Chloroplast Ribosomal Proteins of *Chlamydomonas* Synthesized in the Cytoplasm Are Made as Precursors

ROBERT J. SCHMIDT, ALAN M. MYERS, NICHOLAS W. GILLHAM, and JOHN E. BOYNTON
Departments of Botany and Zoology, Duke University, Durham, North Carolina 27706

ABSTRACT Polyadenylated RNA from *Chlamydomonas* was translated in a cell-free rabbit reticulocyte system that employed [^{35}S]methionine. Antibodies made to four chloroplast ribosomal proteins synthesized in the cytoplasm and imported into the organelle were used for indirect immunoprecipitation of the labeled translation products, which were subsequently visualized on fluorographs of SDS gels. The cytoplasmically synthesized chloroplast ribosomal proteins were first seen as precursors with apparent molecular weights of 1,000 to 6,000 greater than their respective mature forms. Processing of the ribosomal protein precursors to mature proteins was effected by adding a postribosomal supernatant that had been extracted from cells of *Chlamydomonas*. In contrast to the chloroplast ribosomal proteins synthesized in the cytoplasm, two such proteins made within the chloroplast were found to be synthesized in mature form in cell-free wheat germ translation systems programmed with nonpolyadenylated RNA.

Several chloroplast proteins synthesized on cytoplasmic ribosomes are made as precursors that are processed posttranslationally into mature products upon transport into the organelle (6, 8, 31). Precursors ~4,000–6,000 daltons larger than mature proteins have been demonstrated in the case of the small subunit of ribulose-1,5-bisphosphate carboxylase (RuBPCase[1]; 5, 7, 13, 16), the chlorophyll *a/b* binding complex polypeptides 15 and 16 (1, 25), plastocyanin (2), and ferredoxin (18). Westhoff et al. (34) have found that the gamma and delta subunits of the spinach CF_1 complex are synthesized as precursors of 9,000 to 10,000 daltons larger than their mature forms, and Grossman et al. (15) have found precursors of ferredoxin-$NADP^+$ oxidoreductase and plastocyanin that are 8,000 and 15,000 daltons larger than the mature products, respectively. The case of plastocyanin shows that the size of the precursor for a specific polypeptide can vary in different species, since the size of the transit peptide for the plastocyanin precursor moiety is 4,000 daltons in the chlorophyte *Scenedesmus* (2), but 15,000 daltons in pea (15).

Although the sizes of the transit peptides of several of the chloroplast proteins characterized to date are similar, one does not know whether they share a common processing reaction. The amino-terminal sequence of the precursor to the RuBPCase small subunit in pea shows no homology to that from *Chlamydomonas* (10, 26). The algal small subunit precursor was previously reported not to be taken up or processed by pea chloroplasts in vitro, although the pea precursor was processed normally in the homologous in vitro system (6). Recently, Mishkind et al. (21) have reported that this algal small subunit precursor is taken up, but incompletely processed by isolated chloroplasts from vascular plants. Whether or not any similarities exist in the transit peptides of different cytoplasmically synthesized chloroplast proteins from the same species is not yet known. The number of processing enzymes involved in the maturation of these precursors, as well as the coding sites of these enzymes, remain to be ascertained.

These questions are especially pertinent for studies of the biogenesis of chloroplast ribisomes. At least 43 of the 64 chloroplast ribosomal proteins are synthesized on cytoplasmic ribosomes in *Chlamydomonas*, whereas 19 or 20 are made within the chloroplast (28). Since these proteins presumably are required in stoichiometric amounts to assemble functional chloroplast ribosomes, we are interested in knowing whether or not the cytoplasmically synthesized proteins are initially made as precursors, and share a common transport mechanism responsible for their uptake by chloroplasts. Likewise we wish to determine whether or not ribosomal proteins

[1] *Abbreviations used in this paper:* ddH_2O, double distilled water; HS, high salt medium without an organic carbon source; SHA, containing sodium acetate; -N, lacking nitrogen; RuBPCase, ribulose-1,5-bisphosphate carboxylase.

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synthesized within the chloroplast are made initially as mature forms or as precursors. To begin to answer these questions we have isolated polyadenylated and nonpolyadenylated RNA from *Chlamydomonas* cells that were induced to synthesize chloroplast and cytoplasmic ribosomes rapidly without cell division, translated this material in vitro, and immunoprecipitated the translation products with antisera raised against six specific chloroplast ribosomal proteins.

MATERIALS AND METHODS

Strains: The wild type *Chlamydomonas reinhardtii* stock CC-126 and the erythromycin resistant stock CC-504, which carries the nuclear mutation *ery-M1b* (11, 22), were obtained from the *Chlamydomonas* Genetics Center, ℅ Dr. Elizabeth Harris, Department of Botany, Duke University, Durham, NC.

Media and Growth Conditions: High salt medium (32) was used without an organic carbon source (HS) or containing 29.4 mM Na acetate (HSHA; 29). Solid medium used for stock maintenance (YHA) was HSHA containing 1.5% agar (Meer Corporation, North Bergen, NJ) supplemented with 0.4% yeast extract (Difco Laboratories, Detroit, MI). Suspension of cells in HS or HSHA medium lacking nitrogen (HS-N or HSHA-N) induced gametogenesis, a process that involves degradation of both chloroplast and cytoplasmic ribosomes (20, 30).

