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Identification of *cis*-Acting RNA Leader Elements Required for Chloroplast *psbD* Gene Expression in Chlamydomonas

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The *psbD* mRNA of *Chlamydomonas reinhardtii* is one of the most abundant chloroplast transcripts and encodes the photosystem II reaction center polypeptide D2. This RNA exists in two forms with 5′ untranslated regions of 74 and 47 nucleotides. The shorter form, which is associated with polysomes, is likely to result from processing of the larger RNA. Using site-directed mutagenesis and biolistic transformation, we have identified two major RNA stability determinants within the first 12 nucleotides at the 5′ end and near position −30 relative to the AUG initiation codon of *psbD.* Insertion of a polyguanosine tract at position −60 did not appreciably interfere with translation of *psbD* mRNA. The same poly(G) insertion in the *nac2-26* mutant, which is known to be deficient in *psbD* mRNA accumulation, stabilized the *psbD* RNA. However, the shorter *psbD* RNA did not accumulate, and the other *psbD* RNAs were not translated. Two other elements were found to affect translation but not RNA stability. The first comprises a highly U-rich sequence (positions −20 to −15), and the second, called *PRB1* (positions −14 to −11), is complementary to the 3′ end of the 16S rRNA. Changing the *PRB1* sequence from GGAG to AAAG had no detectable effect on *psbD* mRNA translation. However, changing this sequence to CCUC led to a fourfold diminished rate of D2 synthesis and accumulation. When the *psbD* initiation codon was changed to AUA or AUU, D2 synthesis was no longer detected, and *psbD* RNA accumulated to wild-type levels. The singular organization of the *psbD* 5′ untranslated region could play an important role in the control of initiation of *psbD* mRNA translation.

INTRODUCTION

Chloroplast gene expression has been shown to be regulated at various levels, including transcription and several post-transcriptional steps, such as RNA stabilization, RNA processing, and RNA splicing and translation. Genetic analysis of the unicellular green alga *Chlamydomonas reinhardtii* and higher plants suggests that these processes are controlled in large part by nucleus-encoded factors. These factors are synthesized in the cytosol and subsequently imported into the chloroplast, where they interact with their cognate target sites on either chloroplast RNAs or proteins to fulfill their function (Rochaix, 1996; Sugita and Sugiura, 1996; Goldschmidt-Clermont, 1998).

The availability of a biolistic chloroplast transformation system (Boynton et al., 1988) has allowed us to analyze site-directed chloroplast mutations and to use heterologous reporter systems. This has led to the identification of essential *cis*-acting RNA elements on chloroplast mRNAs that are involved in the regulation of gene expression. As an example, the analysis of chloroplast transformants revealed the importance of 5′ untranslated regions (UTRs) for the regulation of translation of the *psbC* (Zerges and Rochaix, 1994; Zerges et al., 1997), *psbA* (Mayfield et al., 1994), *petD* (Sakamoto et al., 1994), and *psaB* (Stampacchia et al., 1997) mRNAs in Chlamydomonas. They encode subunits of photosynthetic complexes in the thylakoid membrane. In tobacco, light-regulated synthesis of D1, one of the reaction center polypeptides of photosystem II, also was shown to be mediated by the *psbA* 5′ leader region (Staub and Maliga, 1994).

In contrast, the *cis* elements involved in RNA metabolism do not appear to be restricted to a unique part of chloroplast transcripts but can be located in both the 5′ and 3′ UTRs. Several chloroplast 3′ UTRs have been shown to contain stem–loop structures. Deletion of this structure leads to a marked reduction in the accumulation of the *atpB* (Stern et al., 1991) and *psaB* mRNAs (Lee et al., 1996). RNA stability can be maintained when the *atpB* 3′ stem–loop is replaced by a poly(G) stretch of 18 residues (Drager et al., 1996). Use of similar G tracts in yeast has been shown to impede the activity of 5′ to 3′ exonucleases (Vreken and Raue, 1992); consistent with these data, recent studies indicate that poly(G) sequences can stabilize chloroplast transcripts when placed into their 5′ UTRs (Drager et al., 1998).

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The analysis of the photosynthetic nuclear mutant *nac2-26*, which is deficient in chloroplast *psbD* mRNA accumulation (Kuchka et al., 1989), revealed that the principal target site for the nucleus-encoded factor is located within its 5′ UTR but not its 3′ UTR (Nickelsen et al., 1994). In wild-type cells, two different 5′ ends of this mRNA can be detected at positions −74 and −47 relative to the AUG start codon. The longer form most likely represents a precursor. The shorter mRNA starting at position −47 represents the predominant *psbD* transcript and is probably generated through a 5′ processing event. Interestingly, a 47-kD protein was shown to interact in vitro only with the longer but not with the shorter form of the *psbD* 5′ UTR. The binding activity of this protein was found to be altered in *nac2-26*, suggesting that it plays a role in the accumulation of *psbD* RNA (Nickelsen et al., 1994).

Here, we have analyzed the *psbD* 5′ UTR to identify *cis* elements involved in the stabilization, processing, and translation of *psbD* transcripts. Using biolistic chloroplast transformation, we created several mutations in the endogenous *psbD* 5′ UTR region. These mutations identify two critical elements involved in the stabilization and translation of the *psbD* mRNA and also suggest that there may be a link between the presence of the short form of *psbD* RNA and its translation.

RESULTS

Construction of Strains with Mutated *psbD* 5′ UTR Regions

To identify the *cis*-acting RNA elements required for expression of the *psbD* gene in Chlamydomonas, we introduced several site-directed mutations into the *psbD* 5′ region by biolistic transformation of wild-type cells. The plasmid used for transformation contained the *psbD* region with the appropriate mutation and, in addition, the *aadA* expression cassette inserted upstream of *psbD* in the opposite orientation (Figure 1A). This cassette allowed us to select the transformants based on their acquired resistance to spectinomycin (Goldschmidt-Clermont, 1991).

To analyze the function of the first 27 nucleotides of the *psbD* leader that are unique to the longer *psbD* mRNA starting at position −74, we introduced a set of mutations into this portion of the transcript. Either the entire region (Figure 1B, Δ74/48) or parts of it (Figure 1B, Δ68/48 and Δ62/46) were deleted. In addition, nucleotides −62 to −57 were changed from AATTAA to GGATCC (BamHI site), thereby reducing the AT content in the middle part of this leader (Figure 1B, 57B). The putative processing site around position −47 was changed from AATAAA to a BamHI site (Figure 1B, 46B). Further changes at this site resulted in mutants 46I1 and 46I2 containing insertions of 4 and 14 bp, respectively (Figure 1B).

A second set of mutations was designed to test the role of sequence elements for translation of *psbD* mRNA. A putative Shine–Dalgarno sequence GGAG located 10 nucleotides upstream of the *psbD* initiation codon (Figure 1B; putative ribosome binding site) was changed in various ways (Figure 1B, mutants PRB1A, PRB1B, and PRB1C). In mutants PRB1A and PRB1B, the sequence upstream of the AUG start codon also was changed to create a NdeI site. In mutant PRB2A, a sequence element called *PRB2*, which is located at approximately position −30, was changed from TGAGTTG to ACTAGTC. The wild-type sequence shows partial complementarity to another element close to the 3′ end of 16S rRNA (UGGAUCA) located upstream of the CUCC motif (complementary to the putative Shine-Dalgarno sequence). Recent sequence analyses of the 3′ region of the 16S rRNA genes in different Chlamydomonas strains revealed the presence of an A residue at position 4 of the upstream element (H. Tourne and J. Nickelsen, unpublished data) rather than a C residue, as reported earlier (Dron et al., 1982). *PRB1* and *PRB2* are separated by a long T-rich tract that was replaced by a BamHI site in the transformant ΔU (Figure 1). A similar set of sequence motifs in the *psbA* 5′ UTR of tobacco complementary to parts of the 3′ terminal region of 16S rRNA was found to be important for translation of the *psbA* mRNA in vitro (Hirose and Sugiura, 1996).

To ensure that the transformants Δ74/48, Δ68/48, Δ62/46, ΔU, 46I1, and 46I2 were homoplasmic, we tested their DNA for the presence of residual wild-type *psbD* gene copies by using either polymerase chain reaction (PCR) amplification or DNA gel blot analysis (data not shown). Because the mutants 57B and 46B contain a new BamHI site, their homoplasmicity was tested by BamHI digestion of PCR products (Figure 1B; data not shown). The other mutants with single or multiple base changes were tested for homoplasmicity by using DNA gel blot analysis with a *psbD*-specific DNA probe and/or by sequencing of the PCR products (data not shown).

