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Changes in Chloroplast mRNA Stability during Leaf Development

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During spinach leaf development, chloroplast-encoded mRNAs accumulate to different steady-state levels. Their relative transcription rates alone, however, cannot account for the changes in mRNA amount. In this study, we examined the importance of mRNA stability for the regulation of plastid mRNA accumulation using an in vivo system to measure mRNA decay in intact leaves by inhibiting transcription with actinomycin D. Decay of *psbA* and *rbcL* mRNAs was assayed in young and mature leaves. The *psbA* mRNA half-life was increased more than twofold in mature leaves compared with young leaves, whereas *rbcL* mRNA decayed with a similar relative half-life at both leaf developmental stages. The direct in vivo measurements demonstrated that differential mRNA stability in higher plant plastids can account for differences in mRNA accumulation during leaf development. The role of polysome association in mRNA decay was also investigated. Using organelle-specific translation inhibitors that force mRNAs into a polysome-bound state or deplete mRNAs of ribosomes, we measured mRNA decay in vivo in either state. The results showed that *rbcL* and *psbA* mRNAs are less stable when bound to polysomes relative to the polysome-depleted mRNAs and that their stabilities are differentially affected by binding to polysomes. The results suggested that ribosome binding and/or translation of the *psbA* and *rbcL* mRNAs may function to modulate the rate of their decay in chloroplasts.

INTRODUCTION

The development of proplastids into mature chloroplasts is characterized by major morphological changes, the most obvious of which is the rapid synthesis and assembly of the photosynthetic thylakoid membranes. In spinach and other plants, plastid-encoded mRNAs accumulate during this developmental process. The kinetics of accumulation during development, however, are different for several mRNAs (for review, see Tobin and Silverthorne, 1984; Gruissem et al., 1988; Mullet, 1988; Gruissem, 1989). For example, the mRNA of the D1 protein of photosystem II (*psbA*) is present at low levels only in dark-grown spinach cotyledons but accumulates rapidly after illumination to the same level as the mRNA for the large subunit of the ribulose-1,5-bisphosphate carboxylase (*rbcL*), which is similar in the dark and light. In young spinach leaves, the *psbA* and *rbcL* mRNAs accumulate to similar levels, but in mature leaves, *psbA* mRNA is more abundant than the *rbcL* mRNA (Deng and Gruissem, 1987). The accumulation pattern of these and other mRNAs for photosynthetic proteins in response to light and during leaf development is similar in mustard, pea, maize, and barley (Link, 1984; Rodermel and Bogorad, 1985; Klein and Mullet, 1987; Sasaki et al., 1987).

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Regulation of accumulation of different mRNAs to their characteristic steady-state levels during leaf development can occur at the transcriptional and/or post-transcriptional level. Earlier studies using plastid run-on transcription assays demonstrated that the general transcriptional activity of the plastid genome changes during leaf development (Deng and Gruissem, 1987; Baumgartner et al., 1989). In spinach chloroplasts, however, there are no major changes in the relative transcriptional activities of several genes, including *psbA* and *rbcL*, comparing young and mature leaves (Deng and Gruissem, 1987; Deng et al., 1987; for review, see Gruissem, 1989). The lack of corresponding transcriptional adjustments suggests, therefore, that the developmental control of mRNA stability can affect the accumulation of specific mRNAs. Although control of mRNA stability is an attractive model for the regulation of chloroplast mRNA accumulation, direct evidence of changes in mRNA half-lives has not been demonstrated in higher plants.

It is now generally accepted that post-transcriptional events that affect mRNA processing, transport, and stability are significant factors in the control of gene expression (Brawerman, 1989). Differential mRNA stability as a mechanism of post-transcriptional control during development or as a response to exogenous inducers has already been described for prokaryotic systems (Nilsson et al.,

1984) and for mammalian cells (Brock and Shapiro, 1983; Müller and Kühn, 1988). The molecular mechanisms by which the control of differential mRNA stability is achieved, however, are still largely unknown. In spinach chloroplasts, the post-transcriptional regulation of differential mRNA accumulation may occur at the level of mRNA maturation and/or the level of mRNA stability. Plastid protein-coding genes in monocistronic and polycistronic transcription units are generally flanked by inverted repeats in their 3′ untranslated regions. The inverted repeats form stable stem-loop structures in vitro and are processing signals for precise mRNA 3′ end formation (Stern and Gruissem, 1987; Stern et al., 1989). They are also required for the stabilization of 5′ mRNA segments. Deletion or point mutations that alter the structure of the inverted repeats significantly affect the stability of 5′ mRNA segments in vitro (Stern et al., 1989; Adams and Stern, 1990) and in vivo (Stern et al., 1991). It is unknown, however, whether the inverted repeats also have a function in the differential stabilization of plastid mRNAs or only act to enhance their general stability, e.g., by protecting mRNAs against 3′ exonucleolytic attack. In addition to 3′ inverted repeats as *cis*-acting regulatory sequences, the assembly of plastid mRNAs into polysomes may also contribute to their differential stability. It is not known, however, whether and how translation in chloroplasts affects the stability of individual mRNAs.

The purpose of our present study was twofold. First, we wanted to obtain evidence in vivo that the observed differences in spinach *psbA* and *rbcL* mRNA accumulation during leaf development are due to changes in the stability of the two mRNAs. Second, we were interested in determining the role of translation and polysome association in the specific decay of *psbA* and *rbcL* mRNAs. The direct application of a transcription inhibitor to spinach leaves has allowed us to provide the first demonstration that half-lives of plant chloroplast mRNAs do change during development. Furthermore, using translation inhibitors that enable us to dissect the decay of polysome-bound mRNA and mRNA that is associated only with monosomes or packaged into ribonucleoprotein particles, we provide evidence that ribosome association of the *psbA* and *rbcL* mRNAs is not required to increase their stability, but rather that ribosome loading may be necessary to initiate and/or facilitate the turnover of both mRNAs. The results also suggest that the decay of *psbA* and *rbcL* mRNAs may be regulated by different mechanisms.

RESULTS

Chloroplast mRNA Decay in Vivo: Relative Half-Lives of Two mRNAs Differ in Young and Mature Leaves

The first question we addressed is whether a measurable difference in plastid mRNA turnover during spinach leaf development can account for the differences in chloroplast mRNA accumulation. We chose to analyze the *psbA* and *rbcL* mRNAs, which are transcribed from monocistronic transcription units in the spinach chloroplast genome and which encode the D1 protein of photosystem II and the large subunit of ribulose-1,5-bisphosphate carboxylase, respectively. These mRNAs differ in size: the *psbA* mRNA is approximately 1.2 kb and the *rbcL* mRNA 1.75 kb in length. Two 5′ ends are detectable for the *rbcL* mRNA, but the significance of the processing event that establishes the two 5′ ends is unknown (Mullet et al., 1985; Deng and Gruissem, 1988). The accumulation kinetics of the *psbA* and *rbcL* mRNAs differ in young and mature spinach leaves. In young leaves, both mRNAs accumulate to approximately similar levels, although the transcription rate of the *psbA* gene is approximately 50% higher than that of the *rbcL* gene, as determined in a plastid transcription run-on assay (Deng and Gruissem, 1987). In contrast, in mature leaves the *psbA* mRNA accumulates to approximately threefold to fourfold higher levels than the *rbcL* mRNA. This is not reflected in the relative transcription rates of their genes, which in fact show a small decrease of 10% to 15% for the *psbA* gene relative to the *rbcL* gene in mature leaves (Deng and Gruissem, 1987). Considering the discrepancy between transcription rates and mRNA accumulation, it is possible that the differences in *psbA* and *rbcL* mRNA accumulation in young and mature leaves are controlled by changes in mRNA stability. Thus, the *psbA* and *rbcL* mRNAs provide a suitable experimental system to test this possibility in vivo.

