Plant Cell Physiol. 29(1): 1–7 (1988)

Quantification of mRNA in Chloroplasts of *Chlamydomonas reinhardii*: Equal Distribution of mRNA for a Soluble and a Membrane Polypeptide in Stroma and Thylakoids

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The relative contents of the mRNAs were analyzed for the 32 kDa herbicide-binding protein and for the large subunit of ribulose-1,5-bisphosphate carboxylase in the membrane fraction and in the soluble fraction of chloroplasts from *Chlamydomonas reinhardii*. The presence of mRNA for the two proteins in both subchloroplast fractions was demonstrated by in vitro translation of isolated RNA in the reticulocyte lysate. The relative amounts of the two mRNAs were measured by hybridizations with cloned chloroplast DNA probes at two stages of the cell cycle. Both mRNAs were distributed in the same ratio between membrane and soluble fractions, about 75% of both mRNAs being in the membrane and 25% in the soluble fraction. Therefore, in chloroplasts the accumulation of mRNAs on thylakoid membranes does not reflect the final localization of soluble and membrane proteins.

Key words: *Chlamydomonas reinhardii* — Chloroplast (Polysome) — mRNA content — 32 kDa herbicide-binding protein — Ribulose-1,5-bisphosphate carboxylase (Large subunit).

In chloroplasts, thylakoid-bound polysomes are present (Margulies and Michaels 1975, Bolli et al. 1981) in varying amounts during the cell cycle (Bolli et al. 1981, Chua et al. 1976). The functional significance of the membrane-binding is not clear. By translating polysomes bound to thylakoids of *Chlamydomonas*, Margulies et al. (1975) found that essentially all terminated polypeptide chains remained with thylakoids, and thus were incorporated cotranslationally into the membranes as they were synthesized. The comparison of the translational capabilities of free and bound chloroplast ribosomes from pea suggested a functional difference between these polysome species (Ellis 1977, 1981). After incubation of the membrane-free, soluble chloroplast fraction with ATP, GTP and ^{35}S-methionine, most of the translation products of free chloroplast ribosomes were released as soluble proteins, the main product being the LS. In contrast, all radioactive products synthesized by thylakoid-bound ribosomes remained with the membrane even after puromycin treatment, and hence, were thought to be hydrophobic polypeptides. Subsequently, several workers (Alscher et al. 1978, Herrin et al. 1981, Herrin and Michaels 1985, Margulies 1983, Bhaya and Jagendorf 1984) confirmed that the "function of thylakoid-bound ribosomes is to synthesize thylakoid membrane proteins" (Herrin et al. 1981). The finding that the extrinsic α- and β-subunits of CF_1 are synthesized on both free and membrane-bound polysomes of pea chloroplasts (Bhaya and Jagendorf 1985) supported the idea that the hydrophobicity of the proteins determines their site of synthesis.

A new aspect arose from the work of Leu et al. (1984) and Minami and Watanabe (1984). They found different protein products after so-called run-off translation of thylakoid-bound polysomes and of stromal polysomes and ribosomes. However, no difference among the product patterns was observed, when RNA extracted from thylakoids or stroma was translated in a heterologous translation system. With both extracts the LS and the 32 kDa protein were produced. Recently, Hattori and Margulies (1986) also found that the LS was produced by thylakoids

Abbreviations: CF_1, coupling factor 1; 32 kDa protein, 32 kDa herbicide-binding membrane protein; LS, large subunit of ribulose-1,5-bisphosphate carboxylase; Rubisco, ribulose-1,5-bisphosphate carboxylase; SSPE, 0.18 M NaCl, 1 mM EDTA, 10 mM Na-phosphate pH 7.4.

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of spinach in an *E. coli* translation system and concluded translatable mRNA for LS to be present in thylakoid-bound polysomes. Unfortunately, the direct quantitative determination of mRNA via its translation product is not accurate, what we demonstrate in the present paper. Interestingly, Marzuki and Hibbs (1986) also found that in yeast mitochondria a hydrophilic protein, the var 1 protein, is synthesized on membrane-associated mitochondrial ribosomes and that these ribosomes cannot be distinguished from those responsible for the synthesis of the hydrophobic mitochondrial translation products.

To clarify the questions on mRNA localization in chloroplast, the mRNA contents for a membrane and a soluble chloroplast protein should be measured simultaneously in stroma and thylakoid fractions from isolated chloroplasts. Since evidence has been presented for the variation of total amount of chloroplast RNAs and mRNAs during the cell cycle of *Chlamydomonas* (Herrin et al. 1986, Howell and Walker 1977), the mRNA contents should be determined for at least two different stages of the cell cycle.