As a first measure of the effects of the introduced leader mutations, we analyzed the phenotypes of the homoplasmic transformants by determining their fluorescence transients (Fenton and Crofts, 1990) and by testing their ability to grow photoautotrophically. Mutants 57B, 46B, Δ62/46, 46I1, 46I2, PRB1A, PRB1B, and PRB1C grew on minimal medium, with PRB1C exhibiting altered fluorescence transients, whereas mutants Δ74/48, Δ68/48, ΔU, PRB2A, PRB1/2, IC1, and IC2 did not. These strains had fluorescence transients characteristic for mutants defective in electron transfer in photosystem II (Figure 1B; data not shown). As a next step, all mutants were characterized in more detail.

Characterization of *psbD* mRNA in Chloroplast Mutants

Levels of *psbD* mRNA in the various mutant strains were quantitated by comparative RNA gel blot analysis, and 5′

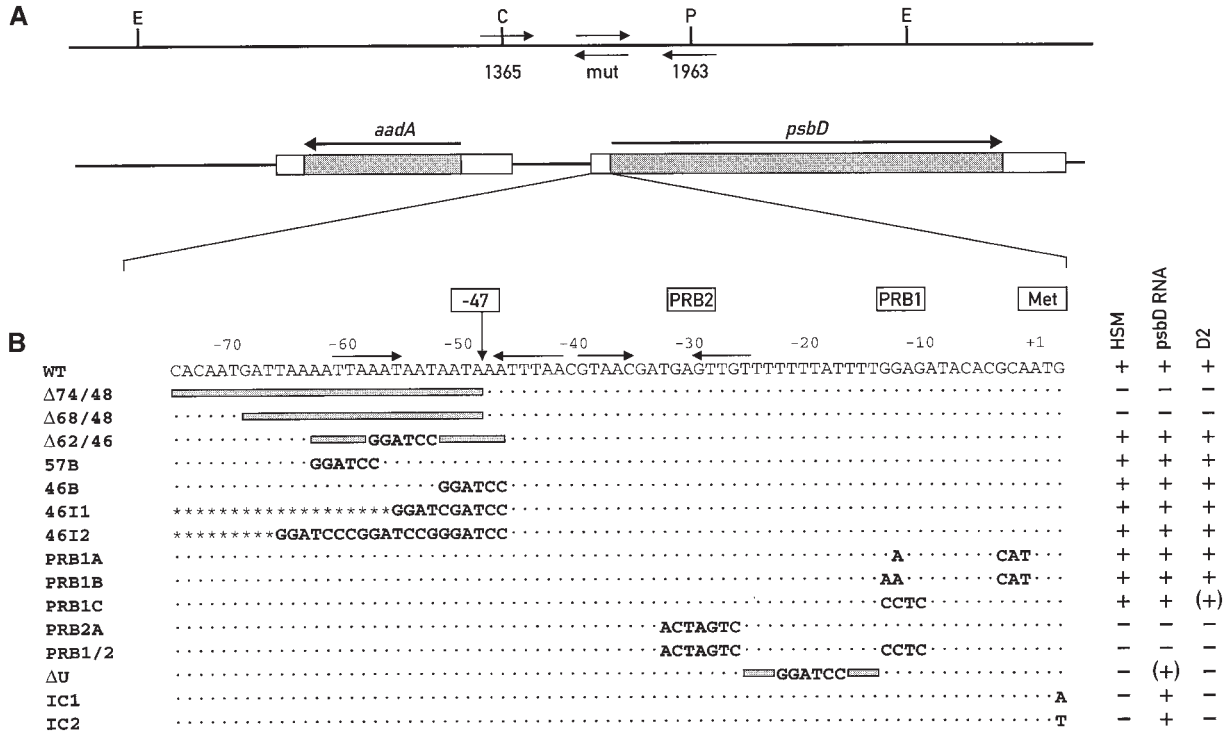

Figure 1. Site-Directed Mutagenesis of the *psbD* 5′ UTR of Chlamydomonas.

(A) *psbD* region with the location of the *aadA* expression cassette and several restriction sites used in this study. Coding regions and 5′ and 3′ UTRs are indicated by filled and open boxes, respectively. The direction of transcription is indicated by long horizontal arrows. On the line at top, oligonucleotides 1365 and 1963, which were used for amplifying the ClaI-PvuII fragment, are indicated together with the mutagenic oligonucleotide (mut). C, ClaI; E, EcoRI; P, PvuII.

(B) Alignment of the *psbD* leader sequences from the wild type (WT) and the different mutants. Conserved residues are indicated by dots, deletions are marked by thin filled boxes, and altered sequences are in boldface. The regions marked by asterisks in 46I1 and 46I2 indicate that in both cases, the mutant region is preceded by the *psbD* 5′ UTR upstream of −52. The processing site at position −47 (boxed) and the *PRB1* and *PRB2* elements as well as the initiation codon (Met) are marked. The arrows overline potential stem–loop structures. Growth (+) or absence of growth (−) of the mutants on HSM medium is indicated under HSM. The amount of *psbD* RNA and D2 protein is indicated by + (80 to 100% of wild-type level), (+) (20 to 30% of wild-type level), and − (<5 to 10% of wild-type level). For all mutants examined, the rate of D2 synthesis parallels that of D2 accumulation, except for Δ74/48 and Δ68/48, for which D2 synthesis was not determined. The indications for the mutants were derived from the data shown in Figures 2, 6, and 7.

end maturation was followed by primer extension studies. No or only minor effects on *psbD* RNA accumulation were observed in mutants 57B, 46B, Δ62/46, 46I1, and 46I2 (Figure 2A, lanes 3, 4, and 6 to 8), which harbor changes upstream of and at the putative processing site at position −47, the *PRB1* mutants PRB1B, PRB1A, and PRB1C (Figure 2B, lanes 1 to 3), as well as the AUG initiation codon mutants IC1 and IC2 (Figure 2B, lanes 9 and 10). A significant decrease in *psbD* mRNA accumulation (25 to 50% of wild-type level) was found in the U-tract deletion mutant ΔU (Figure 2A, lane 5). The RNA 5′ maturation reaction at position −47 was not affected in any of these mutants, except for the *PRB2* mutants (Figure 3, see below). Even the deletion of sequences upstream of the putative processing site in Δ62/46 or the insertion of fragments of different lengths at position −47 in 46I1 and 46I2 allowed the accumulation of wild-type levels of the short form of the *psbD* mRNA (Figure 3, lanes 9 to 11). The data further indicate that the photosystem II–deficient phenotype of the U track deletion mutant ΔU (Figure 3, lane 7) and the AUG initiation codon mutants IC1 and IC2 (Figure 3, lanes 19 and 20) is not caused by a marked reduction in the level of *psbD* RNA or faulty 5′ end maturation. Instead, these three mutants appear to have defects in *psbD* mRNA translation (see below).

Significant effects on *psbD* transcript accumulation were observed when the first 27 nucleotides of the *psbD* leader were deleted (Δ74/48; Figures 1 and 2A, lane 1). Less than 1% of the wild-type *psbD* mRNA was detectable in Δ74/48.

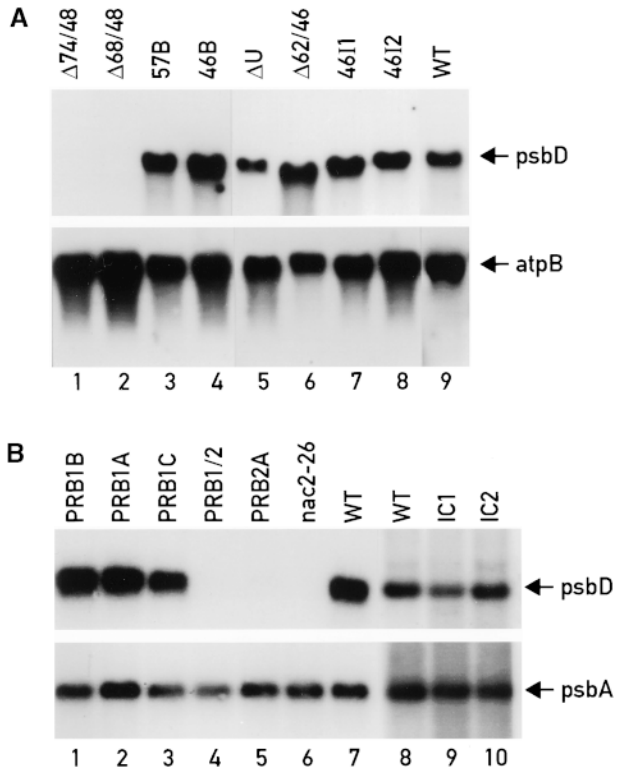

Figure 2. Accumulation of *psbD* RNA in the 5′ UTR Mutant Strains.