Cells were grown at 25°C in 6-liter cultures of HS or HSHA in 9-liter carboys mixed with a magnetic stirrer. HSHA cultures were bubbled with air under indirect fluorescent light (dim light, ~10 $\mu E\ m^{-2}s^{-1}$ photosynthetically active radiation [PAR]), whereas HS cultures were bubbled with air plus 5% CO_2 directly under high intensity cool white fluorescent lights (bright light, ~200 $\mu E\ m^{-2}s^{-1}$ PAR). Gametogenesis and ribosome regeneration (see below) were always under bright light conditions, regardless of the medium employed.

To begin the gametogenesis-ribosome regeneration regime, log phase cultures were harvested under sterile conditions by centrifugation at 10,000 *g* for 5 min at 20°C. Cells were washed once with growth medium lacking nitrogen, resuspended at 3.5×10^6 cells/ml in 6 liters of HS-N or HSHA-N, and stirred under bright light with 5% CO_2 for 18 h. Each culture then received 20 ml of 2.8 M NH_4Cl, which returned the nitrogen concentration to that of HS medium (9.3 mM) and initiated the regeneration process. Wildtype cells grown in HSHA medium and both wild type and *ery-M1b* cells grown in HS medium were lysed 4 h after the beginning of ribosome biogenesis.

Preparation of Antisera against Chloroplast Ribosomal Proteins: Antibodies were raised in rabbits against isolated chloroplast ribosomal proteins L-1, L-6, L-13, L-18, L-21, L-26, and L-29 of Schmidt et al. (28), and characterized as described in reference 27.

Isolation of Polyadenylated and Nonpolyadenylated RNA: Cells were harvested by centrifugation as described above. Total cellular RNA was extracted according to previously described methods (4, 14). Cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5/150 mM NaCl/15 mM EDTA/2% SDS/40 µg/ml Proteinase K) to a density of 2×10^8 cells/ml. The suspension was gently shaken at room temperature for 20 min. RNA was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (50:50:1) and precipitated over night at −20°C with 2 vol of cold ethanol. The RNA was resuspended in sterile double distilled water (ddH_2O) and precipitated twice with 2 vol of ethanol at −20°C, once with an equal volume of 4 M LiCl at 0°C, and again with 2 vol of ethanol at −20°C.

The precipitated RNA was pelleted by centrifugation at 10,000 *g*, for 10 min at 2°C, dried under vacuum, and resuspended in 10 mM Tris-HCl pH 7.4/5 mM EDTA/0.2% SDS to a final concentration of 50 A_{260} U/ml or less. Polyadenylated RNA was separated from nonpolyadenylated RNA by affinity chromatography using poly U Sepharose-4B (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) equilibrated in a buffer consisting of 10 mM Tris HCl pH 7.4/5 mM EDTA/0.4 M NaCl/0.2% SDS (14). The absorbance at 280 nm of the effluent from this column was monitored using a continuous flow cell in an Aminco DW-2 spectrophotometer. After adding 1/10th volume of 4 M NaCl to the RNA solution, it was passed through the affinity column and nonpolyadenylated RNA was collected and precipitated at −20°C with 2 vol of cold ethanol. Bound polyadenylated RNA was washed with several column volumes of equilibration buffer and then eluted in this same buffer without NaCl, but containing 90% deionized formamide. The eluted RNA in formamide was diluted with an equal volume of sterile ddH_2O, brought to 0.4 M Na^+ by the addition of 4.0 M NaCl, and precipitated with ethanol. Both the polyadenylated and nonpolyadenylated RNA fractions were purified further by repeating the entire affinity chromatography procedure.

Precipitated RNA was pelleted as above, dried under vacuum, resuspended in 0.1 to 1.0 ml ddH_2O, and the concentration of RNA in these solutions was determined by measuring absorbance at 260 nm. The polyadenylated and nonpolyadenylated RNA samples were diluted to 0.5 and 6.13 mg/ml respectively, and were stored in 0.05-ml aliquots at −70°C.

Translation of RNA In Vitro: Both polyadenylated and nonpolyadenylated RNA were translated in the rabbit reticulocyte lysate system (23) supplied by New England Nuclear (Boston, MA) or Bethesda Research Laboratories (Gaithersburg, MD), or in a wheat germ cell-free system (19) supplied by Bethesda Research Laboratories. All reaction conditions were those specified by the commercial suppliers of the translation systems except that reaction volumes were often increased by a factor of two to twelve. The radioactive amino acid used in the translation reactions was [^{35}S]methionine (1,200–1,400 Ci/mmol) from New England Nuclear or Amersham Corp. (Arlington Heights, IL). Concentrations of polyadenylated and nonpolyadenylated RNA in the translation reactions were 40–80 and 250 µg/ml, respectively. Activity of the polyadenylated RNA translated in the rabbit reticulocyte system was 10- to 15-fold over the background of tubes lacking RNA, whereas activity of the nonpolyadenylated RNA translated in the wheat germ system was 2- to 3-fold over background.

The polyadenylated RNA isolated from wild type cells grown in HS medium was 15 to 25% less active in the rabbit reticulocyte in vitro translation system than was RNA from cells of the same genotype grown in HSHA, although both samples produced qualitatively identical translation profiles after immunoprecipitation with antiserum raised against chloroplast ribosomal protein L-6 (data not shown). Therefore, we are able to compare the translation profiles of RNA from wild type cells grown in HSHA with those of RNA from *ery-M1b* cells grown in HS.

