc1-F34*, *tbc2-F64*, or *tbc3-rb1* (Rochaix *et al.* 1989; Zerges and Rochaix 1994; Zerges *et al.* 1997). Growth tests were performed by spotting 10 μl of each culture (10^3 cells) on media solidified with agar (Difco) and varying concentrations of spectinomycin [spc (Sigma, St. Louis); corrected for its partial purity] in petri plates and observed for growth and photographed after 5–7 days of incubation at 24° under dim light. Growth is defined as the appearance of an even distribution of cells throughout the circular area that received the inoculated cells. Cell growth was equal to that observed for cells grown in the absence of spectinomycin. Papillary growth occurring over longer periods was excluded and considered as nongrowth. To distinguish wild-type and mutant progeny for *TBC3*, *mt*− progeny were testcrossed to *FuD34 (mt+)* and their progeny were tested for phototrophic growth on minimal medium.

RNA-gel blot analyses: Samples (10 μg) of total RNA prepared with Tri-reagent (Sigma) were electrophoresed through a 1% agarose gel (containing formaldehyde), transferred to Hybond-C+ membrane (Amersham, Buckinghamshire, UK), and probed with double-stranded DNA probes, which had been labeled with random primers using [α-^{32}P]dATP (Sambrook and Russell 2001). The *aad*A probe was a 0.81-kb *Nco*I-*Pst*I cloned DNA fragment corresponding to the *aad*A structural gene (Goldschmidt-Clermont 1991). The relative amounts of the RNA in the samples were standardized by probing the blots with either ^{32}P-labeled DNA fragments derived from *psbC* [0.95-kb *Hin*dIII fragment from the chloroplast DNA fragment R9 (Rochaix 1978)] or a *Pst*I cDNA fragment from the nuclear *rbcS2* gene (Goldschmidt-Clermont and Rahire 1986).

RESULTS

To identify regions of the 547-nt *psbC* 5′ leader that promote translation and control it through interactions



B					
TBC1	wt	m	wt	wt	
TBC2	wt	wt	m	wt	RNA
TBC3	wt	wt	wt	m	
wt	5000	<50	<50	>1000	+
Δ3-222	<50	<50	<50	n.d.	–
Δ27-93	50	<50	<50	50	+/–
Δ95-222	50	<50	n.d.	50	+
Δ95-167	50	<50	<50	50	+
Δ168-222	50	<50	<50	50	+
Δ223-320	<50	<50	<50	250	+
Δ321-391	100	100	<50	1000	+
Δ321-467	100	n.d.	n.d.	n.d.	+
Δ321-519	500	n.d.	n.d.	n.d.	+
Δ321-532	50	50	n.d.	n.d.	+
Δ484-532	<50	n.d.	n.d.	250	+
Δ475-519	2000	50	50	n.d.	+
Δ408-519	1000	100	50	n.d.	+
Δ379-519	2500	50	50	3000	+
Δ379-391	3000	100	<50	n.d.	+
ΔSD	100	<50	<50	100	+

FIGURE 2.—Analyses of the effects of *psbC* 5′ leader deletions on spc resistance conferred by chimeric *psbC-aadA-rbcL* reporter genes. (A) The predicted stem-loop structure (diamond-shaped) and the target regions of *TBC1*, *TBC2*, and *TBC3* are indicated at the top. The *psbC* 5′ leader is shown with the series of deletion mutants. (B) Levels of spc resistance are given for strains that are wild type (wt) or mutant (m) for *TBC1*, *TBC2*, or *TBC3*. n.d., not determined. RNA levels are indicated by + (wild-type level), +/− (low), and − (undetectable).

with the products of *TBC1*, *TBC2i*, and *TBC3*, a series of mutant 5′ leaders was generated (Figure 2A). Each was fused to the *aadA* reporter gene, which was linked previously to the 3′ UTR of the chloroplast *rbcL* gene (ZERGES and ROCHAIX 1994). The resulting chimeric *psbC-aadA-rbcL* reporter genes were introduced into the chloroplast genome by biolistic transformation (see MATERIALS AND METHODS).