In intact plants, determination of mRNA decay by pulse labeling of RNA is complicated by the relatively slow uptake of radioactive precursors and the difficulty of obtaining information on local pool sizes of nucleotide triphosphates. In our hands, short-term in vivo labeling of spinach leaves by application of radioactive UTP or orthophosphate to wounded leaves or by uptake did not yield reproducible results (data not shown). The application of transcription inhibitors such as actinomycin D, which has been shown to inhibit transcription in chloroplasts of several plant species, including spinach (Crouse et al., 1984), provides a compromise for a first estimation of mRNA half-lives in vivo. Also, in contrast to isolated chloroplasts, measurements of chloroplast mRNA decay in intact leaves in the presence of actinomycin D ensures the presence of nuclear factors that may affect chloroplast mRNA half-lives (such as RNases, RNA-binding proteins, ribosomal proteins, etc.) for at least the lengths of the half-lives of the factors and their mRNA themselves.

To measure relative *psbA* and *rbcL* mRNA decay in chloroplasts in vivo, mature leaves from hydroponic spinach plants were cut under water and kept in water containing 200 μg/mL actinomycin D; to treat young leaves with actinomycin D, roots of intact young plants were cut under water to a length of approximately 2 cm. The leaves were pretreated for 30 min, after which total RNA was isolated at different time points. The decay kinetics of

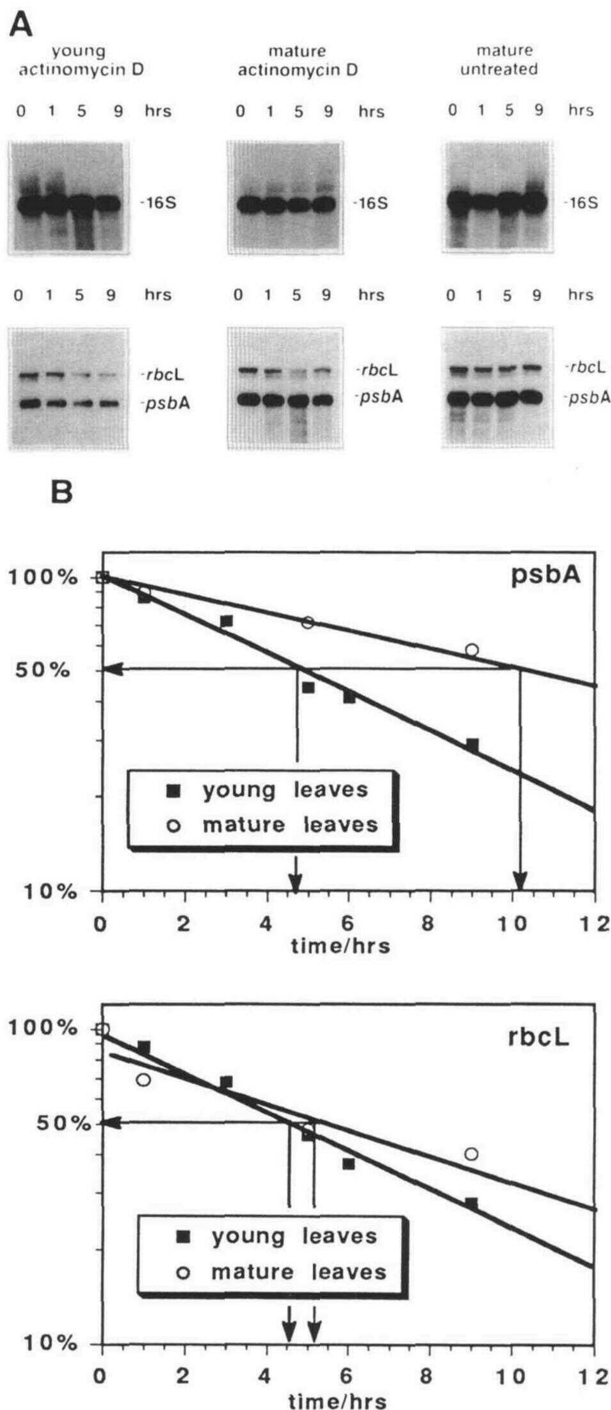

Figure 1. Decay of *psbA* and *rbcL* mRNAs in Vivo.

Chloroplast mRNA decay was measured in young and mature leaves from hydroponic spinach plants. Leaves were treated with 200 μg/mL actinomycin D; a set of control leaves was treated with water only. Total RNA was extracted after 0 hr, 1 hr, 3 hr, 5 hr, 6 hr, and 9 hr from young leaves and 0 hr, 1 hr, 5 hr, and 9 hr from mature leaves.

(A) RNA gel blot analysis. For each time point, 10 μg of total RNA was separated on denaturating 1.2% agarose-formaldehyde gels and transferred to nylon membranes. *psbA* and *rbcL* mRNAs and 16S ribosomal RNA were detected by hybridization using gene-specific probes. The RNA gel blot analysis of RNA from young leaves is shown only for the 0 hr, 1 hr, 5 hr, and 9 hr time points.
(B) Quantitation. After RNA blot analysis, the bands corresponding to *psbA* mRNA, *rbcL* mRNA, and 16S RNA were excised and radioactivity was measured by scintillation counting. The amount of *psbA* and *rbcL* mRNA was normalized to 16S RNA for each time point. The data collected from three independent sets of experiments were subjected to a linear regression analysis. The graphs were plotted using a modified linear regression alogarithm for $y = ax + b$, with each time point being weighted equally.

psbA and *rbcL* mRNAs were analyzed by hybridization of equal amounts of total cellular RNA for each time point to gene-specific probes labeled to similar specific activities. The amount of chloroplast 16S ribosomal RNA was used as an internal standard (see Methods). Figure 1A shows the decay of *rbcL* and *psbA* mRNA in actinomycin D-treated young and mature spinach leaves relative to untreated control leaves. In young leaves, both mRNAs decayed with similar kinetics, but in mature leaves the stability of *psbA* mRNA was increased relative to *rbcL* mRNA. No decay was detectable in the untreated control leaves. To obtain a more quantitative estimation of the levels of both mRNAs at each time point, the hybridized material in the bands representing *psbA* mRNA, *rbcL* mRNA, and 16S RNA was excised from the membrane, and radioactivity was measured by scintillation counting. The amount of *psbA* mRNA and *rbcL* mRNA was normalized to the amount of 16S RNA for each time point. The results in Figure 1B show that under the experimental conditions *rbcL* mRNA decayed with approximate half-lives of 4.5 hr and 5.2 hr in young and mature leaves, respectively. In contrast, the relative half-life of *psbA* mRNA, which was approximately 4.7 hr in young leaves, increased to more than 10 hr in mature leaves. The increase in *psbA* mRNA half-life relative to the *rbcL* mRNA half-life in mature leaves was consistent with the difference in the accumulation of both mRNAs and the lack of corresponding significant changes in their relative transcription rates at these two developmental stages.