Total RNA from the indicated mutant strains was separated electrophoretically, transferred to nylon membranes, and hybridized with a *psbD*-specific probe. WT, wild type.

(A) As loading control, blots were rehybridized with an *atpB*-specific probe. The lower position of the *psbD* RNA band in lane 6 is due to a blotting artifact.

(B) As a loading control, blots were rehybridized with a *psbA*-specific probe.

A similar phenotype was observed when the region between −68 and −48 was deleted, thereby leaving the first six nucleotides of the *psbD* mRNA intact (Δ68/48; Figures 1 and 2A, lane 2). In contrast, the small deletion from position −62 to −46 accompanied by the insertion of a BamHI site in mutant strain Δ62/46 did not affect *psbD* RNA accumulation (Figures 1 and 2A, lane 6). This indicates that one essential *cis* element for *psbD* RNA accumulation is located within the first 12 nucleotides of its leader region.

One surprising result of the analysis of these site-directed chloroplast mutations was the marked reduction of *psbD* transcripts in the PRB2A mutant (Figure 2B, lane 5). Interestingly, no short mRNA starting at position −47 accumulated in PRB2A, whereas some of the long-form *psbD* mRNA was detectable (Figure 3, lane 14). Furthermore, a signal around position −62 was found to be enhanced with variable intensities in different RNA preparations from PRB2A and also from PRB1C (Figure 3, lanes 13 and 14). In the double mutant PRB1/2, no further effect on the *psbD* transcript level was observed (Figure 3, lane 15). Thus, the *PRB2* motif—besides the first 12 nucleotides of the leader—appears to represent a second *cis* element required for the stable accumulation of *psbD* mRNA, especially the short form. Whether this region also is involved in RNA translation remains to be clarified.

Transcriptional Activity in Mutants Δ74/48 and PRB2A

The loss of *psbD* RNA in the mutants Δ74/48 and PRB2A could be due to a deficiency either in RNA stability or in the *psbD* promoter. To distinguish between these two possibilities, the relative *psbD* transcription rates in these mutants were determined by using a chloroplast run-on transcription assay. The levels of *psbD* transcription in Δ74/48 and PRB2A were estimated at 40 and 27% relative to the wild type (Figure 4, lanes 2, 4, and 6). There is thus a significant decrease that cannot account for the almost complete absence of *psbD* transcripts in these mutants (Figures 2A, lane 1, and 2B, lane 5). We conclude from these data that the first 12 nucleotides of the *psbD* leader and the *PRB2* motif are required for the stabilization of *psbD* transcripts. Furthermore, these data strongly suggest that the short *psbD* RNA starting at position −47 is generated through a 5′ processing event and does not represent a primary transcript, because a putative promoter driving the transcription of the abundant short form of *psbD* RNA most likely would have been deleted in the Δ74/48 mutant. It is clear from these data that the transcription rate is not diminished to the same extent as the stability of the RNA in these mutants.

Polysome Formation with *psbD* mRNA

The destabilization of *psbD* transcripts in the 5′ deletion mutant Δ74/48 suggests that newly synthesized *psbD* RNA has to follow an obligate 5′ processing pathway to be stabilized and subsequently translated. Another possibility is that the short *psbD* mRNA form represents an inactive product of the longer form, although the short form is very abundant. To test this hypothesis, we examined the distribution of this RNA on polysome gradients. The short *psbD* mRNA form starting at position −47 was detected throughout the entire gradient if Mg^{2+} ions stabilizing polysomal complexes were present (Figure 5A). The distribution of *psbD* RNA in the gradient was altered when EDTA was included instead of $MgCl_2$, and the short form of the *psbD* mRNA was detected mainly in the top fractions of the gradient containing the bulk cellular RNA (Figure 5B). Thus, these data show that the short *psbD* mRNA form represents an actively translated transcript and not an inactive product. Because of its low abundance, it was not possible to follow reliably the long form of *psbD* RNA in these polysome gradients.

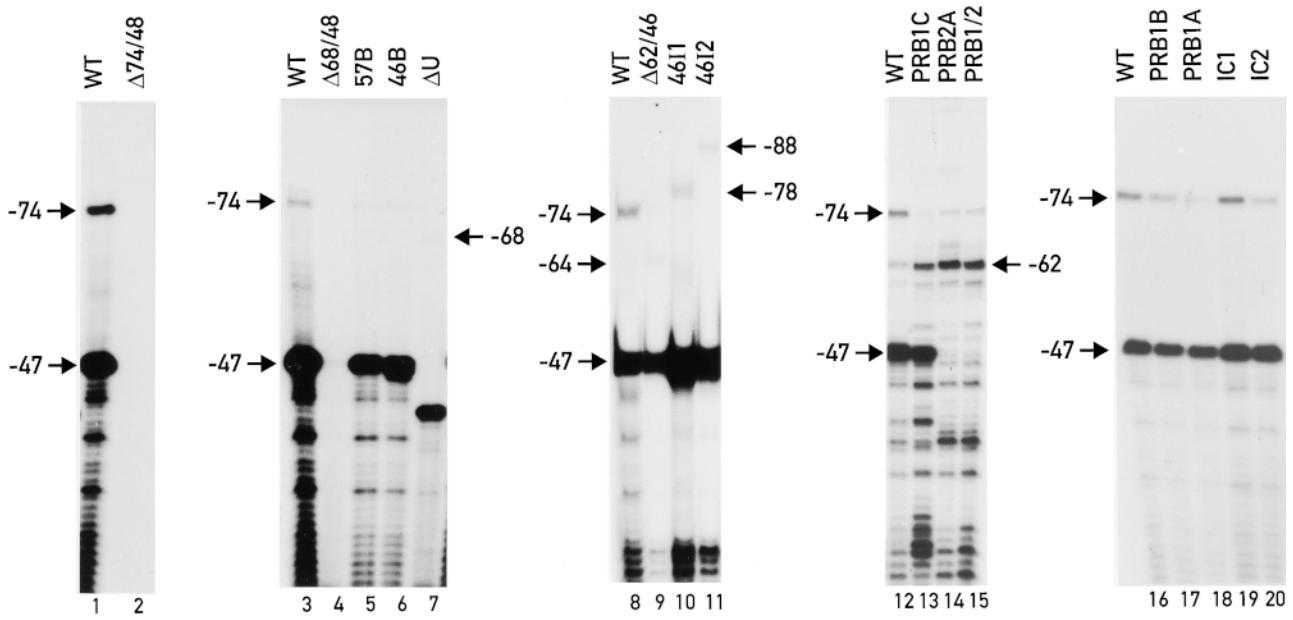

Figure 3. Primer Extension Analysis of Mutant *psbD* mRNA 5′ Ends.

Primer extension analysis was performed by using oligonucleotide 3131. The different mutant strains are indicated at the top. Arrows point to the 5′ ends of *psbD* transcripts, with their positions given relative to the AUG start codon (see Figure 1B). WT, wild type.

Synthesis and Accumulation of D2 in the Mutants

The amount of photosystem II accumulating in the different mutants was determined by immunoblotting by using an antibody raised against D1 as a probe. It has been shown previously that absence of D2 leads to the destabilization of the photosytem II complex and that D1 and D2 accumulate to the same level (Erickson et al., 1986; Kuchka et al., 1988). As a control, the accumulation of the PsaD polypeptide of photosystem I was analyzed at the same time. It can be seen in Figure 6 that all mutants that are able to grow photoautotrophically (PRB1A, PRB1B, 57B, 46B, Δ62/46, 46I1, and 46I2) accumulate wild-type levels of photosystem II, indicating that the introduced alterations of the *psbD* leader do not influence *psbD* gene expression. The only exception is the PRB1C mutant in which D1 accumulates to ~25% that of the wild-type level (Figure 6A, lane 5), which is sufficient for growth on minimal medium. However, the significant reduction of photosystem II in PRB1C indicates an important role of the *PRB1* motif for *psbD* expression.

As expected, no photosystem II was detected in the mutants lacking detectable amounts of *psbD* mRNA, that is, PRB1/2, PRB2A, and Δ74/48 (Figures 6A, lanes 6 and 7, and 6B, lanes 1 and 2). Small amounts of photosystem II were, however, detected in Δ68/48, although no *psbD* mRNA was detectable in this mutant. In the U-tract deletion mutant ΔU and in both initiation codon mutants IC1 (ATA) and IC2 (ATT), which are deficient in photosynthesis but accumulate 50 to 100% of wild-type levels of *psbD* RNA, no D1 was detectable, strongly suggesting that they are affected at the level of translation (Figures 6A, lanes 1 and 2, and 6B, lane 5).