We decided to use *aadA* as a reporter for this study because detection of P6 synthesis by *in vivo* pulse labeling does not provide a very sensitive assay as the signal drops below background levels when the rate of synthesis of P6 is <20–30% of the wild-type level. In contrast, spc resistance conferred by *aadA* provides a highly sensitive assay. We acknowledge that the lack of a calibration curve between spectinomycin resistance and *aadA* expression at low resistance levels makes some of our measurements qualitative. Nevertheless, the results described below demonstrate clear and reproducible differences in spc resistance levels between wild-type and mutant nuclear backgrounds, which reveal differences in requirements for 5′ UTR sequences for the interactions with the three nucleus-encoded functions.

To distinguish translational effects from effects on earlier steps of gene expression, it was necessary to determine the level of accumulation and the size of each chimeric mRNA expressed in the chloroplast of the transformants. As seen in Figure 3, RNA-gel blot analyses revealed that the deletions alter the electrophoretic mobility of the chimeric mRNAs consistent with the lengths of the deleted regions. Deletions of sequences between positions 95 and 532 do not significantly reduce the steady-state levels of the chimeric mRNAs (Figure 3). However, reduced mRNA levels were detected from the chimeric genes with deletions Δ3–222 and, to a lesser extent, Δ27–93 (Figure 3). Thus, sequences within the

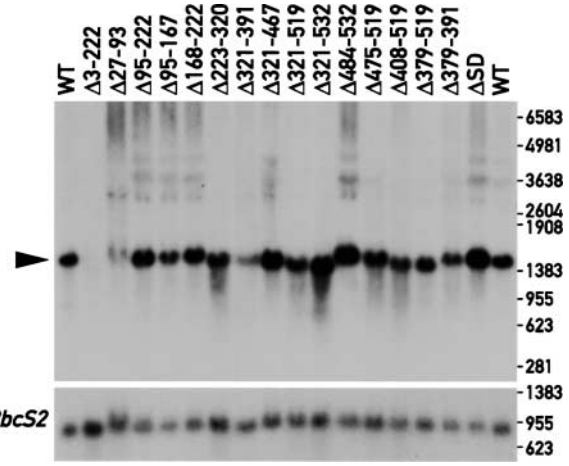


FIGURE 3.—RNA-gel blot analysis of the *psbC-aadA-rbcL* chimeric mRNAs. Total RNA isolated from homoplasmic transformants was fractionated by agarose gel electrophoresis, blotted, and hybridized with a probe corresponding to the *aadA* coding sequence to detect the *psbC-aadA-rbcL* chimeric mRNA (indicated by an arrowhead). To control for the amounts of RNA loaded in each lane, the filter was hybridized with a *RbcS2* probe.

5′-most 93 nt of the 5′ leader are required for transcription, 5′ end processing (ROCHAIX 1996), and/or mRNA stabilization (NICKELSEN 1998). It appears that the mRNA with the Δ27–93 deletion is partially active in translation because *aadA* expression was detected (Figure 2). Replacement of the SD-like sequence (GGAGG, positions 535–539) in some experiments was observed to moderately increase the level of the mRNA (Figure 3), while in other trials no increase was observed (data not shown).

Two regions of the *psbC* 5′ leader are required for translation and are separated by a dispensable region: To identify regions of the *psbC* 5′ leader that control translation from its initiation codon, the level of *aadA* reporter gene expression was determined by assaying the degree of spc resistance of at least five transformants for each chimeric reporter gene. Spc resistance is a measure of *aadA* expression because it correlates with levels of the streptomycin/spectinomycin adenyltransferase protein and its activity (ZERGES *et al.* 1997; FARGO *et al.* 1998, 1999, 2001). [Our antiserum against this protein (ZERGES and ROCHAIX 1994) lost its specificity during storage and could not be used.] Growth was assayed in the presence of spc at concentrations of 50, 100, 250, 500, 750, and 1000 μg/ml. Strains resistant to 1000 μg/ml spc were tested for growth in the presence of 1500, 2000, 3000, 4000, 5000, and 6000 μg/ml spc. The maximum spc concentration to which each chimeric reporter gene conferred resistance is shown in the first column of Figure 2B. For example, the chimeric gene with the wild-type *psbC* 5′ leader confers resistance to 5000 μg/ml spc, but not to 6000 μg/ml. Mutations that alter spc resistance without producing a corresponding change in the steady-state level of the chimeric mRNA should affect translation from the *psbC* 5′ leader.