To substantiate the developmental differences in *psbA* and *rbcL* mRNA half-lives,we included several controls. First, we used 16S chloroplast ribosomal RNA as an internal standard for the following reason: in spinach, transcription of the *rrn*, *psbA*, and *rbcL* loci are affected similarly by changes in overall plastid transcription activity in young leaves compared with mature leaves (Deng and Gruissem, 1987). Therefore, any changes in RNA accumulation due to transcriptional changes in young and mature spinach leaves will most likely not affect the measured half-lives when standardized against 16S chloroplast ribosomal RNA.

Second, RNA in 4-cm to 6-cm spinach leaves treated with actinomycin or water only was directly labeled with radioactive orthophosphate at time point 6 hr for 1 hr. No substantial incorporation into total RNA or polyA$^+$ RNA was detected in the actinomycin-treated leaves (data not shown). In addition, actinomycin prevented the accumulation of the mRNA for the spinach light-harvesting chlorophyll *a*/*b*-binding protein during the duration of the time course in treated leaves but not control leaves (data not shown).

Third, hybridization of a probe complementary to the intron of the *pet*D mRNA (coding for subunit IV of the cytochrome b_6/f complex) was used as a control to determine the efficiency of the actinomycin D treatment in chloroplasts of intact leaves. Excised intron sequences are generally less stable than mature mRNAs as revealed by the relative ratios of mature mRNAs to excised introns at steady state. RNA gel blots were probed with a *pet*D intron-specific probe, as shown in Figure 2. The *pet*D gene is part of the *psb*B operon, which encodes the genes for the 51-kD chlorophyll apoprotein of photosystem II (*psb*B), the 10-kD phosphoprotein of photosystem II (*psb*H), as well as the cytochrome b_6 (*pet*B) and subunit IV (*pet*D) of the cytochrome b_6/f complex (Rock et al., 1987; Tanaka et al., 1987; Kohchi et al., 1988; Westhoff and Herrmann, 1988). Both *pet*B and *pet*D are interrupted by a single intron. In spinach, the operon is transcribed into one 5.6-kb primary RNA, which is processed in a complex pathway yielding 17 different precursors and mature RNAs, all of which accumulate to specific levels (Westhoff and Herrmann, 1988). The complex hybridization pattern in Figure 2, therefore, represents the RNAs that include the intron sequence. It is difficult to estimate the relative half-life of the excised intron sequence because it continues to be synthesized by splicing even after transcription is inhibited. For the purpose of this control, however, the kinetics of intron degradation and disappearance of the intron-containing RNA precursors are similar in actinomycin D-treated young and mature leaves compared with untreated control plants, indicating that the inhibitor is also efficient in blocking transcription in chloroplasts of both leaf types. This control was included in all decay experiments shown in this study.

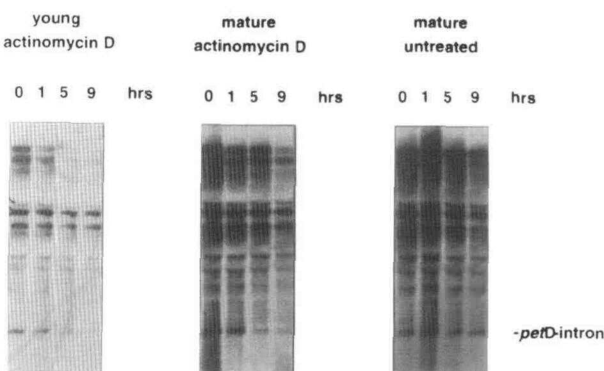

Figure 2. Decay of the *pet*D Intron in Actinomycin D-Treated Leaves.

RNA blot analysis of the intron sequence of the chloroplast *pet D* (cytochrome b_6/f complex)-mRNA was performed. Ten micrograms of total RNA from each time point of actinomycin D-treated young and mature leaves as well as untreated mature control leaves was separated on denaturating 1.2% agarose-formaldehyde gels, and transferred to nylon membrane. The *pet*D intron was detected by a ^{32}P-labeled, 320-bp intron-specific DNA probe. The additional RNAs detected by hybridization with the *pet*D intron-specific DNA probe represent partially processed RNAs of the polycistronic *psb*B-*psb*H-*pet*B-*pet*D transcript (Westhoff and Herrmann, 1988).

The Levels of *psb*A and *rbc*L mRNAs Are not Affected by Depletion of Nuclear Factors

Actinomycin D interacts with the DNA and, therefore, inhibits transcription in the nucleus as well (Dani et al., 1984). It is possible, therefore, that during the extended treatment of young and mature spinach leaves with actinomycin D, nuclear-encoded proteins become depleted that are required for stabilization of chloroplast mRNAs. The turnover of such factors may affect the half-lives of *psb*A and *rbc*L mRNAs. To control for this possibility, leaves were treated with 25 μg/mL cycloheximide for 15 hr to inhibit cytoplasmic translation. If the mRNA decay observed during the 9-hr treatment of leaves with actinomycin D is a result of depletion of nuclear-encoded gene products, a similar effect on the steady-state levels of the mRNAs should be detectable in plants treated with cycloheximide alone. The efficiency of the translational block by cycloheximide in young and mature leaves was monitored by in vivo protein labeling using ^{35}S-methionine. Total RNA was isolated from the same leaves and analyzed on RNA gel blots for the steady-state level of 16S ribosomal RNA, *rbc*L mRNA, and *psb*A mRNA. The results shown in Figure 3A demonstrated that cycloheximide treatment for 15 hr inhibits the synthesis of most proteins compared with the water control; those proteins that incorporate radioactivity are, among others, mainly the gene products of *rbc*L and *psb*A, indicating that the translational block is specific. The results from the RNA blot analysis shown in Figure 3B demonstrated that in both young and mature leaves no significant changes in the steady-state levels of 16S RNA, *psb*A mRNA, and *rbc*L mRNA are detectable as a consequence of the cycloheximide treatment. This control allowed us to conclude that the measured decay of *psb*A and *rbc*L mRNAs after treatment with actinomycin D as a transcription inhibitor is not simply a result of the change

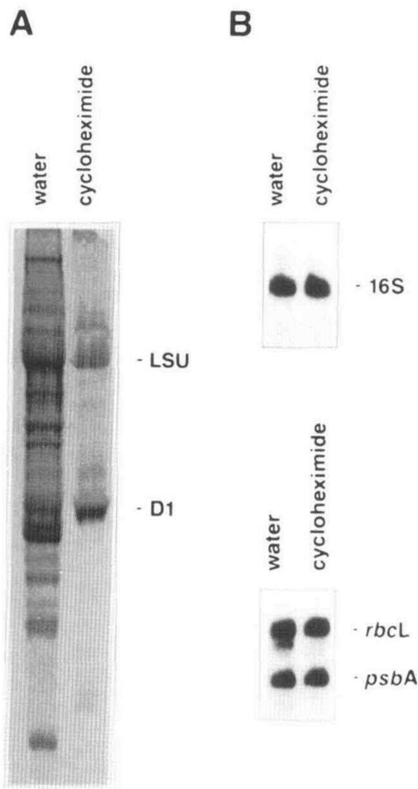

Figure 3. Effect of Cycloheximide Treatment on *psbA* and *rbcL* mRNA Steady-State Levels and Translation.