Therefore, at half time and at the end of the light period of synchronized cultures of *Chlamydomonas reinhardii* we determined the amounts of mRNAs for LS and 32 kDa protein by hybridization with cloned DNA-fragments, in order to compare the distribution of mRNAs between stroma (free polysomes) and thylakoids (membrane-bound polysomes), and to estimate the extent of mRNA binding to thylakoids.

Materials and Methods

Chloroplast isolation—Chloroplasts were isolated as previously described (Mendiola-Morgenthaler et al. 1985) at the 6th and 13th hour of a light period from synchronized cultures (14 h light/10 h dark) of *Chlamydomonas reinhardii* cw-15 (stock CC-1615 from Chlamydomonas Genetics Center, Duke University, Durham, NC). The cells were incubated with 200 μg/ml chloramphenicol for 20 min before harvesting. The homogenization medium contained 100 μg/ml chloramphenicol. The isolated chloroplasts were washed twice in the homogenization medium without chloramphenicol prior to fractionation. The ribosomal subunit composition of chloroplasts and chloroplast fractions was analyzed as previously described (Bolli et al. 1981).

Fractionation of chloroplasts—Isolated chloroplasts were sedimented and carefully resuspended in 25 mM Mg-acetate; 25 mM KCl; 12.5 mM Tris/HCl, pH 7.6, to a final concentration of 1 mg/ml chlorophyll. After they had been broken in the Yeda-Press at 25–30 atmospheres ($2.5–3 \times 10^6$ Pa), the resulting homogenate was adjusted to 25 mM Mg-acetate; 200 mM KCl; 40 mM Tris/HCl pH 7.6; 10 mM 2-mercaptoethanol. The supernatant obtained by centrifugation at $39{,}000 \times g$ for 15 min was free of chlorophyll and called the soluble chloroplast fraction. The pellet was washed twice and resuspended to the original volume in the same buffer (membrane fraction).

Extraction of RNA—Chloroplasts or chloroplast fractions were solubilized in 1% sodium laurylsarcosinate and extracted once with phenol (equilibrated with Tris/HCl, pH 8.0, and containing 0.1% hydroxyquinoline and 0.2% 2-mercaptoethanol), once with phenol/chloroform/isoamyl alcohol 25 : 24 : 1 (v/v/v) and once with chloroform/isoamyl alcohol 24 : 1 (v/v). The RNA was precipitated with ethanol. The dry RNA was dissolved in double distilled water. The amounts of RNA were measured spectrophotometrically and expressed as A_{260} units.

In vitro translation—In vitro translation in the reticulocyte lysate was done following the instructions of the manufacturer (NEN). Products were analyzed by polyacrylamide gel electrophoresis followed by fluorography (Leu et al. 1984). The intensity of labeling of the two main products was determined by integration of the densitograms obtained after different exposure times.

Cloned DNA probes—Plasmids pEC8 and pEC23 containing the *Chlamydomonas* chloroplast DNA EcoRI fragment R15 and R14, respectively, were isolated and characterized by Herrin and Michaels (1985 and personal communication). Plasmid R 15/4 was obtained from J.-D. Rochaix (University of Geneva) and contains a HindIII internal fragment of the *Chlamydomonas* LS gene (Dron et al. 1982).

Northern blot and dot blot analysis—RNA was separated in 1.5% agarose/6% formaldehyde gels (Maniatis et al. 1982), transferred immediately onto nitrocellulose filter and hybridized with labeled DNA probes (Herrin and Michaels 1984). As molecular weight standards rRNA of *E. coli* were separated on the same gel and stained with Acridine Orange. For dot blot hybridization, the RNA was denatured with 50% formamide, 6% formaldehyde in SSPE at 50°C. Subsequently, the buffer concentration was adjusted to 4.4 × SSPE and the solution was applied to nitrocellulose filter using a Bio-Dot microfiltration apparatus (Bio Rad).