The fact that the introduced mutations are located in the 5′ UTR of the *psbD* mRNA suggests that impaired photosystem II accumulation is due to defects in the synthesis but not in the stability of the D2 protein. This was tested by pulse labeling the proteins of these mutants with ^{35}S-sulfate in the presence of cycloheximide to inhibit cytosolic protein synthesis. D2 synthesis was <5% of wild-type levels in the U-tract deletion mutant ΔU (Figure 7A, lane 1) and the ATG mutants IC2 and IC1 (Figure 7A, lanes 6 and 7). In contrast, D2 was synthesized at the same rate as in the wild type in mutants PRB1A and PRB1B that have only minor changes in the *PRB1* region and accumulate 100% of photosystem II (Figure 6A, lanes 3 and 4, and Figure 7A, lanes 4 and 5). D2 synthesis was reduced approximately fourfold in the PRB1C mutant in which the *PRB1* GGAG motif was changed to CCTC (Figure 7B, lane 1). This is consistent with an approximate fourfold reduction of photosystem II in this mutant (Figure 6A, lane 5). As expected, the RNA stability mutants PRB1/2 and PRB2A were completely deficient in D2 synthesis (Figure 7B, lanes 2 and 3). It is noticeable that in these mutants, synthesis not only of D2 but also of D1 is reduced, suggesting some type of coupling between the synthesis and/or stability of these two reaction center proteins of photosystem II, a property that has been noted previously (Erickson et al., 1986; De Vitry et al., 1989).

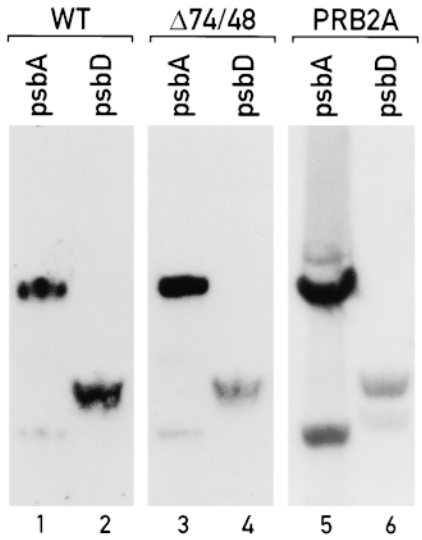

Figure 4. Chloroplast Run-on Transcription.

Radioactively labeled run-on transcripts from the wild type (WT; lanes 1 and 2) and the mutants Δ74/48 (lanes 3 and 4) and PRB2A (lanes 5 and 6) were hybridized with filter-immobilized DNA fragments of the *psbA* and *psbD* genes.

Insertion of a Poly(G) Tract in the *psbD* 5′ UTR Restores *psbD* RNA Stability but Not Translation in the Absence of Nac2 Function

The *nac2-26* mutant previously has been shown to be specifically deficient in *psbD* RNA accumulation (Kuchka et al., 1989). The target site of the Nac2 function appears to be the 5′ UTR of *psbD* RNA (Nickelsen et al., 1994). Poly(G) tracts consisting of 18 consecutive G residues inserted into transcripts have been shown to impede exoribonucleolytic activity in yeast and the chloroplast of Chlamydomonas (Vreken and Raue, 1992; Decker and Parker, 1993; Muhlrad et al., 1994; Drager et al., 1996, 1998). To determine the role of 5′ exoribonucleases in the degradation of the *psbD* mRNA, we inserted a poly$(G)_{18}$ tract at positions −60 (G60) and −14 (G14) relative to the *psbD* initiation codon both in the wild-type (G60/WT) and *nac2-26* (G60/nac2) strains. Figure 8A (lanes 5 and 6) shows that *psbD* RNA is restored nearly to the wild-type level in the G60/nac2 mutant. Partial restoration of *psbD* RNA also occurs in the G14/nac2 mutant (Figure 8A, lane 9). The amount of *psbD* RNA is vastly increased in the G14/WT strain (Figure 8A, lanes 7 and 8) but is not markedly affected in the G60/WT strain (Figure 8A, lanes 3 and 4). Although the *psbD* RNA is stabilized in G60/nac2, no D2 protein is detectable by immunoblotting (Figure 8B, lane 4), indicating that translation is inhibited. In contrast, D2 is produced in the G60/WT strain, although it only accumulates to 20 to 30% of the wild-type level. The fact that no D2 protein is detectable in the G14/WT and G14/nac2 strains is not surprising because the U-rich tract required for translation has been deleted in these strains (see Figures 6 and 7).

If a 5′ exonuclease is involved in the degradation of the 5′ end of *psbD* mRNA in the *nac2-26* mutant, the 5′ end of the stabilized *psbD* RNA would be expected to map near the 5′ end of the poly(G) sequence. We tested this by performing primer extensions on total RNA by using reverse transcriptase and a primer near the ATG initiation codon. A strong signal corresponding to the 5′ end of and within the poly(G) sequence can indeed be detected with *nac2-26* RNA and, surprisingly, also with wild-type RNA (Figure 9A, lanes 3 and 4, and Figure 9B). Furthermore, weak signals corresponding to the −74 transcript are nearly of equal strength in the presence or absence of the Nac2 function. These results indicate that degradation of the *psbD* RNA is mediated by a 5′ exonuclease but that its activity is not enhanced by the *nac2-26* mutation. The equally strong signal corresponding to the 3′ end of the poly(G) sequence is due to the fact that reverse transcriptase is stopped by the poly(G) tract sequence (F. Vaistij, M. Goldschmidt-Clermont, and J.-D. Rochaix, unpublished results). The striking difference between the wild-type and *nac2* strains is that the mature −47 *psbD* transcript is missing in the mutant (Figure 9A, lane 4). The Nac2 function thus is required for the accumulation of this mRNA. Similar patterns were obtained with the G14/WT and G14/nac2 strains, that is, comparable signals corresponding to the −74 transcript, a nearly undetectable signal with the −47 *psbD* RNA in the mutant, and strong signals corresponding to the 5′ end of the poly(G) sequence in both the wild-type and mutant strains (Figure 9A, lanes 5 and 6, and Figure 9B).

DISCUSSION

cis Elements Involved in *psbD* mRNA Stabilization

Our analysis of the *psbD* leader region of Chlamydomonas by site-directed mutagenesis has revealed the existence of at least two distinct regions required for the stable accumulation of *psbD* transcripts. One is located within the first 12 nucleotides of the leader region and thus is specific to the long form of *psbD* RNA. A second element is localized within the 7-mer UGAGUUG located near position −30 and therefore present in both the long and the short leader regions. Interestingly, this sequence shows partial complementarity to the sequence UGGAUCA near the 3′ end of the 16S rRNA upstream of the CUCC motif that could interact with the Shine-Dalgarno–like element GGAG within the *psbD* leader. This raises the possibility that pairing with the 16S rRNA could stabilize the *psbD* mRNA. In addition, the presence of this second RNA stability element could explain why the short *psbD* mRNA is stable when it is generated via 5′ processing from the longer form but is unstable when it is expressed as the mature form in the deletion mutant Δ74/68

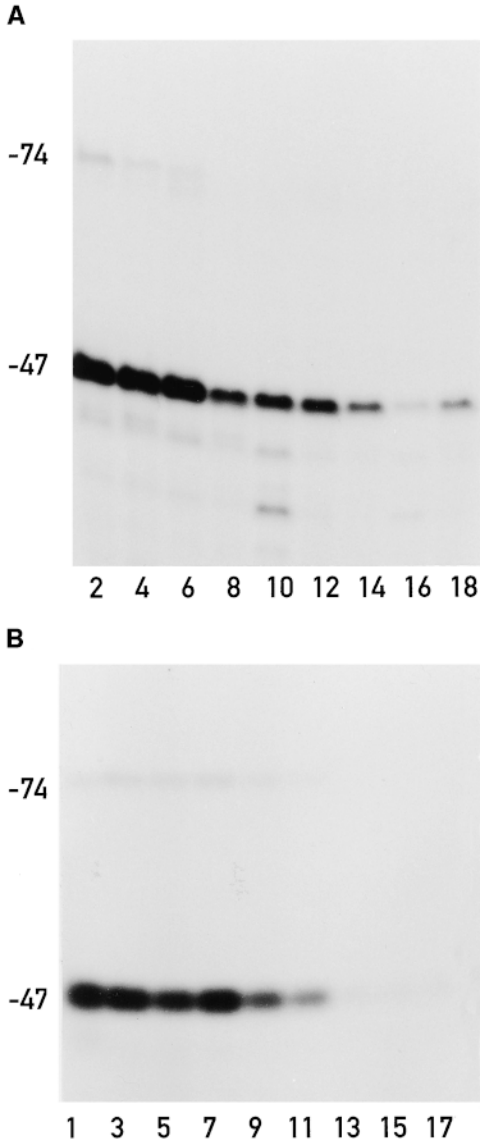

Figure 5. Distribution of Wild-Type *psbD* mRNA on a Polysome Gradient.