Immunoprecipitation of Translation Products: The translation reactions were stopped after 90 min by the addition of 1/10th volume of 100 µg/ml cycloheximide. Each reaction received 2 vol of denaturing solution (4% SDS/5 mM EDTA/40 mM Tris-HCl, pH 7.4) and the mixtures were boiled for 5 min. They were then diluted with immunoprecipitation buffer (40 mM Tris-HCl, pH 7.4/2% Triton X-100/2 mM EDTA/154 mM NaCl) to a final SDS concentration of 0.4%. The immunoprecipitation buffer was brought to a concentration of 50 µg/ml Aprotinin (Sigma Chemical Co., Inc., St. Louis, MO, 15.2 trypsin inhibitor U/mg of solid). Preimmune serum was added to each of the samples, and the solutions were allowed to stand at room temperature for 30 min. Antigen bound to IgG was isolated from the solution by adding 0.02 ml of a slurry of Sepharose CL-4B beads covalently bound to *Staphylococcus aureus* protein A (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) in immunoprecipitation buffer, and gently mixing the solutions for 2 h at 4°C (17). The beads were separated from the mixture by brief low speed centrifugation, and the immune serum was added to a concentration of 75 to 150 µg IgG/ml depending on the serum sample used. After 30 min at room temperature, fresh protein A-Sepharose CL-4B beads were added to repeat the immunoprecipitation procedure. These beads were pelleted from the labeled solution after 2 h of gentle mixing at 4°C. In many cases, sequential immunoprecipitations were made from a single translation reaction, by adding appropriate amounts of the different antisera one at a time and repeating the above procedure. The affinity of protein A for IgG in this buffer system was calculated empirically to be 730 µg per 0.1 ml of Sepharose bead slurry (data not shown). Therefore, the binding capacity of the beads used for immunoprecipitation is in excess of the total IgG added to each sample. All samples of Sepharose beads were washed 5 times in immunoprecipitation buffer and 5 times in 0.85% NaCl, following which the IgG-antigen complexes were eluted from protein A in 0.6 ml of 1 M acetic acid, and lyophilized.

Aliquots of translation mixtures containing polyadenylated RNA were treated with a *Chlamydomonas* postribosomal supernatant upon termination of the in vitro translation reaction. In Fig. 2, one aliquot of a 4-× translation mixture received the denaturation treatment as described above, whereas the other aliquot received an equal volume of a postribosomal (S-122) supernatant from *C. reinhardtii*, prepared as described in (9). The sample containing the postribosomal supernatant was incubated at 37°C for either 60 or 90 min at a final concentration of 4.25 A_{280} U of S-122/ml. The samples were then prepared for immunoprecipitation as described above, except that ½ volume of 2-× concentrated denaturing solution was added, yielding the same final SDS concentration as was present in the samples not treated with S-122. In Fig. 3, a 12-× translation mixture was divided into six equal aliquots upon termination of the translation reaction, and treated to assess processing as described in the legend of Fig. 3. In those experiments examined, we ascertained that immunoprecipitates of large subunit proteins averaged <0.08% of the total counts in the translation mixtures. In the case of polyadenylated RNA, from 4 to 12 separate in vitro translation reactions were immunoprecipitated with specific antibodies depending on the protein being analyzed. Two different translation reactions containing nonpolyadenylated RNA were analyzed by immunoprecipitation.

In Vivo Labeling: After 8 h of ribosome regeneration in HS medium where the $MgSO_4$ was replaced with an equimolar amount of $MgCl_2$, the cells

were pelleted by centrifugation (13,000 g, 4 min, 20°C), resuspended in 20 ml of the same medium to a density of 1.0×10^8 cells/ml, and returned to the shaker under bright light for 30 min. Carrier-free $H_2{}^{35}SO_4$ (ICN, Irving, CA) was added to a final concentration of 400 μCi/ml, and labeling was allowed to proceed for 30 min. 6 ml of the cell suspension was removed for processing, and unlabeled sulfate was added to the remaining suspension to a final concentration of 10 mM to begin the chase period. 6-ml aliquots were harvested after 45 min of chase.

Each aliquot of cells was pelleted in a 30-ml Corex centrifuge tube in a swinging bucket rotor at 16,000 g, 20°C, for 1 min, mixed with 0.4 ml of denaturating solution, and boiled for 5 min. 6.5 ml of immunoprecipitation buffer was added to each tube and mixed with the lysed cells. The lysates were then transferred to 12-ml thick walled glass centrifuge tubes and spun at 40,000 g, 20°C, for 30 min in an angle head rotor. The supernatants from this centrifugation were transferred to 10 ml lyophilization vials for immunoprecipitation, which was carried out as described above except that the volumes of antiserum and protein A Sepharose beads were increased proportionately.

Polyacrylamide Gel Electrophoresis: Immunoprecipitated proteins were lyophilized, resuspended in SDS dithiothreitol sample buffer (2% SDS/20% sucrose/10 mM dithiothreitol/32 mM Tris-H_2SO_4, pH 6.1) and separated by electrophoresis using the second dimension SDS gel system of (28) utilizing an upper reservoir buffer of 0.082 M Tris/0.04 M Boric acid/0.1% SDS, pH 8.6, and a lower reservoir buffer of 0.42 M Tris-HCl, pH 8.9. The stacking gel consisted of 6% acrylamide-bisacrylamide (30%:0.8%)/0.1% SDS in 0.054 M Tris-H_2SO_4, pH 6.1, not the 0.54 M Tris incorrectly stated in (28). Occasionally this same gel system containing 8 M urea was employed. Gels containing radiolabeled proteins were prepared for fluorography (3) and exposed to XAR-5 X-ray film (Eastman-Kodak, Rochester, NY) at −70°C for 3 d to 4 wk. Molecular weight estimates were obtained using the same molecular weight standards and methods described in (28).