(A) Inhibition of cytoplasmic translation by cycloheximide. Proteins were labeled in vivo with ^{35}S-methionine after 15 hr of treatment with 25 μg/mL cycloheximide and in a water control. Proteins were separated by SDS gel electrophoresis, transferred to nitrocellulose filters, and exposed to x-ray film. The autoradiograph of proteins from three leaves, treated as described above, is shown. D1, gene product of the *psbA* gene; LSU, large subunit of the ribulose-1,5-bisphosphate carboxylase gene product of the *rbcL* gene.
(B) RNA gel blot analysis of total RNA isolated from cycloheximide-treated and nontreated leaves. Ten micrograms of total RNA was separated on a 1.1% agarose-formaldehyde gel, transferred to nylon membranes, and hybridized with a DNA probe specific for 16S ribosomal RNA and RNA probes specific for *psbA* and *rbcL* mRNA.

in steady-state mRNA level due to the absence of nuclear-encoded proteins.

Taken together, the results indicated that the relative half-lives of *psbA* and *rbcL* mRNAs change during the maturation of the spinach leaf. These data strongly suggest that differential mRNA stability during spinach leaf development is a post-transcriptional mechanism that is involved, at least in part, in the control of plastid mRNA accumulation.

The Change in Stability of the *psbA* mRNA Is not Correlated with Its Polysomal Distribution

One factor that could affect the relative stabilities of *psbA* and *rbcL* mRNAs during leaf development is the recruitment of the mRNAs onto polysomes. Polysome assembly could result in a different degree of protection against nucleolytic degradation. To test this hypothesis, we first analyzed the extent to which *psbA* and *rbcL* mRNAs are assembled into polysomes in young and mature leaves. We took advantage of the sedimentation properties of polysomes, which have higher sedimentation constants than free RNAs or ribonucleoprotein particles and which allow us to distinguish between polysome-bound and nonpolysomal RNA. Crude lysates of young and mature spinach leaves were layered onto 5-mL analytical 15% to 55% sucrose gradients and centrifuged to separate the polysomes from nonpolysomal RNA. A control lysate in which ribosomes were dissociated into subunits by EDTA was centrifuged separately. Ten 0.5-mL fractions were collected and analyzed for *psbA* and *rbcL* mRNA by RNA blot hybridization. The results in Figure 4 show that the polysomal distribution of *rbcL* and *psbA* mRNA differed from each other in young and mature spinach leaves. Most of the *rbcL* mRNA was associated with polysomes, whereas a significant amount of the *psbA* mRNA sedimented at the top of the gradient and was not assembled into polysomes. In the EDTA-treated control lysate, both mRNAs shifted toward the top of the gradient as expected. A similar polysomal distribution of *rbcL* and *psbA* mRNAs also has been reported for barley seedlings (Klein et al., 1988). In spinach, the sedimentation profiles of both mRNAs were similar for lysates from young and mature leaves. These data showed that there is no significant developmental change in the recruitment of both mRNAs onto polysomes. They also suggested that the increase in *psbA* mRNA stability in mature leaves is not due simply to a shift in the polysomal distribution of the *psbA* mRNA at this developmental stage.

Ribosome Association Affects mRNA Decay in Chloroplasts

Although we could exclude a change in polysomal distribution as a mechanism to increase *psbA* mRNA half-life during leaf development, it is still possible that polysome association may be critical to the differential degradation of chloroplast mRNAs. The importance of polysome association for mRNA turnover has been reported for other systems, such as mammalian histone mRNAs (Graves et al., 1987), the MATα1 mRNA in yeast (Parker and Jacobson, 1990), the mammalian β-globin mRNA (Bandyopadhyay et al., 1990), or the β-lactamase and the outer membrane protein mRNA of *Escherichia coli* (Nilsson et

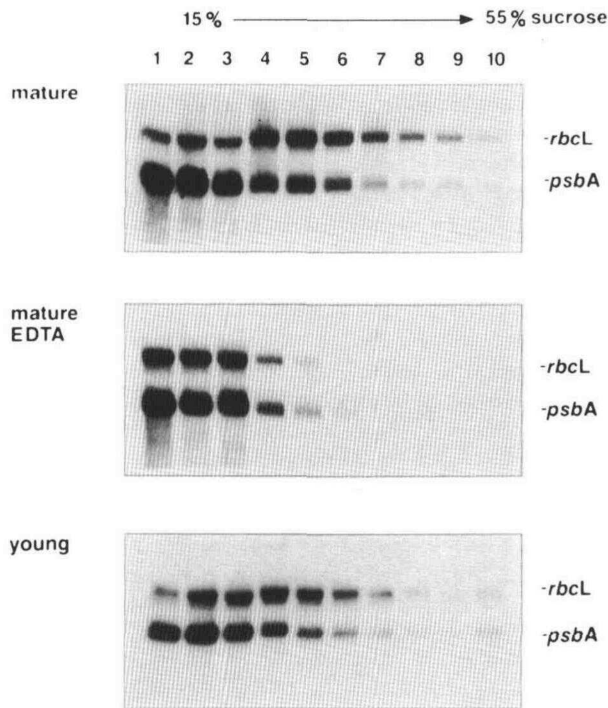

Figure 4. Distribution of *psbA* and *rbcL* mRNAs on Polysomes in Young and Mature Leaves.

Crude leaf lysates from young and mature leaves were size fractionated on analytical 15% to 55% sucrose gradients (Sorvall AH650, 45,000 rpm, 65 min), including one lysate from mature leaves that was treated with EDTA. Ten fractions of 0.5 mL each were collected, and the RNA purified from each fraction was assayed for *psbA* and *rbcL* mRNA levels by RNA gel blot hybridization.

al., 1987), but there are no reports for any plant or organelle mRNAs. The following experiments were designed to examine the general effect of ribosome binding to *psbA* and *rbcL* mRNAs on their decay and differential stability in young and mature leaves. As tools to force mRNAs in vivo into the polysome-bound or the polysome-depleted state, translation inhibitors specific to 70S ribosomes were used. The first, lincomycin, inhibits the early steps in peptide bond formation before polysome assembly and therefore allows polysome runoff without affecting initiation (Vazquez, 1979). The second, chloramphenicol, inhibits the peptidyl-transferase center of free and RNA-bound ribosomes with the same efficiency, thus stalling elongating ribosomes on the mRNA (Vazquez, 1979).

Hydroponic spinach plants were treated for 15 hr with 300 μg/mL lincomycin or 400 μg/mL chloramphenicol. Inhibition of chloroplast translation was monitored by SDS-gel electrophoresis and autoradiography of proteins labeled in vivo with ^{35}S-methionine, as shown in Figure 5. A prominent labeled band from control leaves represents the 32-kD D1 protein of photosystem II, which is the product of the chloroplast *psbA* gene. This protein is synthesized very rapidly in the light and therefore is highly labeled (Hoffman-Falk et al., 1982). Comparison of membrane proteins from inhibitor-treated and control plants demonstrated that the protein is labeled only in the control plant, indicating that both inhibitors effectively reduce chloroplast translation.

The distribution of *psbA* and *rbcL* mRNAs on polysomes in lincomycin-treated and chloramphenicol-treated mature leaves was analyzed by centrifugation of cell lysates in analytical sucrose gradients. Figure 6 shows the results from the RNA gel blot analysis of *psbA* and *rbcL* mRNA levels in the gradient fractions. In lincomycin-treated leaves, both mRNAs were shifted toward the top of the gradient as expected and similar to the profile of RNAs

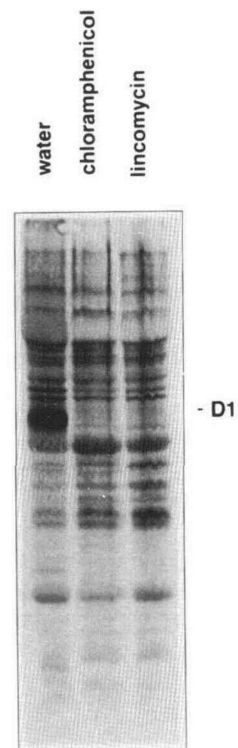

Figure 5. Effect of Chloroplast Translational Inhibitors on Protein Synthesis in Vivo.