(A) Primer extension products from RNA of different gradient fractions obtained with oligonucleotide 3131 are shown. Numbers of fractions from the top (left) to the bottom (right) of the tubes are indicated. The 5′ ends of transcripts at positions −74 and −47 are marked.

(B) Control containing EDTA rather than $MgCl_2$ to destabilize polysomal complexes is shown. Numbers of fractions from the top (left) to the bottom (right) of the tubes are indicated. The 5′ ends of transcripts at positions −74 and −47 are marked.

(Figure 1B). One possibility is that an early phase of translation initiation, for example, the binding of the 30S ribosomal subunit, requires the long precursor form. This RNA is stabilized by a mechanism involving the first 12 nucleotides of the leader. Once ribosome binding has taken place, the RNA could be stabilized via the 7-mer motif after the 5′ extension has been removed. If only the short form is synthesized, ribosomal loading could not occur, and consequently, stabilization of the RNA mediated by the 7-mer motif would not be possible.

Another possibility is that the *PRB2* motif serves as the recognition site for a yet unidentified *trans*-acting factor that is distinct from the 30S ribosomal subunit. Finally, alterations of the *PRB2* motif might destroy a putative stem–loop structure that is predicted by computer-assisted RNA secondary structure analysis in the region around position −30 (Figure 1B). However, such a secondary structure element apparently is not required for *psbD* RNA stabilization, because a similar RNA structure analysis for the *psbD* leader in the U-tract deletion mutant ΔU predicts a stronger stem–loop structure that would prevent the formation of the *PRB2* stem–loop structure (data not shown). Another putative stem–loop structure located around the 5′ processing site at position −47 (Nickelsen et al., 1994; Figure 1B) also appears to play no significant role, because disruption of this structure in mutants Δ62/46 and 46I2 has no effect on *psbD* gene expression. It is interesting to note that a segment of the ribulose biphosphate carboxylase large subunit (*rbcL*) 5′ UTR from tobacco has been shown to stabilize its mRNA in the dark (Shiina et al., 1998).

The exact role of the 5′ processing of the *psbD* mRNA remains to be elucidated. We have shown previously that a 47-kD protein interacts specifically with the long *psbD* 5′ UTR but not with the short form of the leader (Nickelsen et al., 1994). This and other *trans*-acting factors could play an important role in the stabilization of the *psbD* RNA as well as in its proper targeting to the thylakoid membrane and in the initial events of translation. At the same time, it could, however, interfere with the formation of a complete translation initiation complex, for example, the association of the 50S subunit. Removal of the 5′ extension of the *psbD* leader might be required for allowing this step. Alternatively, 5′ processing might be necessary for recruiting other translation factors. An increase in the size of the *rbcL* 5′ UTR induced by methyl jasmonate treatment of barley leaves has been shown to inhibit the initiation of translation of this mRNA (Reinbothe et al., 1993).

Similar to *psbD*, a heterogeneity with regard to leader length has been observed for both *psbA* and *psbB* transcripts in Chlamydomonas (Erickson et al., 1984; Nickelsen et al., 1994; Bruick and Mayfield, 1998; J. Nickelsen, unpublished results). Thus, these three genes appear to follow a related mode of gene expression. It has been shown that their products assemble into an intermediate core complex during photosystem II biogenesis (De Vitry et al., 1989), and a model has been proposed in which mRNA 5′ end formation reflects a coordinate step during expression of the

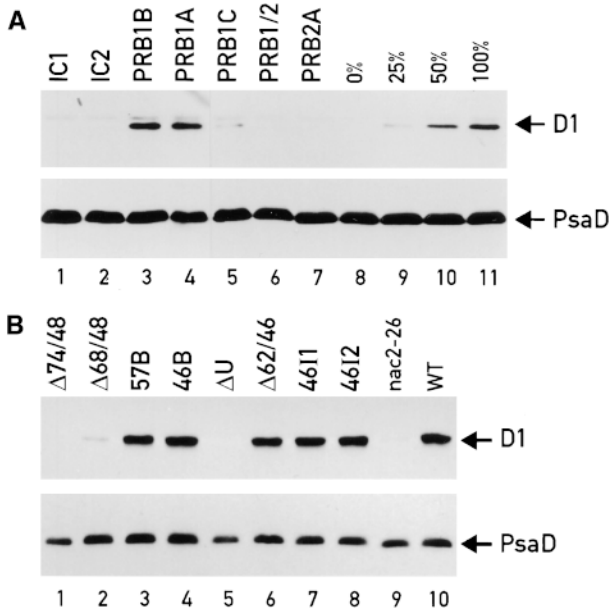

Figure 6. Accumulation of the D1 and PsaD Proteins from the *psbD* 5′ UTR Mutants.

Total proteins from the indicated Chlamydomonas mutant strains corresponding to 2 μg of chlorophyll were separated by SDS-PAGE, immunodecorated with antibodies raised against D1 or PsaD, and visualized using chemiluminescence.

(A) Immunoblots of mutants IC1, IC2, PRB1B, PRB1A, PRB1C, PRB1/2, and PRB2A. The 0, 25, 50, and 100% represent serial dilutions of wild-type protein extracts with Fud7 extracts to maintain the total protein content constant. The Fud7 mutant failed to accumulate the photosytem II complex (Bennoun et al., 1986).

(B) Immunoblots of mutants Δ74/48, Δ68/48, 57B, 46B, ΔU, Δ62/46, 46I1, 46I2, *nac2-26*, and the wild type (WT).

psbA, *psbB*, and *psbD* genes (Rochaix, 1996). Recently, a correlation between processing of the *psbA* 5′ UTR and association of the *psbA* mRNA to ribosomes has been proposed (Bruick and Mayfield, 1998). Two different 5′ ends also have been reported for *rbcL* transcripts in Chlamydomonas (Shapira et al., 1997).

Interestingly, the *psbB* mRNA also appears to be stabilized by elements within its leader region (F.E. Vaistij, M. Goldschmidt-Clermont, and J.-D. Rochaix, unpublished results), whereas the analysis of *psbA* leader mutants did not reveal specific elements involved in RNA stabilization, except for mutations that affect a putative Shine-Dalgarno element GGAG (Mayfield et al., 1994).

cis Elements Involved in *psbD* mRNA Translation

It is noticeable that the mutations in the *psbD* initiation codon ATT and ATA appear to strongly inhibit translation of *psbD* mRNA because the corresponding mutants do not accumulate detectable amounts of photosytem II and are unable to grow photoautotrophically. This is in contrast to similar mutations created in the *petD* initiation codon. An ATT *petD* mutant still is able to grow photoautotrophically, although it is temperature sensitive (Chen et al., 1993, 1995).

Aside from the ATG start codon, at least two additional elements involved in translation of the *psbD* message were identified. One is a putative Shine-Dalgarno sequence GGAG that is complementary to the 3′ end of the chloroplast 16S rRNA. Minor changes of this element to either GAAG or AAAG have no effect on *psbD* gene expression. When this element is changed to the sequence CCTC, only 25% of the D2 protein accumulates, which is consistent with a fourfold reduction in the rate of protein synthesis, as measured by protein pulse labeling. This finding is in contrast to a recent analysis suggesting that Shine-Dalgarno–like sequences within the 5′ UTRs of chloroplast transcripts in Chlamydomonas have no significance for gene expression (Fargo et al., 1998). However, different genes (*atpB*, *atpE*, *rps4*, and *rps7*) that are not as highly expressed as *psbD* were analyzed in this study. It is noticeable that even for *psbD*, there seems to be no strict requirement for the presence of the *PRB1* motif, because its drastic change to CCTC in mutant PRB1C still allows photoautotrophic growth of the cells. The second element is represented by a long stretch of 11 consecutive T residues containing a single adenosine. Deletion of this T track completely abolishes D2 synthesis. Interestingly, in *Escherichia coli*, the ribosomal protein S1 binds to a U-rich region upstream of the Shine-Dalgarno sequence during translation initiation (Boni et al., 1991).