Results

Chloroplasts of *Chlamydomonas reinhardii* were isolated in the presence of chloramphenicol and separated into soluble and membrane fractions. From the analysis of the ribosomal subunits in these fractions (Fig. 1) the absence of cytoplasmic ribosomes can be seen. About 55% of the chloroplast ribosomes were found in the membranes, about 35% in the soluble fraction, and the rest was lost during fractionation (Table 1). From isolated chloroplasts, we extracted about 7.1 A_{260} units RNA per mg chlorophyll. When total RNA was extracted from the chloroplast fractions, 63% of the RNA was recovered from

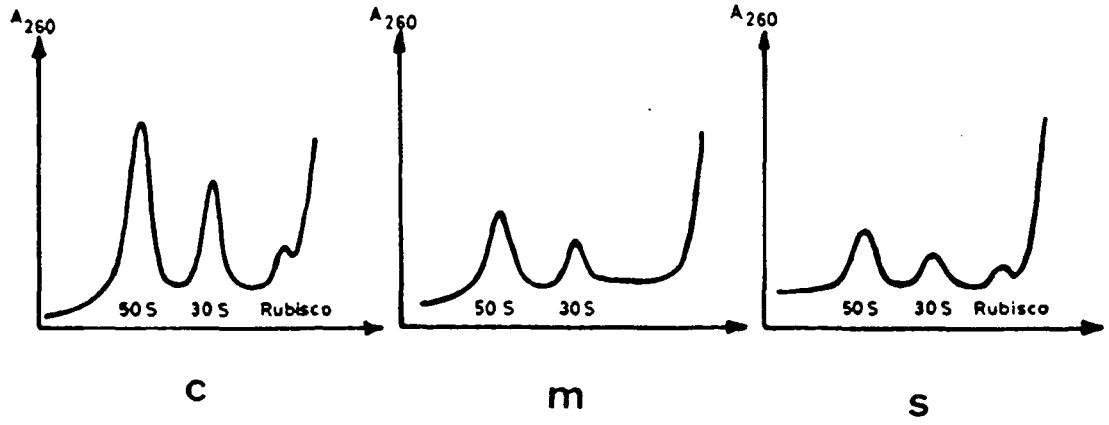

Fig. 1 Analysis of ribosomal subunits of whole chloroplasts (c), chloroplast membranes (m) and soluble fraction (s). The ribosomal subunits were isolated and analyzed by centrifugation on sucrose density gradients.

the thylakoids and 37% from the stroma (Table 1). Thus, within the limits of determination this ratio was comparable to the ratio of ribosomes recovered from the same fractions (Table 1).

The RNAs from whole chloroplasts, membranes and soluble fraction were translated in vitro using a reticulocyte lysate. The main products, previously identified as the LS (55 kDa) and the 34 kDa precursor of the 32 kDa protein (Leu et al. 1984), were labeled by in vitro translation of RNAs from all three fractions (Fig. 2), showing that intact mRNAs for both proteins are present in the soluble and in the membrane fractions. However, depending on the amount of total RNA present in the incubation mixture, the relative labeling intensities of LS and 32 kDa protein precursor were quite different. Therefore, such in vitro translation should not be used for quantitative determinations of mRNA (Fig. 2).

Table 1 Amounts of ribosomes and extracted RNA of chloroplasts (c), membranes (m) and soluble (s) fraction

	Chloroplast ribosomes (%)[a]	Extracted RNA (%)[b]
Whole chloroplasts (c)	100	—
Membrane fraction (m)	55	63
Soluble fraction (s)	35	37
Ratio (m/s)	1.6	1.7

[a] The distribution of chloroplast ribosomes was determined by measuring the area of the peaks of the densitograms of Fig. 1.

[b] RNA was extracted from chloroplast membranes (m) and soluble fraction (s) with the phenol/chloroform procedure. Here, the sum of extracted RNA of (m+s) was taken as 100%.

The relative contents of the mRNAs for the LS and for the 32 kDa protein were determined by hybridization with the cloned EcoRI fragments of chloroplast DNA from *Chlamydomonas reinhardii*, namely pEC8 or 15/4, hybridizing to the LS mRNA (Dron et al. 1982, Rochaix 1981), and pEC23, containing a part of the 32 kDa protein gene (psbA) and hybridizing to the 32 kDa protein mRNA (Harrin and Michaels 1984, 1985, Rochaix 1981). By hybridizing the labeled DNA probes to Northern blots of chloroplast RNA (Fig. 3), labeled bands were visible at 1.5 kb and 1.2 kb, representing the mRNAs for LS and 32 kDa protein, respectively. Furthermore, the autoradiograms clearly demonstrate a higher content of LS mRNA in the membrane fraction than in the soluble fraction (Fig. 3).