Translational effects were observed for most mutations. Deletions affecting sequences 3′ to position 95 do not drastically alter the level of the mRNA and vary in their ability to promote translation, as revealed by the variable levels of spc resistance that they confer. For example, deletions of sequences located between positions 379 and 519 (Δ379–519, Δ408–519, Δ475–519) do not dramatically alter the level of spc resistance and, therefore, the level of translation of the *aadA* reporter gene; transformants expressing these chimeric mRNAs are resistant to spc concentrations between 1000 and 3000 μg/ml (Figure 2B, column 1). Thus, sequences in the 379–519 region are only weakly required for translation (Figure 2).

In contrast, two regions are required for translation. The larger is located between positions 95 and 378 and appears to contain distinct elements, which are required to varying degrees. For example, deletions affecting sequences between positions 95 and 222 (Δ95–222, Δ95–167, and Δ168–222) drastically lower the level of spc resistance to 50 μg/ml, relative to the chimeric mRNA with the wild-type 5′ leader (Figure 2B). The Δ27–93 deletion results in a reduced level of the chimeric mRNA and confers a level of spc resistance similar to that of Δ95–222, Δ95–167, and Δ168–222, suggesting a less stringent requirement for sequences in the 27–93 interval than in the 95–222 interval. As reported previously, the central region of the *psbC* 5′ leader has the potential to form a stable stem-loop structure (ROCHAIX *et al.* 1989) and is required for translation (ZERGES *et al.* 1997). Immediately downstream of this central element are 58 nt that are partially required for translation; deletions removing the bases 321–391 reduce spc resistance (100 μg/ml). The finding that Δ379–519 does not significantly reduce spc resistance delimits this partially required region to positions 321–378. The second region required for translation, located between 519 and 532 close to the GUG initiation codon, is defined by two deletions (Δ321–532 and Δ484–532) that severely reduce spc resistance without affecting the chimeric mRNA levels and deletions (Δ379–519, Δ408–519, and Δ475–519) that still allow for high spc resistance. This required region could extend to the SD-like element (GGAGG, positions 534–538) because its replacement considerably reduces spc resistance (to 100 μg/ml spc, Figure 2B). Translation is probably more drastically reduced because this SD-like sequence replacement was observed in many experiments to augment the level of the mRNA by severalfold (Figures 3).

***cis*-acting targets of *TBC1* and *TBC2*:** The previous finding that the 5′ leader of the *psbC* mRNA can confer all genetic interactions with mutant alleles of *TBC1*, *TBC2*, and *TBC3* upon expression of the *aadA* reporter gene (ZERGES and ROCHAIX 1994; ZERGES *et al.* 1997) indicates that these nuclear loci either encode or con-

trol factors that interact with the *psbC* 5′ leader and are involved in its ability to promote translation from its GUG initiation codon (see Introduction).

To identify regions in the *psbC* 5′ leader that are involved in these interactions, for example, RNA sequences or structures that bind to regulatory factors encoded or controlled by *TBC1* or *TBC2*, we first asked whether the mutant *TBC1* and *TBC2* alleles alter expression of the *aadA* reporter gene from the mutant *psbC* 5′ leaders. It was possible to look only at effects on expression from the mutant 5′ leaders that give a detectable level of expression in the presence of wild-type *TBC1* and *TBC2* alleles. We expected that deletion of a target RNA element could result in independence from the wild-type *TBC1* or *TBC2* functions.

Transformant strains carrying a mutant chimeric reporter gene and wild type for *TBC1* and *TBC2* were crossed individually to strains carrying mutant alleles for either *TBC1* or *TBC2*. As the resulting progeny are haploid and their endogenous *psbC* gene is wild type, the wild-type and mutant progeny for the nuclear locus could be distinguished by their ability to grow photoautotrophically (*i.e.*, in a medium that selects for photosynthesis) or not, respectively, and by analyses of their fluorescence transients (Bennoun and Beal 1997). Moreover, unlike mutations that abolish photosynthesis, 5′ leader mutations that abolish expression of *aadA* produce no effect on viability in the absence of spc and thus impose no selective pressure for reversion.