Proteins were labeled in vivo with ^{35}S-methionine after 15 hr of treatment with 300 μg/mL lincomycin, 400 μg/mL chloramphenicol, or water. Proteins were separated by SDS gel electrophoresis, transferred to nitrocellulose filters, and exposed to x-ray film. The position of the rapidly labeled D1 protein of photosystem II (*psbA* gene product) is indicated and was used to monitor the efficiency of translational inhibition.

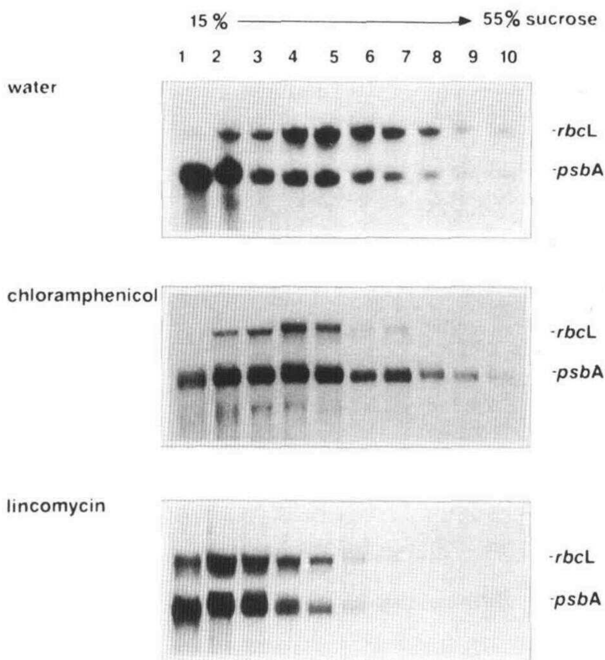

Figure 6. Distribution of *psbA* and *rbcL* mRNAs on Polysomes in Lincomycin-Treated and Chloramphenicol-Treated Leaves.

Crude leaf lysates of mature leaves treated for 15 hr with 300 μg/mL lincomycin or 400 μg/mL chloramphenicol were size fractionated on analytical 15% to 55% sucrose gradients (Sorvall AH650, 45,000 rpm, 65 min). Ten fractions of 0.5 mL each were collected and assayed for *psbA* and *rbcL* mRNAs by RNA gel blot hybridization. The positions of the *psbA* and *rbcL* mRNAs in the gradients are indicated.

from the EDTA-treated lysate (see Figure 4). Furthermore, the sedimentation profiles of both *psbA* and *rbcL* mRNAs were similar after lincomycin treatment and differed from the water control. Treatment of spinach leaves with chloramphenicol had an opposite effect on the distribution of the RNAs. Most of the *psbA* mRNA sedimented at a lower position in the gradient because of the formation of larger polysomes, whereas the sedimentation profile for the *rbcL* mRNA was similar compared with the water control. Interestingly, there was a consistent decrease in the level of the *rbcL* mRNA relative to the *psbA* mRNA in chloramphenicol-treated mature leaves after the analytical gradient centrifugation. This difference, however, was most likely the result of the instability of the *rbcL* mRNA-stalled ribosome complex during polysome isolation or in the sucrose gradient because no significant decrease was observed when the RNA was isolated directly using the guanidinium thiocyanate extraction procedure, as shown in Figure 7B. Based on the specific effects of lincomycin and chloramphenicol, it is possible, therefore, to use these inhibitors to determine the half-lives of both *psbA* and *rbcL* mRNAs in their polysome-bound and polysome-depleted configuration.

Decay measurements were performed using 200 μg/mL actinomycin D to inhibit transcription in leaves that were pretreated for 15 hr with 300 μg/mL lincomycin or 400 μg/mL chloramphenicol. The treatment with the translation inhibitors was continued during the actinomycin D treatment. Control leaves treated with water for 15 hr followed by actinomycin D showed kinetics of *psbA* and *rbcL* mRNA decay essentially identical to the results shown in Figure 1. Figure 7 shows the results from the RNA blot analysis of *psbA* and *rbcL* mRNAs during the actinomycin time course. In all experiments, chloroplast 16S ribosomal RNA was used as an internal standard. After lincomycin treatment (Figures 7A and 7C), the decay of both mRNAs was reduced significantly in young and mature leaves. This result strongly suggests that polysome association per se of *psbA* and *rbcL* mRNAs does not increase the stability of these RNAs.

In contrast to lincomycin-treated leaves, chloramphenicol treatment (i.e., ribosome stalling) caused a more rapid decay of both *psbA* and *rbcL* mRNAs (Figures 7B and 7C). It is important to note, however, that inhibition of chloroplast translation by chloramphenicol also had a stabilizing effect on the *rbcL* mRNA compared with leaves treated with actinomycin alone, whereas this effect of chloramphenicol on the decay of the *psbA* mRNA was detectable only in young leaves (see Figure 1). The basis for this effect of chloramphenicol is not clear, but there is a formal possibility that the translation block affects the synthesis of a short-lived plastid nuclease and/or import of a specific nuclear-encoded nuclease. Control experiments with leaves treated with lincomycin or chloramphenicol, but not actinomycin D, as expected did not reveal significant decay of either *rbcL* or *psbA* mRNA over the 9-hr time course (data not shown). The stabilization observed for the two mRNAs in the presence of the translation inhibitors, however, does not affect the general conclusion that there are apparent differences in half-lives of the spinach *psbA* and *rbcL* mRNAs between their polysome-bound and polysome-depleted configurations. These differences suggest that translation of the two RNAs may be critical to initiate and/or facilitate their turnover and that both mRNAs may decay by different mechanisms.

DISCUSSION

The results presented in this manuscript substantiate and extend the model that post-transcriptional changes in mRNA stability are involved in the differential regulation of spinach chloroplast gene expression. Furthermore, the direct determination of the turnover rates of the spinach *psbA* and *rbcL* mRNAs in vivo provides an estimation of chloroplast mRNA half-lives in plants. The results indicated