Protein Blotting and Purification of Chloroplast Ribosomal Proteins: Following SDS polyacrylamide gel electrophoresis, proteins were electrophoretically transferred to nitrocellulose and treated with antibody and ^{125}I-protein A as described by Schmidt et al. (27). Chloroplast ribosomal proteins used as markers on the polyacrylamide gels were obtained by electrophoretic elution from urea-charge gels of (28) as described in (27). BSA was sometimes used as a carrier.

RESULTS

Polyadenylated RNA isolated from cells of wild type and the *ery-M1b* mutant was translated in vitro in the rabbit reticulocyte system in the presence of [^{35}S]methionine. Polypeptides related to chloroplast ribosomal proteins L-2, L-6, L-18, L-21, and L-29 were separated from other products of the translation mixture by indirect immunoprecipitation with antisera raised against the cytoplasmically synthesized chloroplast ribosomal proteins L-6, L-18, L-21, and L-29 (28). Antisera to chloroplast synthesized ribosomal proteins L-1 and L-13 did not react with any polypeptides from the translation products of polyadenylated RNA. All antisera, with the exception of anti-L-6, are monospecific for their respective large subunit proteins as judged by the sensitive method of [^{125}I]protein A imaging of antibodies reacted with large subunit proteins electrophoretically separated and blotted to nitrocellulose (27). The antiserum raised against ribosomal protein L-6 also cross reacted with chloroplast ribosomal proteins L-2 and L-5 (Fig. 1). With this antiserum, protein L-2 as well as L-6 was nearly always precipitated from [^{35}S]labeled translation products in vivo or in vitro. Consequently, immunoprecipitation with anti-L-6 allowed the simultaneous analysis of both proteins L-2 and L-6 in the experiments described below. For reasons that are unclear, protein L-5, known from the ^{35}S-labeling experiments of Schmidt et al. (28) to be made in the cytoplasm, was not observed among the immunoprecipitation products.

We have examined the [^{35}S]methionine-labeled products of several independent translation reactions programmed with polyadenylated RNA that were indirectly immunoprecipitated with antisera for ribosomal proteins L-6, L-18, L-21, and L-29. Fluorographs of representative SDS gels (Fig. 2), revealed one or more bands of higher molecular weight than each of the respective ribosomal proteins in question. In the case of anti-L-6, three distinct bands were seen with apparent molecular weights of 5,500, 9,600, and 10,300 greater than that of the mature L-6 protein. The largest was 1,000 greater than the mature L-2 protein (Fig. 2, anti-L-6, wildtype, lane *a*). These results suggested that both L-2 and L-6 might be synthesized initially as precursors. To test whether this was the case, the translation mixture was treated with a *Chlamydomonas* postribosomal supernatant (S-122) as was done by Dobberstein et al. (13) to show processing of the precursor for the small subunit of RuBPCase. Indeed, following S-122 incubation, the L-6 antibody precipitated a band that co-migrated with mature L-6 along with a band that co-migrated with mature L-2 (Fig. 2, lane *b*). The appearance of mature L-6 and L-2 in the S-122 treated lane correlates with a decrease in the intensity of the higher molecular weight bands seen in the untreated lane. Thus we presumed that two of the three bands present in the untreated lane (designated *P* in lane *a*) represented precursors of L-6 and L-2, which could be processed by enzyme(s) present in the postribosomal supernatant extracted from cells of *Chlamydomonas*.

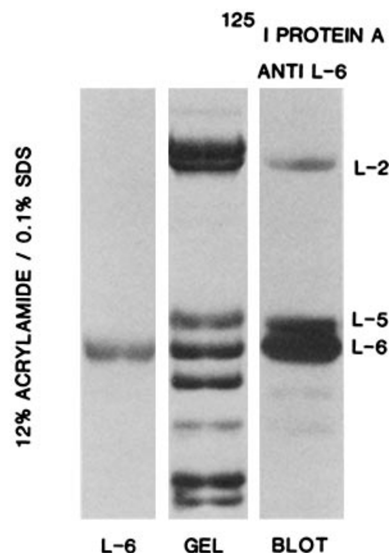

FIGURE 1 Western blot of chloroplast large subunit proteins that reacted with antiserum to protein L-6 of the large subunit. The middle lane represents only the upper half of a Coomassie Blue-stained SDS gel profile of large subunit proteins. The right hand lane is the autoradiogram from an unstained replicate of the middle lane blotted to nitrocellulose, reacted with L-6 antiserum and ^{125}I protein A. This antiserum reacts not only with L-6 but also quite strongly with proteins L-2 and L-5. For further characterization see reference 27. The left hand lane shows the location of purified L-6 protein run on the same gel.

To substantiate our conclusions we used the L-6 antibody to immunoprecipitate the in vitro translation products derived from the polyadenylated RNA of the erythromycin resistant mutant, *ery-M1b*. In this mutant the molecular weight of the L-6 protein is reduced relative to wildtype (11). Accordingly we predicted that the in vitro translation products from the *ery-M1b* message should also be smaller than the products derived from the wildtype message. Immunoprecipitation of the translation mixture with anti-L-6 yielded a polypeptide