The chimeric reporter gene is inherited by >95% of progeny because the chloroplast genome is inherited in a non-Mendelian fashion, predominantly from the mating-type plus (*mt*+) parent. To confirm the presence of the chimeric gene in the progeny analyzed and to exclude possible effects of the *TBC1* or *TBC2* mutations on the level of the chimeric mRNAs, total RNA was prepared from three progeny strains (wild type, *tbc1* mutant, or *tbc2* mutant) and analyzed by RNA-gel blotting. The presence of the *psbC-aadA-rbcL* mRNA revealed that the chimeric reporter gene was inherited by all progeny tested (data not shown). Although some variations in the levels of certain chimeric mRNAs were observed between different experiments and preparations, none could fully account for the effects of the *TBC1* or *TBC2* mutations on spc resistance (as described below). Moreover, the mutant alleles of *TBC1* or *TBC2* were not associated with any reproducible alterations of the electrophoretic mobility of the chimeric mRNAs.

The level of spc resistance was determined for 5–20 progeny strains (from the crosses described above) for the translatable mutant chimeric genes and for either mutant or wild type for *TBC1* or *TBC2* (Figure 2B). Wild-type progeny for *TBC1* and *TBC2* showed the same level of spc resistance as their parental strain did, as both carry the same chimeric reporter gene. Differing levels of spc resistance were observed for the PSII-deficient progeny that had inherited a mutant allele of either *TBC1* or *TBC2* (Figure 2B, columns 2 and 3).

As described above, the deletions that remove sequences between positions 27 and 222 (Δ27–93, Δ95–222, Δ95–167, and Δ168–222) strongly diminish translation. Progeny strains that inherited a mutant allele of either *TBC1* or *TBC2* are sensitive to 50 μg/ml spc (Figure 2B). Thus, that translation from these mutant 5′ leaders still requires the wild-type functions of *TBC1* and *TBC2* indicates that sequences between positions 27 and 222 probably do not contain major *cis*-acting sequences that are required for the interactions with these nuclear gene products. Similarly, the SD-like sequence (positions 534–538, Figure 1) also does not appear to constitute an essential part of the *TBC1* or *TBC2* *cis*-acting targets because the level of spc resistance conferred by the chimeric mRNA without this sequence was abolished by the mutant alleles of *TBC1* and *TBC2* (Figure 2B).

Because the deletions Δ223–320 and Δ484–532 abolish translation of the chimeric mRNAs (neither mRNA confers spc resistance), it was not possible to determine whether the *Tbc1* and *Tbc2* products require these 5′ leader sequences to promote translation. However, the previous finding that the point mutation at position 227 (Figure 1) suppresses the mutant phenotype produced by *tbc1-F34* (but not by a mutant allele of *TBC2*) revealed a functional relationship between this site and *TBC1* function (Rochaix *et al.* 1989; Zerges and Rochaix 1994).

***cis*-acting target sequences of *TBC1* are located between positions 321 and 519 of the *psbC* 5′ leader:** In addition to this genetic interaction of *TBC1* with position 227 of the 5′ leader, sequences located 3′ to the central region also appear to be involved in the interaction with *TBC1*. The 5′ leader with deletion Δ321–391 is exceptional in that the mutant *tbc1* allele does not diminish translation further; both wild-type and mutant progeny for *TBC1* are resistant to 100 μg/ml spc (Figure 2B). The major *TBC1* target within this region appears to be confined between 321 and 378 because Δ379–391 drives only a low level of expression in the *TBC1* mutant background. The importance of the 321–378 region was observed only for *TBC1*, as the mutant *tbc2* allele further reduces translation of the chimeric mRNA with the Δ321–391 deletion: the progeny with the mutant *TBC2* allele are sensitive to the lowest spc concentration tested (50 μg/ml; Figure 2B).