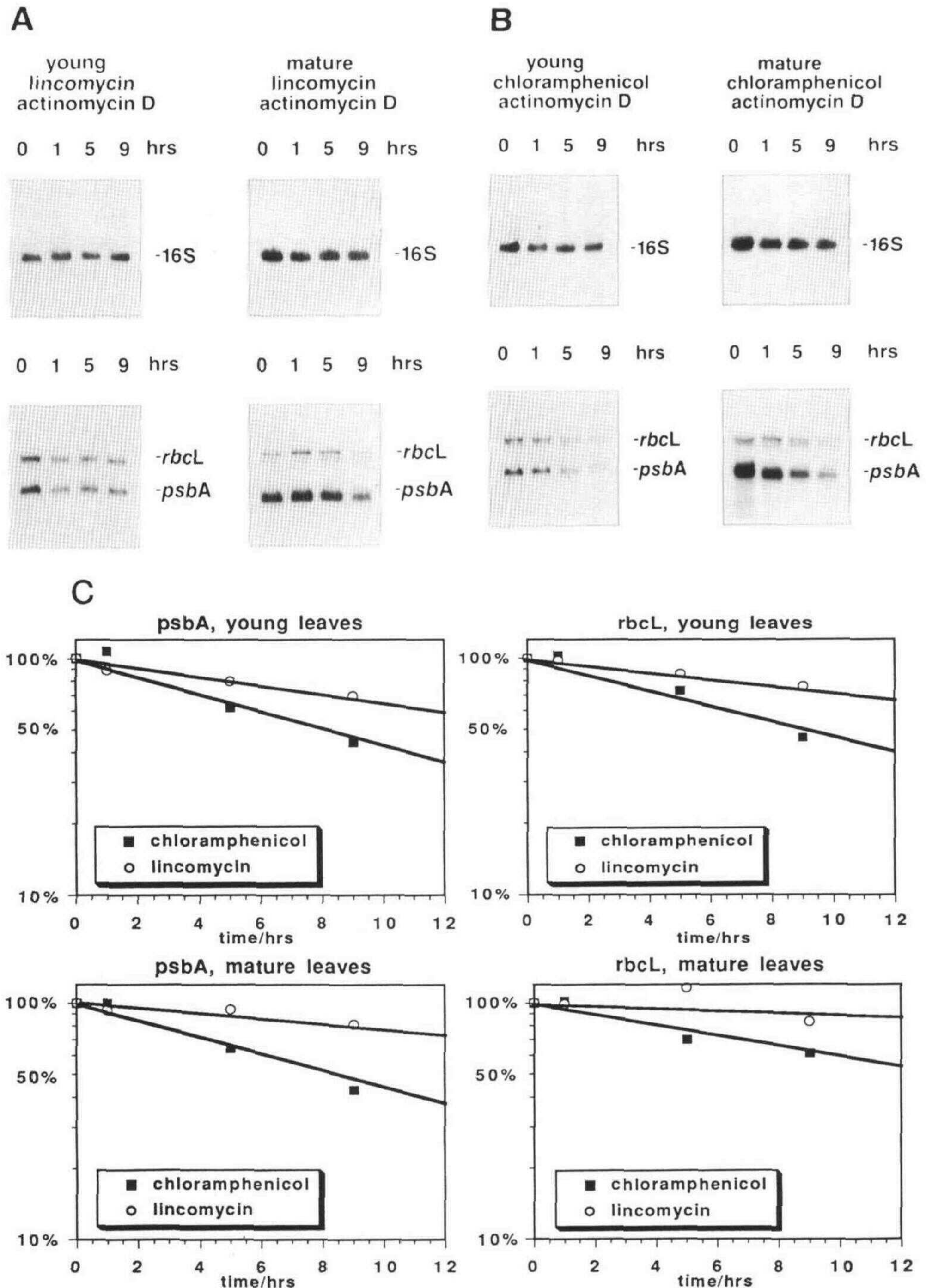

Figure 7. Decay of Polysome-Bound and Polysome-Depleted *psbA* and *rbcL* mRNAs in Leaves.

Chloroplast mRNA decay was measured in young and mature leaves from hydroponic spinach plants after 15 hr of treatment with 400 μg/mL of chloramphenicol or 300 μg/mL lincomycin. The application of the translation inhibitors was continued during the following treatment of leaves with 200 μg/mL actinomycin D. Total RNA was extracted after 0 hr, 1 hr, 5 hr, and 9 hr of actinomycin D treatment.
(A) RNA gel blot analysis of RNA from lincomycin-treated leaves. For each time point, 10 μg of total RNA was separated on denaturating 1.2% agarose-formaldehyde gels and transferred to nylon membranes. *psbA* and *rbcL* mRNAs and 16S ribosomal RNA were detected by hybridization to gene-specific DNA probes.
(B) RNA gel blot analysis of RNA from chloramphenicol-treated leaves.
(C) Quantitation. After RNA blot analysis, the bands of *psbA* mRNA, *rbcL* mRNA, and 16S RNA were excised and radioactivity was measured by scintillation counting. The amount of *psbA* and *rbcL* mRNAs was normalized to 16S RNA for each time point. The graphs represent the averages of data collected from two independent sets of experiments. RNA levels at zero time were fixed to 100%.

that, at least for *psbA* and *rbcL* mRNAs, their half-lives are relatively long compared with the known half-lives of bacterial mRNAs. In addition, the use of translational inhibitors specific to 70S ribosomes strongly suggested that ribosome binding in vivo does not necessarily increase the stability of the two plastid mRNAs. Although chloroplasts have prokaryotic features, these results are in contrast to most bacterial systems in which ribosome protection is an important mechanism to increase the half-lives of mRNAs. Together, our results indicated that the decay of the spinach chloroplast *psbA* and *rbcL* mRNAs is complex and most likely regulated independently by developmentally controlled mechanisms that involve stabilization and/or turnover of free and polysome-bound RNA fractions in the case of *psbA* mRNA and differential stability of both polysome-bound *psbA* and *rbcL* mRNAs.

Previous studies of chloroplast mRNA accumulation and transcription of plastid genes have shown that, in spinach, *psbA* mRNA continues to accumulate relative to *rbcL* mRNA during leaf development despite little change in the relative transcription activity of their genes (Deng and Gruissem, 1987). The direct determination of the decay of these two mRNAs relative to plastid 16S ribosomal RNA in vivo revealed a more than twofold increase in the *psbA* mRNA half-life during spinach leaf development, which is consistent with the observed higher accumulation of *psbA* mRNA relative to *rbcL* mRNA in mature spinach leaves. It should be noted that the application of transcription/translation inhibitors to plants in general poses a significant problem because the extent to which cellular processes are disrupted is difficult to control.

We believe, however, that the observed developmental differences in *psbA* and *rbcL* mRNA half-lives were not simply a consequence of the use of actinomycin D for the following reasons. First, the decay of both *psbA* and *rbcL* mRNAs in young and mature leaves was determined using chloroplast 16S ribosomal RNA as an internal standard. In addition, the comparison of the relative half-lives of *psbA* and *rbcL* mRNAs in young and mature leaves showed the stabilization of *psbA* mRNA relative to *rbcL* mRNA. Second, the efficiency of actinomycin D in chloroplasts of young and mature spinach leaves was controlled by analyzing the decay of the chloroplast *petD* intron sequence, showing that chloroplast transcription was affected by the inhibitor treatment. This control does not prove, however, that the degree to which actinomycin D inhibits transcription in young and mature leaves is identical because the rate of intron synthesis may differ in the two stages because of different rates of splicing. The intron control also indicates that, under the experimental conditions, actinomycin D probably does not inhibit transcription completely because after 5 hr of treatment we could still detect low levels of the *psbB* operon primary transcript. This result suggested that the calculated relative half-lives of *psbA* and *rbcL* mRNAs are most likely underestimated.

Third, we attempted to control the effect of depletion of nuclear-encoded proteins during the actinomycin D treatment on the steady-state levels of the *psbA* and *rbcL* mRNAs. We cannot exclude that the observed developmental differences in *psbA* and *rbcL* mRNA half-lives could in part be controlled by one or several rapidly turning over nuclear-encoded proteins (i.e., proteins with half-lives shorter than the time actinomycin was applied). The application of cycloheximide to block translation of cytoplasmic mRNAs showed, however, that there is no significant effect on the accumulation of both *psbA* and *rbcL* mRNAs for at least 9 hr. It should be noted that the results from the cycloheximide or actinomycin D treatment do not exclude that nuclear-encoded proteins have an important role in the turnover of chloroplast mRNAs. Thus, the measured half-lives for the spinach *psbA* and *rbcL* mRNAs most likely represent their decay rates in mRNA:protein and mRNA:ribosome complexes formed before and during the treatment with actinomycin D.