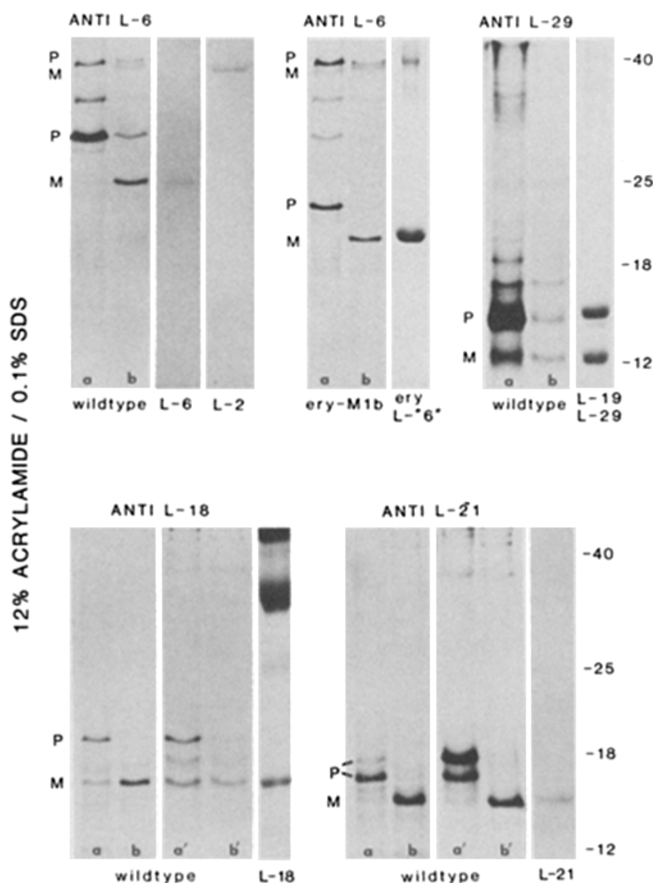

FIGURE 2 Fluorographs of the immunoprecipitates from in vitro translations in a rabbit reticulocyte system programmed with purified, polyadenylated RNA from *Chlamydomonas*. The antiserum used to precipitate the [^{35}S]methionine labeled polypeptides from the translation mixtures is listed along the top left corner of each panel. Immunoprecipitates shown in lanes marked *a* and *b* or *a'* and *b'* are derived from single translation reactions that were divided into two equal aliquots at the end of the translation period. Lanes marked *a* or *a'* represent fluorographs of the immunoprecipitates from ½ of untreated translation mixtures. Lanes marked *b* or *b'* represent fluorographs of immunoprecipitates from the other ½ of the identical translation mixtures treated with an S-122 extract from *Chlamydomonas*. The data shown are representative of the results from seven precursor-processing experiments for protein L-6, two for protein L-29, four for protein L-18, and eight for protein L-21. Precursors to each of the ribosomal proteins are indicated by the letter *P* and the mature forms are indicated by the letter *M*. The immunoprecipitates for lanes *a* and *b* of wildtype L-6, L-29, and lanes *a'* and *b'* of L-21 were obtained sequentially from the same two reaction mixtures. The Coomassie Blue stained gels shown in the right hand lane(s) of each panel show the location of the purified ribosomal proteins run on these gels. Carrier proteins used to precipitate purified L-18 and ery L-"6" are visible towards the top of the stained gels. In the marker lane for anti-L-29, proteins L-19, and L-29 were eluted from a single band in charge gels. Indicators of apparent molecular weights are listed to the right of the L-29 and L-21 panels. Data shown in the upper three panels are all derived from the same polyacrylamide gel. Data shown in the lower two panels are derived from separate gels.

with an apparent molecular weight 2,500 larger than the *ery-M1b* protein (Fig. 2, *ery-M1b*, lane *a*). As is the case for protein L-6 from wildtype, this putative precursor from the mutant was processed by the S-122 supernatant into a polypeptide that co-migrated with the mature form of the mutant protein (Fig. 2).

These data confirm that ribosomal protein L-6 is initially made as a precursor, since a single mutation in the structural gene for this protein reduced both the molecular weight of the mature L-6 protein, and that of its purported precursor. In the same in vitro translation mixture from *ery-M1b*, the precursor to protein L-2 was synthesized and processed into mature L-2 upon treatment with S-122 (Fig. 2, lanes *a* and *b*) as in wildtype. The band appearing between the precursors to L-2 and L-6 in the untreated wildtype lane, and also visible in the same position of the corresponding lane of the *ery-M1b* mutant (Fig. 2), is very likely unrelated to the L-6 protein, and instead may represent as early termination product of the L-2 message. In many experiments this band was absent from the translation products precipitated with anti-L-6. A second faint band in this region seen in the *ery-M1b* lane (Fig. 2, lane *a*) co-migrated with the precursor form of wildtype L-6. This most likely results from the *ery-M1b* RNA preparation being contaminated by residual wildtype RNA from the previous sample fractionated on the poly U-Sepharose column.

In vitro translation mixtures of the polyadenylated RNA were also probed with antisera to proteins L-18, L-21, and L-29. For each of these ribosomal proteins, higher molecular weight precursors also were synthesized (Fig. 2, lanes *a*). In three of eight experiments, immunoprecipitation with anti-L-21 yielded two distinct bands with apparent molecular weights of 16,300 and 17,400–1,700, and 2,800 greater than that of mature L-21 (Fig. 2, anti-L-21, lane *a'*). In half of the experiments the band at 17,400 was barely visible (Fig. 2, anti-L-21, lane *a*), and in one case only the 17,400 band was apparent (data not shown). In all cases, treatment of the translation mixture with S-122 produced a major band that co-migrated with mature L-21 (Fig. 2, lanes *b* and *b'*). That the mature L-21 protein was generated following treatment with S-122 even in the absence of the 17,400-dalton band (Fig. 2, lanes *a* and *b*) or the 16,300-dalton band (data not shown) indicates that both bands can process into a band that co-migrates with mature L-21.