The *tbc1* and *tbc2* mutant alleles showed more complex interactions with chimeric mRNAs with deletions of sequences between positions 379 and 519 (Δ379–519, Δ408–519, Δ475–519). Recall that these deletions only partially lower the level of spc resistance in the presence of wild-type alleles for both *TBC1* and *TBC2* (Figure 2B, column 1). Expression of *aadA* from the chimeric mRNAs with these deletions was drastically reduced in the progeny that inherited the mutant allele of either

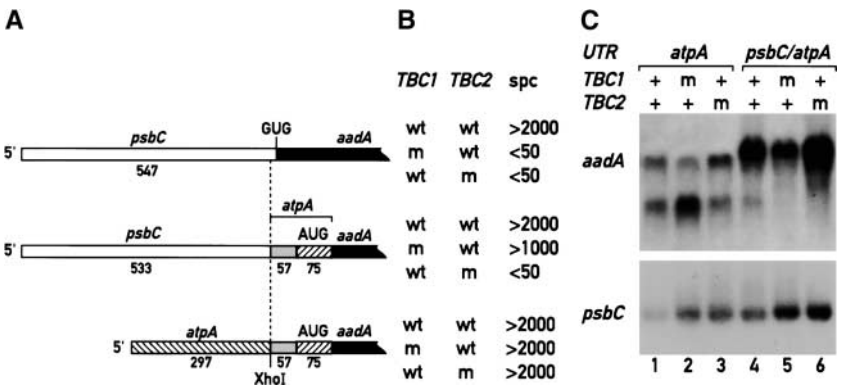


FIGURE 4.—Analysis of chimeric *psbC-atpA* 5′ leaders. (A) Maps show the 5′ leaders of the chimeric genes *psbC-aadA-rbcL*, *psbC-atpA-aadA-rbcL*, and *atpA-aadA-rbcL*. The initiation codons are indicated. The *psbC-atpA* 5′ leader consists of the 533 5′-terminal nt of the *psbC* 5′ leader and the 57 3′-terminal nt of the *atpA* 5′ leader (indicated by a shaded box). It is followed by the 75 5′-terminal nt of the *atpA* coding sequence, which had been fused to *aadA* (GOLDSCHMIDT-CLERMONT 1991). (B) spc resistance levels conferred by the chimeric genes in strains with nuclear backgrounds that are wild type or mutant for *TBC1* or *TBC2*. (C) RNA-gel blot analysis of the chimeric mRNAs of A in the wild-type, *TBC1*, and *TBC2* mutant background. The filter was hybridized with a probe specific for the *psbC* coding region (which is not present in the reporter gene) to control for the amount of total RNA in each lane. The lower RNA band observed in lanes 1–4 is due to some unknown processing event and has been observed previously (GOLDSCHMIDT-CLERMONT 1991).

TBC1 or *TBC2*. But unlike the expression of mRNA bearing the wild-type *psbC* 5′ leader, expression of these mutant mRNAs was not completely abolished by the *trans*-acting nuclear mutations (Figure 2B, columns 2 and 3). These deletions thus weakly suppress the *tbc1* and *tbc2* mutations. The observation that translation from mutant 5′ leaders with sequences between 379 and 519 deleted is only partially dependent upon the wild-type functions *TBC1* and *TBC2* suggests that sequences in this region mediate some of the *trans*-acting effects of these nuclear gene products.

The response to *TBC1*, but not *TBC2*, requires the *psbC* translation initiation region: It was not possible to determine whether deletion of the *psbC* translation initiation region (the 14 nt with the SD-like element and initiation codon, Figure 1) affects the dependencies on the wild-type functions of the nuclear loci because it is fundamentally required for translation. Therefore, these sequences were replaced with the 57 3′-terminal nt of the 5′ leader of the *atpA* mRNA and its ATG initiation codon (Figure 4). The *psbC-atpA-aadA-rbcL* reporter gene was integrated into the *C. reinhardtii* chloroplast genome, homoplasmic transformant strains were obtained, and they were crossed to *mt*− strains that carry a mutant allele for either *TBC1* or *TBC2* (MATERIALS AND METHODS). To control for any effects of the *TBC1* and *TBC2* mutations on translation from the *atpA* AUG initiation codon, transformants for an *atpA-aadA-rbcL* chimeric reporter gene (GOLDSCHMIDT-CLERMONT 1991) were analyzed in parallel.