The mechanisms that control mRNA turnover in chloroplasts of higher plants are unknown. To obtain insights into the pathway of mRNA degradation, we analyzed the decay of *psbA* and *rbcL* mRNAs as polysome-associated complexes in comparison to their polysome-depleted state. The increase in half-lives of both mRNAs after treatment with lincomycin strongly suggested that ribosome association may actually destabilize these two mRNAs in vivo. The results also indicated, although indirectly, that the formation of translation complexes could be a mechanism to initiate and/or facilitate the turnover of both mRNAs.

Our results were in contrast to the situation described for several mRNAs in *E. coli*. First, bacterial mRNA half-lives range from seconds to as long as 20 min (Nilsson et al., 1987); the two chloroplast mRNAs we assayed decayed with half-lives in the range of hours. Second, the half-lives of most bacterial mRNAs are increased by their association with ribosomes in the translation complex. For example, the mRNA from the lactose operon is dramatically destabilized after treatment of the cells with an inhibitor of translation initiation (Schneider et al., 1978). For the β-lactamase mRNA, it has been shown that abortive translation within the first 26 codons destabilizes the mRNA, whereas interruption of translation 3′ to this region does not effect its decay (Nilsson et al., 1987). In this case, *cis*-acting determinants for the decay of the β-lactamase mRNA are confined to specific RNA segments (Nilsson et al., 1988). Third, although our experiments were not designed to determine the role of any nuclear-encoded proteins in chloroplast mRNA turnover, it is likely that nuclear control of plastid mRNA stability is an essential factor. From the complete DNA sequences available for three chloroplast genomes, there is currently no evidence that they encode genes for proteins involved in mRNA binding and degradation (Ohyama et al., 1986; Shinozaki, et al.,

1986; Hiratsuka et al., 1989). Also, *Chlamydomonas* nuclear mutants have been isolated that affect the accumulation of specific chloroplast mRNAs (Kuchka et al., 1989). For these reasons, we believe that models for mRNA decay in chloroplasts cannot be adopted readily from prokaryotic systems.

Several models are possible to explain the significance of polysome association for chloroplast mRNA degradation. First, the polysome complex itself may be important for the degradation process by delivering a specific nuclease. This mechanism has been proposed for the decay of mammalian β-globin mRNA, where polysomes containing the β-globin mRNA copurify with a nuclease activity capable of performing specific cleavages (Bandyopadhyay et al., 1990). Similarly, the coupling of mammalian histone mRNA translation and degradation suggests that a nuclease is associated with the ribosomes involved in translation of this mRNA (Graves et al., 1987). Currently, we have no information on specific nucleases associated with chloroplast ribosomes or chloroplast nucleases in general. Second, the binding of *psb*A and *rbc*L mRNAs to ribosomes may alter the secondary structure of the mRNA, exposing nuclease recognition sites. The exposure of nuclease-sensitive sites may be enhanced by ribosome pausing at rare codons (Wolin and Walter, 1988; Herrick et al., 1990). Third, neither the *psb*A nor the *rbc*L mRNAs but rather their nascent polypeptide chains emerging from the ribosomes may serve as recognition signals for a nuclease, as has been shown for the mammalian tubulin mRNA (Yen et al., 1988).

A more complex mechanism may be possible as well. In yeast, a poly(A)-binding protein interacts with the 3′ end of mRNAs to stabilize those mRNAs in vitro (Bernstein et al., 1989). This protein has also been shown to be required for 60S ribosomal subunit-dependent translational initiation (Sachs and Davis, 1989), suggesting the interaction of a stabilizing factor at the 3′ end of an mRNA with its translation. Both *psb*A and *rbc*L mRNAs, similar to most other chloroplast mRNAs, terminate in an inverted repeat in their 3′ untranslated region, which forms a stable stem-loop structure (Stern et al., 1989). It has been shown that the wild-type 3′ inverted repeat stabilizes ribosome-free upstream segments of RNA against nuclease degradation in vitro relative to linear RNA molecules or mutant 3′ inverted repeats (Stern and Gruissem, 1987; Stern et al., 1989; Adams and Stern, 1990). We have determined that several chloroplast mRNA 3′ inverted repeats interact with a small number of common and distinct proteins (Stern et al., 1989; Schuster and Gruissem, 1991). We do not have evidence of an effect of these 3′ end structures on translation, but they may have a protective function against 3′ exonucleolytic activity when the mRNAs are in their polysome-depleted state after lincomycin treatment. Specific mRNA degradation may than be induced upon assembling the mRNA into polysomes.

Our results provide evidence that chloroplast mRNA decay and its regulation are complex processes that have to take into consideration both polysome-associated and ribosome-free mRNA populations. In addition, our data suggest that *psb*A and *rbc*L mRNAs may decay by different pathways. The comparison of their decay kinetics in lincomycin-treated or chloramphenicol-treated and control plants showed that *rbc*L mRNA is generally stabilized by inhibition of translation, although there is a significant difference between the decay of polysome-bound and polysome-depleted *rbc*L mRNA. The stabilization of *psb*A and *rbc*L mRNAs by the two translation inhibitors was similar in young leaves, but in mature leaves pretreatment with chloramphenicol destabilized the *psb*A mRNA relative to control plants. These data suggested that for both mRNAs polysome association appears to be critical to facilitate specific mRNA degradation. The data also indicated that additional and currently unknown mechanisms and/or factors are involved in regulating the stabilization of the *psb*A mRNA in mature leaves. The stabilization is unlikely to occur at the level of recruitment of the *psb*A mRNA onto polysomes but may involve a developmental change in the stability of the ribosome-free relative to the polysome-associated fraction of the *psb*A mRNA.

METHODS

Plant Material and Inhibitor Treatment

Spinach (*Spinacea oleracea* cv Marathon hybrid) was grown hydroponically under controlled greenhouse conditions. For the application of inhibitors, mature leaves (6 cm and larger) of approximately 6-week-old plants were cut under water and transferred to Eppendorf tubes containing the inhibitor solution. To treat young leaves (1 cm and less), the roots of young, intact plants were cut under water to approximately 2-cm length, and the plants were transferred to Eppendorf tubes containing the inhibitor solution. The inhibitor solution contained 400 μg/mL chloramphenicol (Sigma), 300 μg/mL lincomycin (Sigma), or 25 μg/mL cycloheximide (Sigma) in water as detailed in Results. For the application of actinomycin D (Sigma), the inhibitor concentration was adjusted to 200 μg/mL using a 2 mg/mL stock solution in water. After the appropriate incubation times, which are described in detail in the Results, leaves were harvested into liquid nitrogen.

RNA Preparation

Total RNA was isolated from young and mature leaves as described by Barkan (1989) using a guanidinium thiocyanate extraction procedure. Contaminating DNA in the RNA preparation was removed by digestion with RNase-free DNase (Bethesda Research Laboratories).