Assuming that the 17,400 polypeptide is the actual precursor of L-21, the 16,300 form may be either a true processing intermediate, as was recently reported for the small subunit of ribulose bisphosphate carboxylase from *C. reinhardtii* by Mishkind et al. (21), or a product of a fortuitous proteolytic cleavage near the *N*-terminal or *C*-terminal end of the transit peptide by a nonspecific protease. Alternatively, the 16,300 polypeptide could be the actual precursor of L-21 and the 17,400 form represent an early initiation product generated in our in vitro translation system. Clearly either form can be processed by the postribosomal supernatant into a polypeptide that co-migrates with mature L-21 on gels.

Immunoprecipitation of the translation mixture with anti-L-18 and L-29 yielded a significant amount of the respective mature polypeptides along with the putative precursor forms (Fig. 2, lanes *a* and *a'*). For both of these proteins, treatment of the translation mixture with S-122 (lanes *b* and *b'*) consistently resulted in an increase in the amount of label found in the mature forms relative to that present in the precursor, suggesting that the precursors are being converted into mature products. In three independent translation experiments, a consistent decrease in the total amount of label immunoprecipitated with antiserum to L-29 was seen after treatment

with the S-122 extract. This may result from protein L-29 being more susceptible to nonspecific proteolysis in the presence of S-122 than proteins L-2, L-6, or L-21. Alternatively, this result could be due to less efficient immune precipitation by the L-29 antiserum from the S-122 containing solutions, since the treated samples contain more total proteins per unit immune serum than in the case of translation mixtures not receiving the *Chlamydomonas* high speed supernatant. In some immunoprecipitations with anti-L-18, a band migrating between the precursor and mature form is apparent (compare Fig. 2, lane *a* with *a'*). This band could represent either an early termination product recognized by the L-18 antiserum, or a processing intermediate as discussed previously for L-21.

The presence of mature ribosomal proteins in the immunoprecipitates of untreated translation mixtures is not unique to proteins L-18 and L-29. Overexposure of the fluorographs for L-6 wildtype, *ery-M1b*, and L-21 in Fig. 2 also reveals a small amount of mature protein in the untreated lanes (data not shown). In fact the ratio of mature L-6 to that of its precursor varies in separate translation reactions precipitated with anti-L-6 (compare Fig. 2, lane *a* with Fig. 3, lane *a*). The processing activity is not unique to the rabbit reticulocyte lysate system since qualitatively similar results were obtained for the L-6 protein when polyadenylated RNA was used to program a wheat germ cell-free system (data not shown).

The rabbit reticulocyte lysate itself appears to have little if any processing activity, since addition of an equal volume of this lysate to a completed translation reaction failed to yield an increase in the ratio of mature protein to that of precursor in the case of L-6 (Fig. 3, lanes *a* and *e*) and L-21 (data not shown). The apparent processing capability of the postribosomal supernatant cannot be attributed to a shift in ionic conditions, which increases the affinity of the antibody for mature over the precursor forms of ribosomal proteins already present in the translation mixture. Adjustment of the ionic conditions in the translation mixture to match those present after addition of the supernatant fraction did not affect the ratio of the precursor to mature form of L-6 (Fig. 3, lanes *a*, *b*, and *d*, and L-21, data not shown). The processing capacity

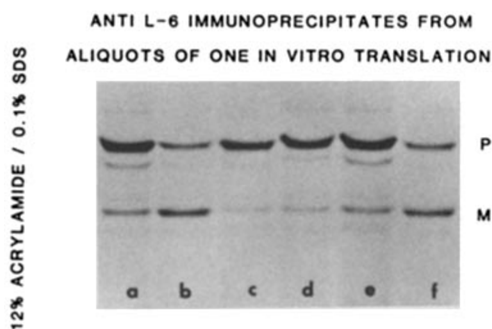

FIGURE 3 Fluorographs of immunoprecipitates obtained following addition of L-6 antiserum to equal aliquots of a single (12 ×) in vitro translation mixture programmed with purified polyadenylated RNA from *Chlamydomonas*, and treated to assess processing as follows; (*a*) untreated aliquot; (*b*) aliquot treated with an equal volume of S-122 extract from *Chlamydomonas*; (*c*) aliquot treated with an equal volume of S-122 extract previously boiled for 5 min; (*d*) aliquot treated with an equal volume buffer used to prepare the S-122; (*e*) aliquot treated with RNAse for 15 min followed by the addition of an equal volume of fresh rabbit reticulocyte reaction mixture; (*f*) as in *b* but incubated at 25°C. All samples except *f* were incubated at 37°C for 60 min during the respective treatments.

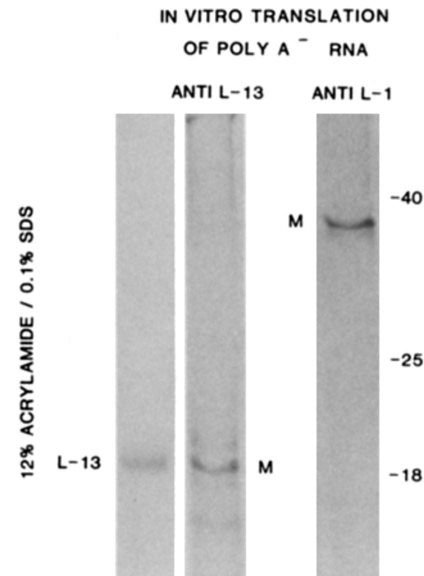

FIGURE 4 Fluorographs of the anti-L-13 and L-1 immunoprecipitates from in vitro translations in a wheat germ system containing [^{35}S]methionine and programmed with purified nonpolyadenylated RNA from *Chlamydomonas*. The Coomassie Blue stained gel shown in the left lane gives the location of purified L-13 run on this gel. Purified L-1 protein was not run in this gel, although the band in the anti-L-1 fluorograph is the correct molecular weight for protein L-1 as described in (28). The data in this figure are all derived from the same polyacrylamide gel and the location of mature L-1 and L-13 on the fluorographs is designated by the letter *M*.

of the postribosomal supernatant is abolished by boiling it for 5 min (Fig. 3, lane *c*) demonstrating that an enzymatic processing activity is indeed supplied by the *Chlamydomonas* fraction.