A model for the synthesis of the D1 protein of photosystem II recently has been proposed based on in vitro translation experiments in tobacco (Hirose and Sugiura, 1996). Two elements of the *psbA* 5′ UTR, called RBS1 and RBS2 (for ribosome binding site), have been proposed to bind to the 3′ end of the 16S rRNA, resulting in a loop-out of an AU-rich region located between RBS1 and RBS2. Similar RNA elements can be recognized in the *psbD* leader of Chlamydomonas, with the Shine-Dalgarno–like motif GGAG at position −13 (*PRB1*) and the 7-mer TGAGTTG near position −30 (*PRB2*) resembling the RBS1 and RBS2 elements, respectively. A U tract would be looped out, similar to the AU-rich region in tobacco. Whereas our data confirm the importance of the GGAG motif and the U tract for translation of the *psbD* mRNA in vivo, it is unclear whether the *PRB2* element is involved in D2 synthesis in Chlamydomonas. The primary in vivo effect of the mutated version of this region is the instability of the *psbD* RNA. Taken together, the similar structure of the *psbD* and *psbA* leaders in Chlamydomonas and tobacco, respectively, suggest a related mode of translational regulation of these two genes. However, in higher plants, no extensive 5′ processing of *psbA* transcripts has been reported except for the formation of a minor RNA, with its 5′ end 16 nucleotides downstream of the primary *psbA* 5′ ter-

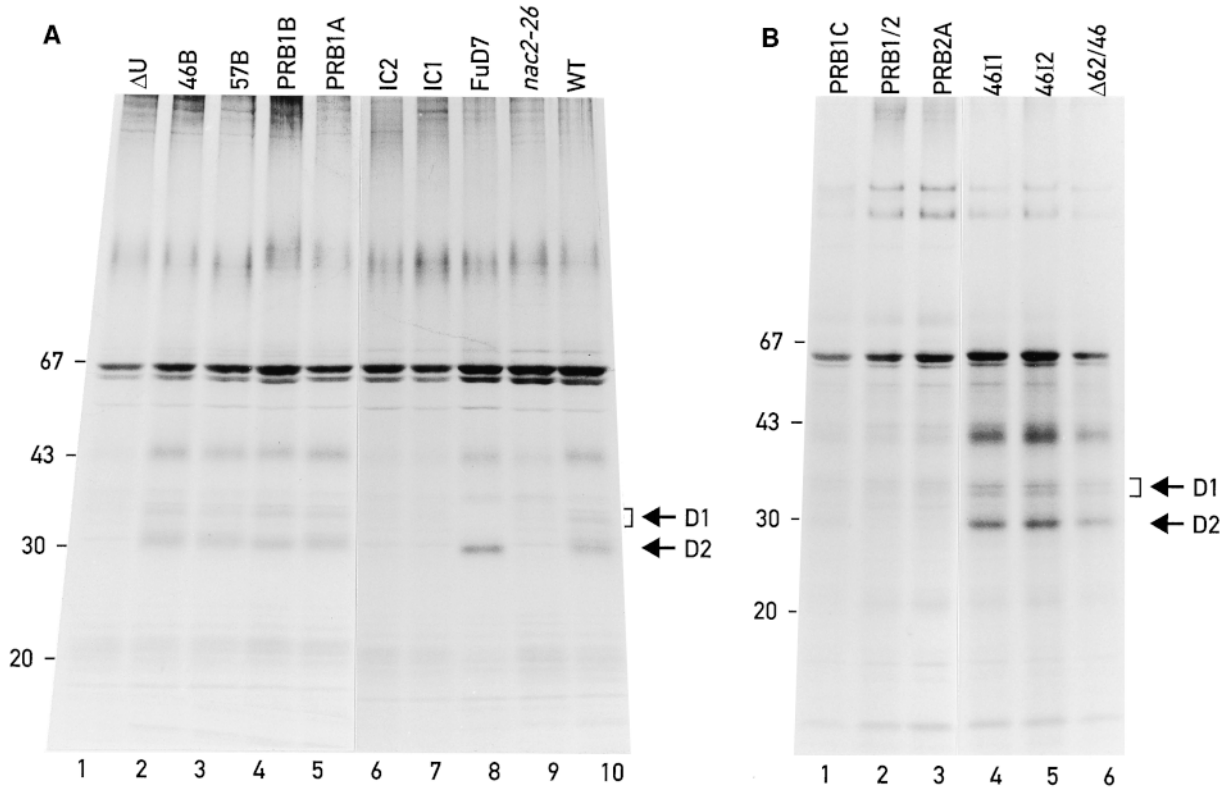

Figure 7. In Vivo Protein ^{35}S-Sulfate Pulse Labeling of the *psbD* 5′ UTR Mutants.

(A) Total proteins from the Chlamydomonas mutant strains indicated were labeled in vivo with ^{35}S-sulfate in the presence of cycloheximide. Extracts were fractionated by SDS-PAGE on a 7.5 to 15% gradient gel and autoradiographed. The D2 and D1 proteins and the molecular masses (in kilodaltons) are indicated at right and left, respectively. WT, wild type.
(B) Total proteins from the Chlamydomonas mutant strains indicated were labeled and fractionated as given in **(A)**.
For **(A)** and **(B)**, electrophoresis was conducted separately.

minus, which occurs exclusively in mRNA associated with membrane-bound polysomes (Kim and Mullet, 1994).

Nac2 Function Is Required for the Formation of the Mature *psbD* mRNA

The levels of the two forms of *psbD* RNA containing 5′ UTRs of 74 and 47 nucleotides, respectively, are not affected to the same extent by the *nac2-26* mutation. Whereas the mature shorter *psbD* RNA is destabilized completely, this clearly is not the case for the larger form (Figure 9B, lane 2). The same holds for the strains containing a poly(G) sequence within the *psbD* 5′ UTR. In these strains, an abundant transcript with the poly(G) sequence at its 5′ end accumulates independently of the presence of the Nac2 function, indicating that the RNA most likely is degraded by a 5′ exonucleolytic activity. However, this activity is not modulated by the Nac2 function. A similar activity involved in the degradation of the *petD* mRNA of Chlamydomonas has been reported by Drager et al. (1998). The Nac2 function appears to be required for the accumulation of the mature *psbD* mRNA with its 5′ end at position −47. Although the poly(G) tract strongly impedes the exonucleolytic activity, it probably does not convey full protection to the downstream region. The Nac2 factor could bind or promote the binding of other proteins near position −47 in the *psbD* 5′ UTR and thereby prevent the progression of the 5′ exonucleolytic activity beyond position −47. Another possibility is that the Nac2 function promotes, directly or indirectly, an endonucleolytic cleavage at position –47 and that this process leads to the stabilization of the *psbD* RNA. This view, however, may be difficult to reconcile with the accumulation of an abundant transcript containing the poly(G) sequence at its 5′ end in the wild-type background, although we cannot

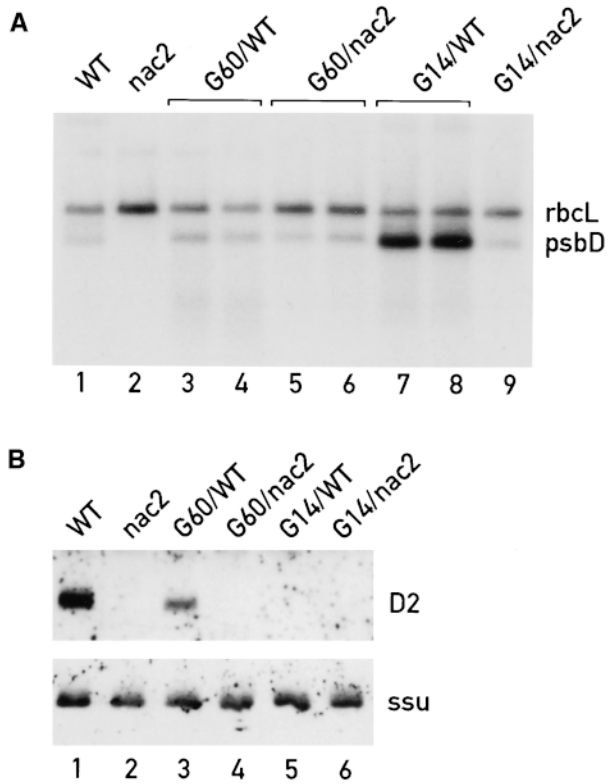

Figure 8. Accumulation of *psbD* RNA and the D2 Protein in Chlamydomonas Mutant Strains Containing a Poly(G)$_{18}$ Tract in the *psbD* 5′ UTR.