Fig. 3 also demonstrates the intactness of isolated mRNA and the specificities of our DNA probes. Therefore, in the following experiments we used dot blot hybridization to measure relative mRNA contents in whole chloroplasts, membranes and soluble fraction at the two indicated stages of the cell cycle. By this method it was possible to quantify a large number of RNA samples under strictly identical conditions to allow direct comparison of the results. The results in Table 2 show, (i) that the mRNAs for the soluble protein LS, and for the 32 kDa membrane protein are present in the same ratio in the RNA extracted from membranes and in the RNA from the soluble fraction, (ii) that these mRNAs are enriched in the RNA from the membrane fraction, while their content in the RNA from the soluble fraction is about half as much as in the thylakoids, and (iii) that these findings are essentialy the same at the 6th and 13th h of the light period.

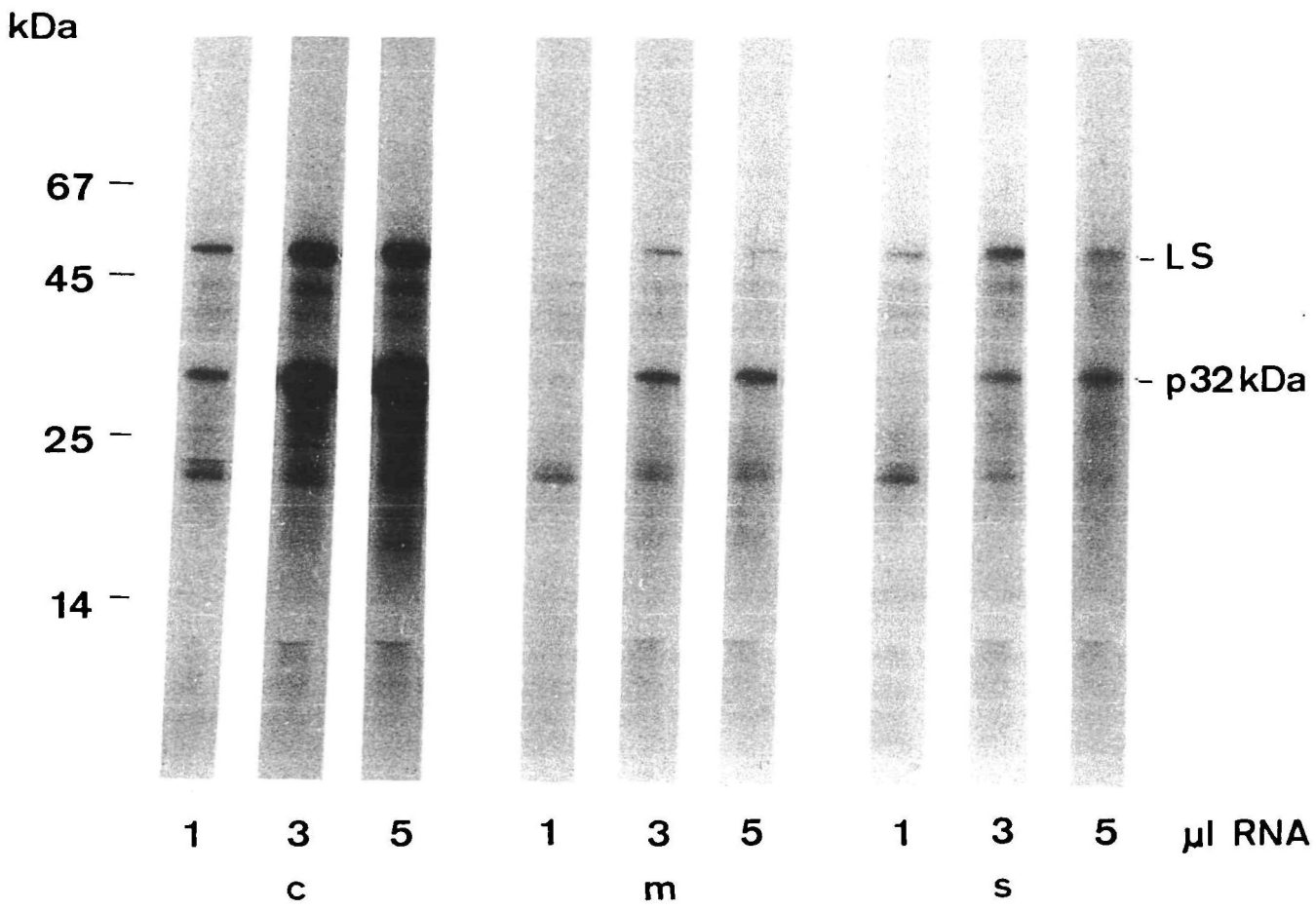

Fig. 2 Fluorogram of the products of in vitro translation in reticulocyte lysate. RNAs were extracted from whole chloroplasts (c), membrane (m) and soluble (s) fractions. For each type of RNA, the product patterns of three incubations with increasing amounts of RNA (0.025, 0.075, 0.125 A_{260} units per 15 µl reaction mixture, respectively) are shown. In controls without added RNA, no radioactive bands were detected.