Preparation of Polysomes

To isolate total polysomes from leaf tissue (Jackson and Larkins, 1976; Barkan, 1989), 0.5 g of leaf material was ground in liquid nitrogen with a mortar and pestle. After addition of 1.5 mL of a buffer containing 0.2 M Tris-HCl, pH 9, 0.2 M KCl, 35 mM $MgCl_2$, 25 mM EGTA, 0.2 M sucrose, 1% Triton-X-100, 2% polyoxyethylene-10-tridecyl ether, 0.5 mg/mL heparin, 100 mM β-mercaptoethanol, 100 μg/mL chloramphenicol, the mixture was ground until thawed. To remove debris, the homogenate was forced through a glass wool plug in a 3-mL syringe, collecting the liquid into an Eppendorf tube. After a 10-min incubation on ice, nuclei and remaining debris were pelleted for 5 min in a microcentrifuge at 4°C. The supernatant was collected and adjusted to 0.5% sodium-deoxycholate and incubated for 5 min on ice. Remaining insoluble material was removed by centrifugation for 15 min at 4°C in a microcentrifuge. Aliquots of 0.5 mL of the supernatant were layered onto 4.5-mL 15% to 55% sucrose gradients in 40 mM Tris-HCl, pH 8.0, 20 mM KCl, 10 mM $MgCl_2$, 0.5 mg/mL heparin, 100 μg/mL chloramphenicol and centrifuged 65 min at 45,000 rpm in a Sorvall AH650 rotor. Ten fractions of 0.5 mL were collected. The RNA in each fraction was purified by the addition of SDS to 0.5%, EDTA to 20 mM, phenol extraction, and precipitation with ethanol. Aliquots of $\frac{1}{12}$ of the original volume of each fraction were subjected to denaturing gel electrophoresis and subsequent analysis of the RNA. To dissociate polysomes, control samples were treated by the addition of EDTA to 20 mM to the leaf lysates before loading onto the gradient. In these gradients 10 mM $MgCl_2$ was substituted by 1 mM EDTA.

Hybridization Probes

The gene-specific spinach probes for *psb*A (1.2-kb BglII-XbaI fragment; Zurawski et al., 1982) and *rbc*L (1.2-kb PstI-EcoRI fragment; Zurawski et al., 1981) were subcloned into the RNA transcription vector pIBI 76 (International Biotechnologies, Inc., New Haven, CT), which allows the transcription of a *psb*A-specific RNA probe using the T7 promoter and of an *rbc*L-specific RNA probe using the SP6 promoter. The spinach gene probe for the chloroplast 16S ribosomal RNA is a 3.0-kb XhoI fragment, which was subcloned from Xho8a (Palmer and Thompson, 1981) into pUC18. The constructs were kindly provided by Dr. Xing-Wang Deng (University of California, Berkeley/USDA Plant Gene Expression Center, Albany, CA). For the spinach *pet*D intron, a 320-bp XbaI-EcoRI fragment (Heinemeyer et al., 1984; Westhoff and Herrmann, 1988) was subcloned into pBluescript SK^+ (Stratagene, Inc.). The *cab*-specific DNA probe was a 0.5-kb HindIII fragment from the coding region of the tomato *cab*3C gene (Pichersky et al., 1987).

For *rbc*L-specific and *psb*A-specific RNA probes, plasmid DNAs containing the gene-specific fragments were linearized with the appropriate enzyme. A standard labeling reaction of 50 μL contained 20 μg/mL linear DNA; 1 mM DTT; 0.5 mM each ATP, GTP, and CTP; 0.05 mM UTP; 50 μCi of α-^{32}P-UTP (800 Ci/mmol, 20 mCi/mL; Amersham); 10 units of SP6 or T7 polymerase (Bethesda Research Laboratories); and buffer supplied by the manufacturer. After incubation for 1 hr at 37°C, 0.5 volume of 7.5 M NH_4OAc and 3 volumes of ethanol were added. After pelleting, the probes were used without further purification.

For 16S RNA-specific, *cab*-specific, and *pet*D intron-specific probes, gel-purified DNA fragments were radiolabeled by the random hexamer priming method. The fragments (150 ng) were boiled for 3 min with 2-μg hexamers (Pharmacia LKB Biotechnology Inc.) and placed on ice. The following reaction contained 50 mM Tris-HCl, pH 7.0; 10 mM $MgCl_2$; 1 mM DTT; 35 μM each dATP, dTTP, and dGTP; 50 μCi of α-^{32}P-dCTP (3000 Ci/mmol, 10 mCi/mL; Amersham); and 5 units of Klenow fragment. After 2 hr at 37°C, 0.5 volume of 7.5 M NH_4OAc and 3 volumes of ethanol were added. After pelleting, the probes were used without further purification.

Filter Hybridization

For RNA gel blot analysis, RNAs were separated on 1.2% agarose-formaldehyde gels (Maniatis et al., 1982). The RNAs were transferred overnight to Hybond N nylon membranes (Amersham) in 5 × SSC, pH 7.0 (1 × SSC: 0.15 M NaCl, 0.015 M Na-citrate). The membrane was UV treated to couple the RNA covalently (120 mJ, Stratalinker, Stratagene). All prehybridizations were performed for 4 hr at 65°C in 6 × SSC, pH 7.0, 5 × Denhardt's solution, 0.5% SDS, and 50 μg/mL denatured salmon sperm DNA (1 × Denhardt's solution: 0.02% BSA, 0.02% Ficoll 400, 0.02% PVP 40). Filters were hybridized at 65°C for at least 16 hr using DNA probes (1×10^8 cpm/μg DNA) or at 68°C for at least 16 hr using RNA probes (1×10^8 cpm/μg DNA template) under conditions of probe excess. Filters hybridized to DNA probes were washed three times for 30 min at 65°C in 0.2 × SSC, pH 7.0, 0.1% SDS. Filters hybridized to RNA probes were washed two times for 30 min at 68°C in 0.2 × SSC, pH 7.0, 0.1% SDS and once for 30 min at 73°C in 0.1 × SSC, pH 7.0, 0.1% SDS, 10% formamide. Filters were exposed to x-ray films for various lengths of time. After autoradiography, the hybridized probes from the appropriate bands were excised from the filters and the hybridized radioactivity was determined by scintillation counting.

In Vivo Labeling of Proteins and RNA

To test for the efficiency of translation inhibitors, proteins were labeled in vivo (Barkan, 1989). A vein in the tip portion of the leaf was injured using a file, and 25 μCi of ^{35}S-methionine (1275 Ci/mmol, 10 mCi/mL; Amersham) were pipetted onto the injured site. Uptake and labeling were allowed to proceed for 1 hr at room temperature, adding water onto the injury after uptake of the label to prevent drying. For protein analysis, the leaf tips (approximately 0.2 g of tissue) were ground in 100 mM Tris-HCl, pH 7.2, 10% sucrose, 5 mM EDTA, 5 mM EGTA, 2 mM PMSF, 40 mM β-mercaptoethanol. To remove debris, the homogenate was forced through a glass wool plug in a 3-mL syringe, while the liquid was collected into an Eppendorf tube. The membranes were pelleted by centrifugation for 3 min in a microcentrifuge. The supernatant containing soluble proteins was transferred to a new tube, and the pelleted membrane material was resuspended in 50 μL of homogenization buffer. This fraction also contains soluble proteins and therefore reflects total protein. Radioactive incorporation was determined by binding to DE81 filters. The same amount of protein of each sample was separated by SDS-gel electrophoresis (Laemmli, 1970). The separated proteins were transferred to nitrocellulose membrane in 50 mM Tris, 38 mM

glycine, 0.04% SDS, 20% methanol using a graphite plate transfer apparatus for 3 hr, 0.8 mA/cm^2 gel. The filters were dried and exposed to x-ray film.

To test the efficiency of actinomycin D, young leaves were cut under water and incubated in 200 μg/mL actinomycin D solution. After 6 hr, carrier-free ^{32}P-orthophosphate was added to 500 μCi/mL, and labeling was continued for 1 hr. After 1 hr, RNA was extracted and analyzed by denaturing gel electrophoresis.

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