Nonpolyadenylated RNA from wild type cells translated in both the reticulocyte and wheat germ cell free systems showed approximately tenfold less activity than that of polyadenylated RNA under these reaction conditions. When the wheat germ translation mixture was immunoprecipitated with antisera raised against the chloroplast synthesized ribosomal proteins L-1 or L-13, no obvious precursor forms of either of these two proteins were observed (Fig. 4). However, due to the low activity of these translation reactions we have not demonstrated conclusively that L-1 and L-13 are synthesized initially as mature ribosomal proteins. For example, precipitation with anti-L-13 did yield a faint band that migrated just above mature L-13 (Fig. 4).

Attempts to immunoprecipitate the precursor to ribosomal protein L-6 from extracts of cells labeled in vivo have proven unsuccessful. Fig. 5 reveals that anti-L-6 precipitates only mature L-6 protein and a higher molecular weight band corresponding to protein L-2. To insure that the higher molecular weight band above L-6 was not a precursor to L-6, we performed partial proteolytic digests with three different proteases, which revealed no peptides in common with those derived from the mature L-6 protein (data not shown).

DISCUSSION

We have demonstrated that five large subunit proteins of the chloroplast ribosome known to be made in the cytoplasm are initially synthesized from polyadenylated RNA as precursors

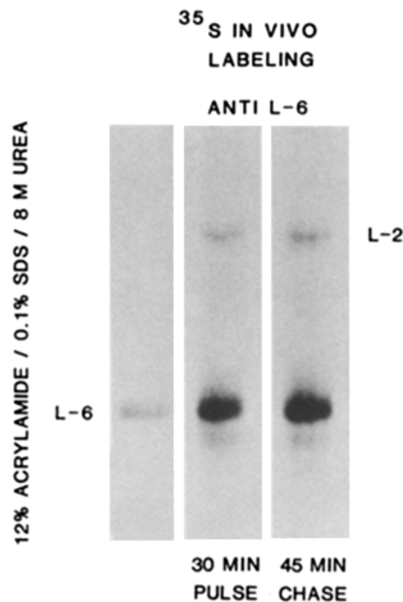

FIGURE 5 Fluorographs of anti-L-6 immunoprecipitates from extracts of *Chlamydomonas* cells labeled with $^{35}SO_4$ for a 30-min pulse. A band co-migrating with protein L-6 and another band corresponding to the molecular weight of L-2 are the only prominent bands appearing in the fluorographs. No precursor to L-6 or L-2 was detected in vivo. The location of purified L-6 run in this gel is indicated in the left lane.

with apparent molecular weights greater than the mature forms of these proteins. In our gel system the apparent molecular weights of these precursors are estimated to be 1,000–6,000 daltons larger than their respective mature products (Table I).

Our data on the in vitro synthesis of chloroplast ribosomal proteins known to be made within the chloroplast are less complete. Chloroplast ribosomal proteins L-1 and L-13 were synthesized in vitro from nonpolyadenylated RNA but not from polyadenylated RNA. This seems consistent with the site of synthesis of these two proteins since the alpha, beta, and epsilon subunit polypeptides of CF_1, the large subunit of RuBPCase and the 32,000-dalton membrane polypeptide, all products of chloroplast protein synthesis, are known to be translated in vitro from nonpolyadenylated RNA (34, 35). Due to the poor expression of the poly A^- RNA under our reaction conditions, we have limited data for these two proteins. L-1 does not appear to be synthesized as a precursor (Fig. 4). However, a faint band does appear above L-13 which may or may not be related to the L-13 protein. Further experiments under conditions of better expression should clarify these observations.

The differences in apparent molecular weight between the precursors of chloroplast ribosomal proteins L-2, L-6, L-18, L-21, and L-29 and their native forms (Table I) are on the average slightly smaller than those found for other chloroplast components known to be made in the cytoplasm (8, 15, 18, 25, 34). As has been shown to be the case for some of the other chloroplast components mentioned above (8, 25) processing of the ribosomal protein precursors occurs separately following their translation.

In most other in vitro translations only precursor forms of cytoplasmically synthesized thylakoid proteins and the small subunit protein of RuBPCase are seen (7, 13, 15, 16, 25, 34). In contrast we observe both the precursor and mature forms of five cytoplasmically synthesized chloroplast ribosomal proteins when polyadenylated RNA is translated in the rabbit reticulocyte system. This observation is not unique to the chloroplast ribosomal proteins. Small amounts of what appear to be mature polypeptides of the chlorophyll *a/b* binding complex can be seen in Fig. 5 of Schmidt et al. (24) along with the precursor forms of these proteins among the immunoprecipitates from pea polyadenylated RNA translated in a rabbit reticulocyte system. Although the basis for this phenomenon is not understood, one possible explanation for the presence of mature ribosomal proteins is that polyA^+ mRNA for the processing enzymes themselves is present in the preparation and is translated by the rabbit reticulocyte lysate.