Discussion

In vitro translation of isolated RNA from chloroplasts and subchloroplast fractions and identification of the two main products (Leu et al. 1984) showed that mRNAs for the soluble (LS) and the membrane protein (32 kDa protein) are present in both the soluble and the membrane fraction of the chloroplast (Fig. 2). However, the amount of mRNAs in each fraction could not be determined quantitatively by translation experiments, because protein synthesis activity was not linearly related to the amount of RNA added (Fig. 2). Therefore, for the determination of the relative mRNA contents, we used hybridization techniques.

If the membrane-bound polysomes predominantly synthesize membrane polypeptides, the polysomes can be expected to be enriched with respect to mRNAs for membrane polypeptides, while free polysomes should predominantly contain mRNAs for soluble proteins. Indeed, in working with whole cells of *Chlamydomonas* instead of isolated chloroplasts, Herrin and Michaels (1985) concluded from their hybridization experiments that the mRNA for the 32 kDa protein is associated with thylakoid-bound polysomes and not present in the soluble cell fraction. However, a comparison of the amounts of RNA isolated from whole cells of *Chlamydomonas* (Herrin and Michaels 1984, Valle et al. 1981) and from isolated chloroplasts (this work) shows that chloroplast RNA is about 10–20% of total cell RNA. Therefore, the large amount of cytoplasmic RNA in their soluble fraction of whole cells should have obscured the presence of mRNA for the 32 kDa protein.