RNA-gel blot analyses of the mRNAs expressed from both chimeric genes, *atpA-aadA-rbcL* and *psbC-atpA-aadA-rbcL*, revealed that these mRNAs accumulate and neither this accumulation nor the molecular weights of the mRNAs are significantly altered by the presence of the mutant alleles of *TBC1* or *TBC2* in the nuclear background (Figure 4). Tests of spc resistance revealed that translation of the *atpA-aadA-rbcL* mRNA is independent of the wild-type *TBC1* and *TBC2* functions: mutant progeny for either loci are as spc resistant as their wild-type siblings (Figure 4). Translation of the *psbC-atpA-aadA-rbcL* chimeric mRNA was also unaltered by the mutant allele of *TBC1*: these progeny are nearly as spc resistant as their wild-type siblings. Thus, replacement of the *psbC* translation initiation region alleviates the requirement for the wild-type *TBC1* function. Strains with the *psbC-atpA-aadA-rbcL* chimeric reporter gene and the mutant *TBC2* allele, however, are severely affected in the translation of the *psbC-atpA-aadA-rbcL* chimeric mRNA; they are sensitive to low levels of spc (Figure 4). Thus, the 5′-terminal 533 nt of the *psbC* 5′ leader can confer the requirement for wild-type *TBC2* function across 57 nt of *atpA* 5′ leader sequences to translation initiation at its AUG initiation codon. This excludes the involvement of the GUG initiation codon, and sequences immediately 5′ to it, in the role of *TBC2*.

Effect of *tbc3-rb1* on mutant *psbC* 5′ leaders: Because *tbc3-rb1* has been characterized only genetically as a dominant suppressor of mutations affecting the *psbC* 5′ leader and the *trans*-acting *TBC1* locus without null or loss-of-function alleles we are unable to make any firm inferences regarding TBC3 function or its mechanism of action. Nevertheless, we were able to localize regions of the *psbC* 5′ leader that contain sequences that interact with TBC3 either directly or indirectly. We tested the ability of the *tbc3-rb1* suppressor mutation to stimulate translation of the *psbC-aadA-rbcL* mRNAs with the deletions that diminish translation and do not prevent accumulation of the mRNA (*i.e.*, Δ95–167, Δ168–222, Δ223–320, Δ321–391, Δ321–319, Δ484–532, ΔSD). Deletions that are not suppressed could affect sequences that are required for some interaction with *TBC3*, while suppression would indicate that the deleted sequences are not required. Transformant strains (*mt*+) carrying a mutant chimeric *psbC-aadA-rbcL* reporter gene were crossed to a *mt*− strain carrying the *tbc3-rb1* mutation. All progeny inherited the chimeric reporter gene in the chloroplast genome and either the wild-type or the mutant *TBC3* allele. To exclude effects of the *tbc3-rb1* suppressor mutation on transcription or stability of the chimeric reporter

mRNAs, RNA-gel blot analyses were performed. For each deletion the levels of RNA were comparable in the presence of the wild-type or *tbc3* mutant allele (data not shown).

As shown previously, the *tbc3-rb1* mutation partially restores translation from *psbC* 5′ leaders with either the Δ223–320 deletion or the *psbC-FuD34* mutation (Rochaix *et al.* 1989; Zerges *et al.* 1997). Similarly, the levels of spc resistance conferred by chimeric genes with the deletions Δ321–391 and Δ484–532 revealed that translation is stimulated by the *tbc3-rb1* suppressor allele in the absence of these sequences (Figure 2B, column 4). In contrast, *tbc3-rb1* was unable to increase translation in mutants affected in two distinct regions of the *psbC* leader: deletions Δ27–93, Δ95–167, Δ168–222, Δ95–222, and the SD-like sequence (Figure 2). In both cases wild-type and mutant progeny for *TBC3* showed similar low, but detectable, levels of spc resistance (Figure 2B, column 4). Thus, the *TBC3* suppressor mutation is able to increase the level of expression from mutant *psbC* leaders affected in the middle part, but not from those with lesions in the 5′- and 3′-terminal part. The mechanistic significance of these data will have to await the molecular identification of the TBC3 product.