(A) RNA gel blot analysis of the wild-type (WT) and mutant strains indicated. Hybridization was with probes specific for *rbcL* and *psbD.*
(B) Immunoblot analysis of the same strains as given in **(A)** with antibodies raised against D2 and the small subunit of ribulose bisphosphate carboxylase (ssu).

exclude the possibility that *psbD* 5′ end maturation depends on both endonucleolytic and exonucleolytic activities.

Although the *psbD* RNA containing the G cassette is stabilized in the absence of the Nac2 function, it is not translated because no D2 protein is detectable. Thus, failure to produce the mature *psbD* mRNA can be correlated with the lack of *psbD* mRNA translation, suggesting that the Nac2 function may be involved not only in the first but also in the second process. How these events are coordinated remains to be determined. It is interesting that several mutations downstream of position −47 prevent the appearance of the mature *psbD* transcript with its 5′ end at position −47, which is replaced by a transcript starting at −62 in the mutant PRB2A (Figure 3). This region thus may be important for the recognition and binding of the protein complex involved in *psbD* mRNA stabilization and initiation of translation.

Aside from *psbD* mRNA, the accumulation of several other chloroplast transcripts, including those of the *petD*, *psbB*, and *psaB* genes, has been shown to be dependent on specific nucleus-encoded functions, the target of which is the 5′ UTR (Drager et al., 1998; Stampacchia et al., 1997; F. Vaistij, M. Goldschmidt-Clermont, and J.-D. Rochaix, unpublished results). In the case of *petD* RNA, the instability caused by the *mcd1-1* mutation occurs through the mature 5′ end of this RNA. Larger transcripts, such as the *petA* or *petD* dicistronic mRNA, accumulate stably in the *mcd1-1* mutant, although they contain all of the *petD* 5′ UTR. There is thus an interesting parallel between the stability of the *petA-petD* RNA in the *mcd1-1* mutant and that of the longer *psbD* RNA starting at position −74 in the *nac2-26* mutant background, both of which give rise to shorter unstable RNAs. However, there could be differences between these two cases. The *petD* mRNA is generated through an endonucleolytic cut of the precursor RNA (although it also can be generated from its own promoter), whereas the mature *psbD* mRNA is more likely to be produced by processive 5′ exonucleolytic digestion of the longer *psbD* RNA. The cloning and characterization of the nucleus-encoded factors involved in chloroplast mRNA stability should provide new insights into the molecular mechanisms underlying chloroplast mRNA accumulation and translation.

METHODS

Oligonucleotides

The oligonucleotides used for the mutagenesis and the analysis of the *psbD* leader region were as follows: oligonucleotide 1365 5′-CCATCGATAAGCTTGATTTTTTATATCATAATAATAAA-3′, positions −261 to −241 relative to the initiation codon; oligonucleotide 1963 5′-AGAAACAGCTGCTGTTAA-3′, positions +237 to +220; oligonucleotide 1392 5′-GTGTGTAAAAAATGCTTA-3′, positions −130 to −113; oligonucleotide 3131 5′-ACCGATCGCAATTGTCAT-3′, positions +18 to +1; oligonucleotide 3894 (mutant Δ68/48) 5′-TTACGTTAAATT-3′* (where asterisk marks position of deletion), 5′-ATTGTGTATTTAATATTATA-3′, positions −36 to −47 Δ −69 to −88; oligonucleotide 3895 (mutant Δ68/48) 5′-TAAATACACAAT*AATTTAACGTAACGATGAGT-3′, position −80 to −69 Δ −47 to −28; oligonucleotide 3896 (mutant 57B) 5′-ATACACAATGATTAAGGATCCATAATAATAAATTTA-3′, positions −77 to −42; oligonucleotide 3897 (mutant 57B) 5′-TAAATTTATTATTATGGATCCTTAATCATTGTGTAT-3′, positions −42 to −77; oligonucleotide 3898 (mutant 46B) 5′-CATCGTTACGTTAAAGGATCCATTATTTAATTTTAA-3′, positions −31 to −66; oligonucleotide 3899 (mutant 46B) 5′-TTAAAATTAAATAATGGATCCTTTAACGTAACGATG-3′, positions −66 to −31; oligonucleotide 3900 (mutant ΔU) 5′-ACGTAACGATGAGTTGGATCCGGAGATACACGCAAT-3′, positions −31 to −26 Δ −14 to +2; oligonucleotide 3901 (mutant ΔU) 5′-ATTGCGTGTATCTCCGGATCCAACTCATCGTTACGT-3′, positions +2 to −14 Δ −26 to −31; oligonucleotide 2569 (mutant Δ74/48) 5′-TAATATTAAATA*AATTTAACGTAACGATGAGT-3′, positions −86 to −75 Δ −47 to −28; oligonucleotide 2570 (mutant Δ74/48) 5′-TTACGTTAAATT*TATTTAATATTATAACTTAT-3′, positions −36 to −47 Δ −75 to −94; oligonucleotide 2567 (mutants PRB1B and PRB1A) 5′-GTTGTTTTTTTATTT-

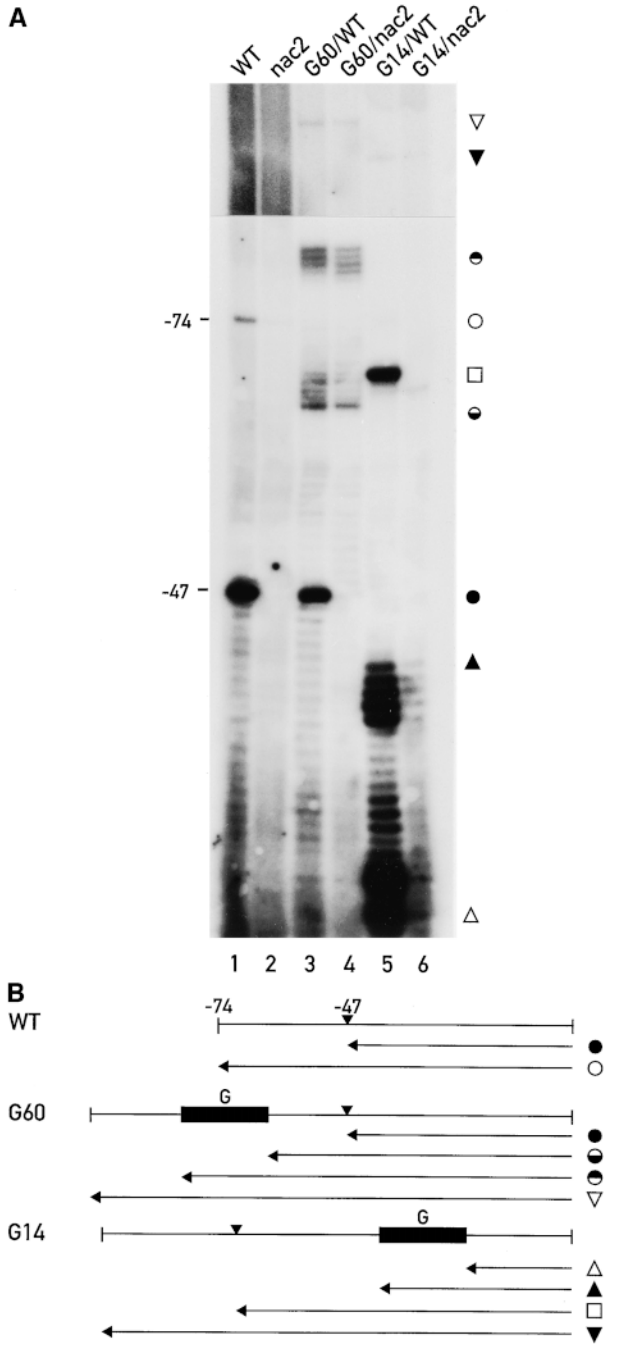

Figure 9. Primer Extension Analysis of *psbD* RNA from Chlamydomonas Mutant Strains Containing a Poly(G)$_{18}$ Tract in the *psbD* 5′ UTR.

(A) Primer extension analysis was performed by using oligonucleotide 3131. Symbols at the right refer to the various transcripts shown in the diagram in **(B)**. The upper part of the autoradiogram has been overexposed to facilitate detection of the longer *psbD* RNA with a 5′ UTR of 74 nucleotides.

(B) Diagrams of transcripts detected in **(A)** in each of the indicated strains. The poly(G) tract is marked by black boxes.

WT, wild type.