We extracted the RNA from isolated chloroplasts and

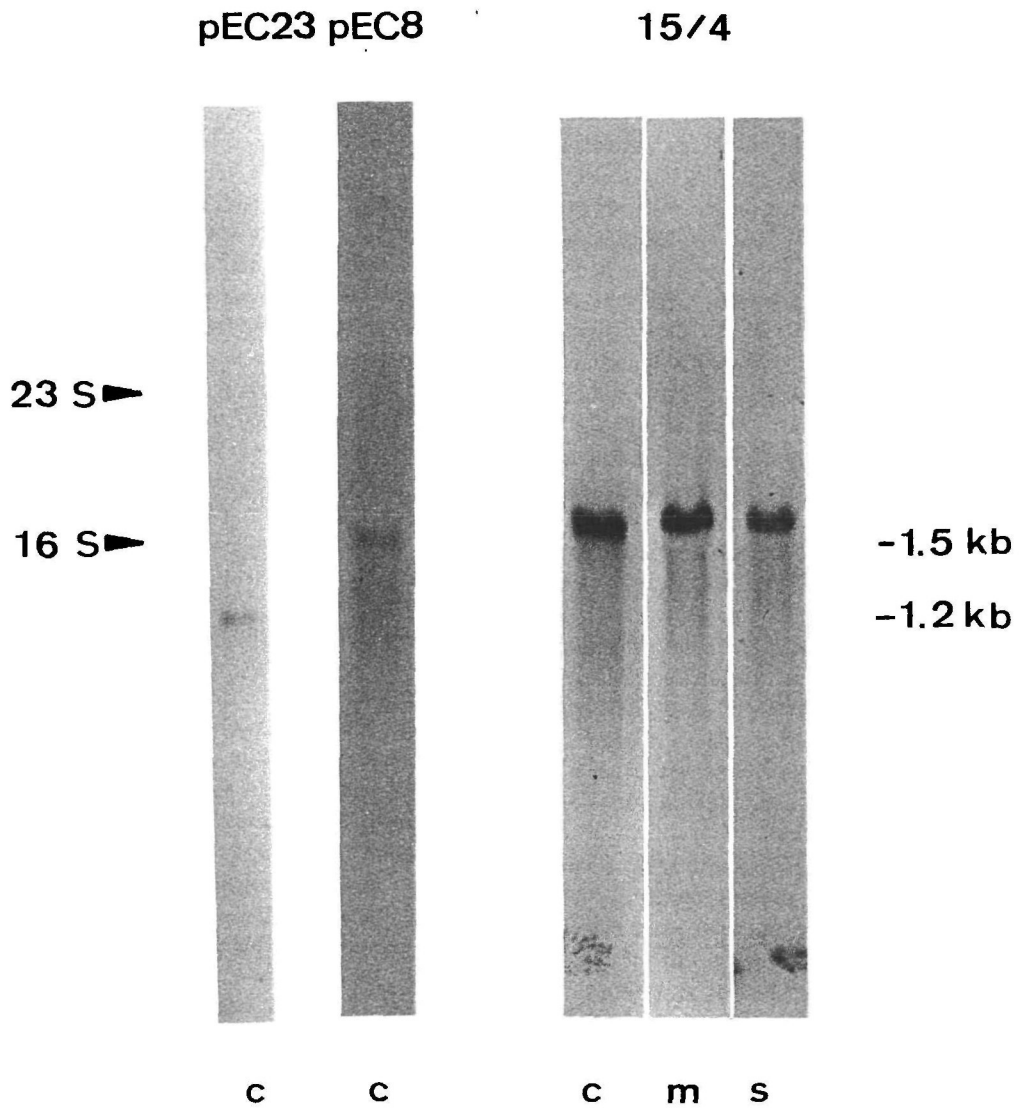

Fig. 3 Autoradiograms of Northern blot hybridizations with nicktranslated plasmids from pEC8 or 15/4 (LS-gene) and pEC23 (32 kDa gene). RNA, 0.125 A_{260} units from chloroplasts (c), membrane fraction (m) and stroma (s) were separated electrophoretically. Arrows indicate the migration distance of rRNA of *E. coli*.

Table 2 Relative mRNA contents in RNAs extracted from whole chloroplasts and from the membrane and soluble fractions as determined by dot-blot hybridization

Harvesting of cells:	6th h of light period			13th h of light period	
	32 kDa (pEC23)	LS (pEC8)	LS (15/4)	32 kDa (pEC23)	LS (15/4)
Whole chloroplast	1	1	1	1	1
Membrane	1.21 (0.09)	1.36 (0.11)	1.23	1.21 (0.05)	1.44 (0.10)
Soluble	0.47 (0.03)	0.53 (0.10)	0.59	0.66 (0.11)	0.62 (0.20)
No. of experiments	3	5	1	3	3

Of each RNA 0.01 and 0.05 A_{260} units were blotted on nitrocellulose filter. The mRNA contents of chloroplast RNA, determined with each DNA probe, were normalized to unity. The values for the RNAs of the chloroplast fractions were calculated relative to unity. In parenthesis: standard deviation.

our results show, that the mRNAs for the LS and for the 32 kDa protein occur in about the same ratio in the soluble and the membrane fractions (Table 2), suggesting that the distribution of mRNAs for membrane and soluble proteins does not differ between the soluble and the membrane chloroplast compartments. This agrees with the results of run-off translation showing that some membrane polypeptides can be synthesized by free polysomes (Bhaya and Jagendorf 1985) and that a soluble polypeptide can be synthesized by membrane-bound polysomes (Hattori and Margulies 1986).

Furthermore, we have shown that in RNA extracted from chloroplast membranes the mRNA content is about twice that in RNA from the soluble fraction (Table 2). Together with the fact that the membrane fraction contains also about 1.6 times more RNA as the soluble fraction (Table 1), we calculate that in chloroplasts, about 75% of the mRNAs for both the soluble and membrane polypeptides occur in membrane-bound form, while only about 25% seem to occur in the stroma.

Finally, during the second half of the light period of the cell cycle, when potein synthesis declines gradually as measured by $^{35}SO_4$ incorporation (Howell et al. 1977), the mRNA distribution and the relative mRNA contents in membrane and soluble fractions are constant (Table 2). Whether this constancy is maintained during the whole cells cycle, remains to be determined.

In the light of these results, thylakoids seem to be a most important site of chloroplast protein synthesis. Furthermore, since there are differences in the products labeled by free and membrane-bound polysomes in run-off translation (Ellis 1977, Leu et al. 1984, Minami and Watanabe 1984), there must be a mechanism in each of these chloroplast compartments by which the translation of selected mRNAs is controlled.

We thank D. Herrin, Tampa/Athens and J.-D. Rochaix, Geneva for their generous gifts of the cloned DNA probes. This work was supported by the Swiss National Foundation for Scientific Research. E. B. received a fellowship from the Roche Research Foundation, Basel.

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(Received April 28, 1987; Accepted September 29, 1987)