DISCUSSION

In prokaryotes, translation is most frequently regulated by repressive *cis*-acting elements, which form secondary structures or bind to proteins that block access of the small ribosomal subunit to the translation initiation region (Gold 1988; Kozak 1999). This led us to expect repressive sequences in the *psbC* 5′ leader, which, when deleted, would result in constitutive translation of the mRNA that is independent of the wild-type functions of the *TBC1* and *TBC2* loci (Rochaix *et al.* 1989). However, similar to results of analyses of other chloroplast 5′ leaders (Sakamoto *et al.* 1994; Higgs *et al.* 1999; Nickelsen *et al.* 1999), we found no repressive sequences; none of the 5′ leader deletions resulted in an increased level of expression in otherwise wild-type strains (Figure 2). In addition, insertion of overlapping *psbC* 5′ leader regions and of the entire 5′ leader into the *atpA* leader did not render *aadA* expression dependent upon wild-type *TBC1* and *TBC2* loci (W. Zerges and J. D. Rochaix, unpublished data). These data suggest that *TBC1* and *TBC2* and their *cis*-acting target sequences activate translation (and that these functions can be replaced by analogous activators in the *atpA* 5′ leader). Thus, unlike eubacterial translational regulatory elements, sequences throughout the *psbC* 5′ leader are required for translation, the largest region being separated from the translation initiation region by 140 nt of dispensable sequences within the 379–519 interval.

Five regions containing *cis*-acting sequences that are required for translation were identified. Partial requirements were found for sequences in three regions: 95–222, 321–378, and the SD-like sequence GGAGG, located 10 nt upstream of the GUG initiation codon (positions 534–538; see Figure 1). Two regions are important for translation: the central region between positions 223 and 320 (Zerges *et al.* 1997) and, at the 3′ end of the 5′ leader, sequences between positions 519 and 532.

The translational requirement for the SD-like sequence indicates that it could function by base pairing with the 3′ end of the 16S rRNA to position the small ribosomal subunit at the initiation codon. However, this sequence could be part of a larger translational element comprising the 28 nt 5′ to the initiation codon, as sequences immediately 5′ to the SD-like sequence are strictly required for translation (Figures 1 and 2).

Site-directed replacement mutations of the SD-like sequences of five mRNAs [*petD* (Sakamoto *et al.* 1994), *atpB*, *atpE*, *rps4*, and *rps7* (Fargo *et al.* 1998)] had little or no effect on their translation *in vivo*. Deletion of a SD-like sequence in the *psbA* 5′ leader of the chloroplast *psbA* mRNA (encoding the D1 subunit of the photosystem II reaction center) abolished translation, but also severely reduced the level of the mRNA (Mayfield *et al.* 1994). Similar to results reported here for *psbC*, replacement of the SD-like sequence in the 5′ leader of the *C. reinhardtii psbD* mRNA reduced translation, but did not abolish it (Nickelsen *et al.* 1999). SD sequences could have a more prominent role in translation of highly expressed mRNAs of cyanobacteria (Osada *et al.* 1999) and chloroplasts (Nickelsen *et al.* 1999) and from initiation codons with otherwise suboptimal contexts for small subunit binding (Esposito *et al.* 2001).

Several sequence elements within the 5′ leaders of chloroplast mRNAs have been found to promote their translation. A sequence of 17 nt located near the 5′ end of the tobacco *psbA* mRNA has a positive influence on translation (Eibl *et al.* 1999). A predicted stem-loop structure in the 5′ leader of the *C. reinhardtii psbA* mRNA has been proposed to interact with translational activation factors and to regulate translation of the *psbA* mRNA in response to light (Mayfield *et al.* 1994). However, this sequence is present only in a minor form of the *psbA* RNA, but absent from the mature mRNA. In the 5′ leader of the *C. reinhardtii* chloroplast *rps7* mRNA several mutations that affect the predicted folding pattern of the RNA also block translation, suggesting that the folding pattern of the RNA is important for its ability to drive translation (Fargo *et al.* 1999). The organization of the *cis*-acting translational elements in the 5′ leader of the *C. reinhardtii* chloroplast *petD* mRNA is most similar to that of the *psbC* 5′ leader. Translation from both 5′ leaders requires three distinct sequence elements (Sakamoto *et al.* 1994; Higgs *et al.* 1999). Translation from the 5′ leader of the *psbD* mRNA in *C. reinhardtii* requires a U-rich sequence 15–20 nt distal to the initiation codon and a SD-like sequence (Nickelsen *et al.* 1999). Translation from the *psbC* 5′ leader requires

an element 16–28 nt 5′ to the GUG initiation codon; however, this element is not U-rich (Figure 1).