T(G,A)AAGATACACCATATGACAATTGCGAT-3′, positions −29 to +14; oligonucleotide 2568 (mutants PRB1B and PRB1A) 5′-ATCGCAATTGTCATATGGTGTATCTT(C,T)AAAATAAAAAAACAAC-3′, positions +14 to −29; oligonucleotide 4561 (mutant PRB1C) 5′-TTGTTTTTTTATTTTCCTCATACACGCAATGACA-3′, positions −28 to +6; oligonucleotide 4560 (mutant PRB2A) 5′-AATTTAACGTAACGAACTAGTCTTTTTTTTATTT-3′, positions −47 to −15; oligonucleotide 4562 (mutant PRB1/2) 5′-AATTTAACGTAACGAACTAGTCTTTTTTTATTTTCCTCATACACGCAATGACA-3′, positions −47 to +6; oligonucleotide 2055 (mutant IC1 for initial codon) 5′-TGGGAGATACACGCAAT(C,A,T)ACAATTGCGATCGGT-3′, positions −14 to +18; oligonucleotide 2056 (mutant IC1 for initial codon) 5′-ACCGATCGCAATTGT(G,T,A)ATTGCGTGTATCTCCAA-3′, positions +18 to −15; oligonucleotide 2640 (mutant IC2) 5′-TTGGAGATACACGCAA(T,C)TACAATTGCGATCGGT-3′, positions −15 to +18; and oligonucleotide 2641 (mutant IC2) 5′-ACCGATCGCAATTGTA(A,G)TTGCGTGTATCTCCAA-3′, positions +8 to −15.

Strains and Chloroplast Transformation

The wild-type strain 137C *mt*$^+$ was maintained on Tris–acetate–phosphate medium (TAP; Gorman and Levine, 1965) at 25°C. Chloroplast transformations were performed as described previously (Fischer et al., 1996) by using a helium-driven particle gun and with selection of the transformants on TAP plates containing spectinomycin (100 μg/mL). After at least three consecutive cell cloning steps, the transformants were tested for homoplasmicity by either DNA blot or polymerase chain reaction (PCR) analysis. Fluorescence transients of individual transformants were determined as described previously (Fenton and Crofts, 1990). Photoautotrophic growth of transformants was tested on HSM plates (Rochaix et al., 1988).

Plasmid Constructions

For each mutation, two complementary oligonucleotides were generated and used separately for PCRs by using the chloroplast EcoRI fragment R3 (containing the 5′ region of *psbD*) as template. One mutagenesis oligonucleotide was used with oligonucleotide 1963 spanning the PvuII site within *psbD* for amplifying the 3′ part of the ClaI-PvuII fragment (Figure 1A). The complementary mutagenesis oligonucleotide was used, with oligonucleotide 1365 containing a ClaI site for amplifying the 5′ part of the ClaI-PvuII fragment (Figure 1A). The two PCR products were used as one template for a PCR primed with oligonucleotides 1365 and 1963. The amplified products were sequenced and used for replacing the ClaI-PvuII fragment in plasmid p108.14. This plasmid contains the *aadA* cassette (Goldschmidt-Clermont, 1991) inserted (in the opposite orientation to *psbD*) at position −263 relative to the ATG initiation codon of *psbD* within the EcoRI fragment R3 (Figure 1).

The deletion from positions −60 to −47 of the *psbD* leader was created by inserting the 200-bp ClaI-BamHI fragment of plasmid 57B into the unique ClaI and BamHI sites of plasmid 46B, which gave rise to plasmid Δ62/46. The BamHI site in plasmid 46B also was used to insert the sequence GATC by digesting with BamHI, filling with the Klenow fragment of DNA polymerase I and religation, giving rise to plasmid 46I1. Plasmid 46I2 was constructed by inserting a BamHI linker (CCGGATCCGGGATC) into the BamHI site of plasmid 46B. Plasmids 57B-60G and ΔU-14G were constructed by digesting plasmids 57B and ΔU, respectively, with BamHI and ligating them to

annealed oligonucleotides BglII-poly(G) (5′-CTAGTTTGGGGGGGG-GGGGGGGGGGAGATCTA-3′) and BglII-poly(C) (5′-CTAGTAGAT-CTCCCCCCCCCCCCCCCCCCAAA-3′).

Analysis of Nucleic Acids

Total DNA from *Chlamydomonas reinhardtii* was isolated as described previously (Rochaix et al., 1988). DNA blot analysis and PCR amplification were performed by using standard procedures (Sambrook et al., 1989).

Total algal RNA was isolated by centrifugation through CsCl cushions (Rochaix et al., 1988). Subsequent RNA gel blot analysis and primer extension assays in which oligonucleotide 3131 was used were as described previously (Nickelsen et al., 1994). Polysomes were separated on linear sucrose gradients according to Baumgartel and Howell (1976). A 400-mL culture of cw15 cells (2×10^6 cells per mL) was separated into two 200-mL fractions that were harvested by centrifugation and resuspended in either 1 mL of lysis buffer containing 200 mM Tris-HCl, pH 7.9, 150 mM NaCl, 50 mM $MgCl_2$, 20 mM EDTA, 50 mM 2-mercaptoethanol, 0.5 mg/mL heparin, 2% Nonidet P-40, 8.55% sucrose, and 100 μg/mL chloramphenicol or, as a control, 1 mL of the same buffer containing no $MgCl_2$. Lysates were centrifuged for 2 min in a microcentrifuge, and subsequently, 0.5 mL of the supernatants was loaded onto either a linear 12.5-mL sucrose gradient (15 to 50%) in 40 mM Tris-HCl, pH 7.9, 30 mM NaCl, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.5 mg/mL heparin, and 100 μg/mL chloramphenicol or, for the control, a gradient in which 2.5 mM EDTA was substituted for the 10 mM $MgCl_2$. After centrifugation at 35,000 rpm for 80 min in an SW40 rotor (Beckman, Palo Alto, CA), 600-μL fractions were collected from the top, immediately adjusted to 1% SDS, and phenol/chloroform extracted. RNA was ethanol precipitated and analyzed by primer extension (Nickelsen et al., 1994).

RNA secondary structure calculations were performed by using the RNAdraw software (Matzura and Wennborg, 1996).

Chloroplast Run-on Transcription

To measure transcription rates, cells were permeabilized by repeated freeze and thaw cycles (Gagne and Guertin, 1992) and subsequently incubated with 100 μCi of ^{32}P-UTP in transcription buffer for 7 min (Sakamoto et al., 1993). RNA was phenol/chloroform extracted, ethanol precipitated, and used for hybridization to nylon filter–immobilized DNA fragments of the *psbA* (10 μg of plasmid R14 digested with EcoRI and HindIII) and *psbD* (10 μg of plasmid R3 digested with HinfI) genes (Monod et al., 1992).

Pulse Labeling of Proteins with ^{14}C-Acetate

Chlamydomonas cells were inoculated in TAP minus NH_4Cl, $CaCl_2$, and $MgSO_4$ at 10^5 cells per mL and incubated for 16 hr at 25°C with agitation. Cells were pelleted, washed, and resuspended in TAP–sulfate (20 mM Tris-acetate, pH 7.0, and 1 mM K_2HPO_4/KH_2PO_4, pH 7.0) for 2 hr. Cells were washed again and resuspended in TAP–sulfate at a concentration of 40 μg of chlorophyll per mL for 5 min. After the addition of cycloheximide to a final concentration of 6 μg/mL for 10 min, 100 μCi of $Na_2{}^{35}SO_4$ was added per milliliter of culture, and the labeling was performed for 15 min at room temperature under low light. Finally, labeled proteins were separated on SDS-PAGE and detected by autoradiography as described previously (Sambrook et al., 1989).

Immunoblot Analysis

Exponentially growing Chlamydomonas cells were pelleted, resuspended in lysis buffer (50 mM Tris-HCl, pH 6.8, and 2% SDS) for 15 min at 37°C, and centrifuged again for 2 min in a microcentrifuge. Chlorophyll concentration of the supernatant was measured according to Porra et al. (1989). Finally, the supernatant was analyzed by SDS-PAGE. Protein gel blotting and immunodetection were performed as described previously (Sambrook et al., 1989).

Quantitation of Autoradiograms

The signals of the autoradiograms were quantified either by comparison of a wild-type dilution series (see Figure 6) or by scanning the autoradiograms with an Apple (Cupertino, CA) Color One scanner 1200/30 and by using the National Institutes of Health Image 1.61 software for quantitative densitometry. Autoradiograms in Figures 2 and 4 were digitalized with an ICU-1 unit (Herolab, Wiesloch, Germany) and quantitated by using the ImageDoc/EASY Win§2 software (Herolab). The signals were compared with internal standards (Figures 2, 4, and 6). In Figure 7, the double band near 67 kD was used as an internal standard. Similar results were obtained by quantitation of the data of Figure 7 with a PhosphorImager (Bio-Rad, Hercules, CA).

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