Using the mutant *psbC* 5′ leaders with deletions that allow a detectable level of translation, it was possible to determine whether the deleted sequences mediate the *trans*-acting functions of *TBC1* or *TBC2*. For example, dependence upon the wild-type *TBC1* function was abolished by deletion Δ321–391, but not by Δ379–391 (Figure 2). Therefore, sequences in the 321–378 interval are required for the interaction with factor(s) encoded by or controlled by *TBC1*. The *TBC1* independence of translation from the hybrid *psbC-atpA* 5′ leader (Figure 4) revealed that *TBC1* requires the 3′ end of the 5′ leader to promote translation (and an element in the 57 nt of the *atpA* 5′ leader can substitute for this interaction). A less probable explanation is that *TBC1* relieves repression by an unidentified sequence in the translation initiation region (the 14 nt, including the SD-like sequence and the GUG initiation codon).

Each of the translatable 5′ leaders still requires *TBC2*. Thus, much of the 5′ leader could be excluded as containing *TBC2* target sequences; the intervals 95–222 and 321–391 are not involved (Figures 1 and 2). However, a weak requirement for *TBC2* function could be identified for the 391–519 region. These results could reflect repeated *TBC2* target sequences, which are dispersed throughout the 5′ leader such that elements located outside of any of the deletions can confer *TBC2* dependence. More probably, however, the main *TBC2* target sequence could be within an essential region (223–320 or 519–532) so that there is insufficient translation from these mutant 5′ leaders to assay for *TBC2* independence. The presence of major *TBC2* targets in the translation initiation region was excluded by the ability of the rest of the *psbC* 5′ leader to confer *TBC2* dependence on translation from the *atpA* translation initiation region (Figure 4). It is intriguing that this effect occurs over the 57 3′-terminal nt of the *atpA* 5′ leader.

Our current model proposes that *psbC* translation requires interactions between *TBC1* and the predicted secondary structure (positions 223–320) and sequences immediately 5′ to the initiation codon. Whether *TBC3* functions in conjunction with *TBC1*, probably through sequences in the 95–222 region and the SD-like sequence, or whether it is involved in other functions remains to be explored. Because *TBC3* is defined by a single dominant allele that suppresses the effects of two different *cis*-acting mutations within the *psbC* leader as well as the effects of the nuclear *tbc1-F34* mutation, we cannot exclude the possibility that *tbc3-rb1* represents a gain-of-function mutation that confers a new RNA-binding specificity to a protein that normally binds elsewhere and is not involved in *psbC* mRNA translation. *TBC2* appears to function independently of these components, possibly in a distinct pathway. Translation of *psbC* mRNA requires both pathways.

Most studies of translational control have used reconstituted systems *in vitro*, and few have taken genetic approaches. Of eubacterial systems, predominantly *Escherichia coli* has been used in these studies. Thus, an understanding of the translational control by the direct or indirect interactions of the *Tbc1*, *Tbc2*, and *Tbc3* products and the *psbC* 5′ leader could lead to new insights into the general mechanisms of translational control and reveal the signal transduction pathways and genetic regulatory systems that control translation in plastids. A class of mRNA-specific translational regulators has been identified for mRNAs encoding central subunits of the oxidoreductase complexes of the electron transport chains in both chloroplasts of land plants and *C. reinhardtii* (Goldschmidt-Clermont 1998; Barkan and Goldschmidt-Clermont 2000; Zerges 2000) and the mitochondria of *Saccharomyces cerevisiae* (Fox 1996). These regulatory functions have been proposed to control the assembly of the polypeptide products of the target organelle mRNA into an integral membrane complex (Zerges 2000, 2002; Choquet *et al.* 2001).

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