A *Chlamydomonas* postribosomal supernatant, thought to contain only soluble proteins, is active in processing the precursors to five chloroplast ribosomal proteins discussed in this paper as well as the small subunit of RuBPCase (13) from *Chlamydomonas.* If one assumes that the postribosomal supernatant contains the same processing enzymes for chloroplast ribosomal proteins that function in vivo, then these enzymes may need not be inserted into the chloroplast envelope or thylakoid membrane to function. However, the possibility that small fragments of thylakoid membrane or chloroplast envelopes are present in this fraction cannot be ruled out. Smith and Ellis (31) have shown that the soluble portion of pea chloroplasts contains the activity responsible for processing the precursor of the small subunit of RuBPCase.

When viewed in the context of other studies (7, 15, 16, 25), our data suggest the molecular weight differences between precursors and mature forms of the chloroplast proteins are due to processing of an *N*-terminal transit peptide, although we cannot exclude other explanations. For instance, the possibility that the transit peptide is located somewhere other than the *N*-terminus, or that the apparent molecular weight

TABLE I

Summary of Apparent Molecular Weights of Precursors of Ribosomal Proteins from the Large Subunit of Chloroplast Ribosomes of Chlamydomonas

Protein	Average apparent molecular weight of mature protein	No. of Gels	Average apparent molecular weight of precursor form	Calculated molecular weight of transit peptide
L-2	38,400	2	39,200	800
L-6	24,400	5	30,100	5,700
ery L-"6"	19,900*	1	22,400*	2,500*
L-18	15,400	2	18,500	3,100
L-21**	14,400	5	17,300	2,900
			16,000	1,600
L-29	12,200	2	14,400	2,200

Estimates of the apparent molecular weights of these proteins are from the indicated number of separate 12% polyacrylamide/0.1% SDS slab gels. A protein mixture containing cytochrome c (12,300), B-lactoglobulin (18,400), a-chymotrypsinogen (25,700), ovalbumin (43,000), BSA (68,000), and phosphorylase B (92,500) was run in adjacent lanes as molecular weight standards. Apparent molecular weights of the ribosomal proteins were calculated from a regression analysis of a plot of log molecular weight vs. mobility for these standards. For any given protein, a single gel would often contain the immunoprecipitates from more than one in vitro translation reaction. Since the molecular weight estimates on any one gel are identical, such data are recorded only once in the above table.

* Value not an average.

** Values for both precursors are given.

differences observed between precursor and mature proteins arise from some secondary modification of the proteins, is not ruled out by our data. If our molecular weight estimates are correct, and the differences in molecular weight between precursors and mature forms reflect the presence of an *N*-terminal transit peptide, then the size of the transit peptides of the five ribosomal proteins would all be different, spanning a range of apparent molecular weights from 800 to 5,700 (Table I). Recently, Van Loon et al. (33) reported that three of the five cytoplasmically synthesized polypeptides of the ubiquinol-cytochrome c reductase complex in yeast mitochondria are made in vitro as precursors with apparent molecular weights 500 to 9,000 larger than their respective mature forms. As is the case for the chloroplast ribosomal proteins, the largest mature cytochrome protein had the transit peptide with the lowest apparent molecular weight. This may indicate that transport of proteins into chloroplasts or mitochondria that belong to a given complex is not regulated by a common precursor sequence, and may involve more than one processing enzyme.

Processing of the ribosomal precursors in vivo must be complete and occur fairly rapidly since no precursor to L-6 could be detected in extracts from cells pulse labeled in vivo (Fig. 5). Indeed, we have found no other reports of precursors to chloroplast proteins being detected in extracts from cells pulse labeled in vivo. In contrast, the processing observed in our in vitro system is often incomplete, whether it be the processing that occurs in untreated reaction mixtures, or that derived from addition of a postribosomal supernatant. In translation reactions in vitro that do not receive postribosomal supernatant, the extent of processing seen after 90 min was not increased further with increasing lengths of incubation prior to SDS denaturation (data not shown).

The difference in apparent molecular weight between the mature and the precursor form of protein L-6 from wildtype and from the *ery-M1b* mutant is marked (Table I). The reason for this paradox is unclear. Possibly our estimates of the apparent molecular weights for these ribosomal proteins are incorrect. Alternatively, the *ery-M1b* mutant might contain a deletion that spans the carboxy-terminal end of the transit peptide and the amino-terminus of the mature protein. Although the precise nature of the *ery-M1b* mutation is not known (11), genetic studies of Davidson and Bogorad (12) failed to yield true revertants, suggesting that the *ery-M1b* mutation is a deletion rather than a nonsense mutation. The possibility that over half of the wildtype transit peptide can be deleted in the *ery-M1b* mutant protein, without disrupting the sequence for directing transport to and processing within the chloroplast, remains to be proven. Interestingly, the transit peptide of the wildtype L-6 protein has an apparent molecular weight nearly twice as large as the next largest transit sequence that we have found among the other ribosomal proteins investigated thus far. If the transit peptide of the L-6 protein requires two processing events, as has been suggested for the *Chlamydomonas* RuBPCase small subunit (21), then the existence of a single deletion spanning the processing site at the *N*-terminus of the protein could still allow for another processing event to occur. This would offer an explanation that is consistent with our observations. Another explanation for the molecular weight alterations seen in the L-"6" protein from *ery-M1b*, which would retain the processing site at the *N*-terminus of the protein, requires two noncontiguous deletions in this nuclear mutation. Further investigations are now under way to address the question of whether much of the 6,000-dalton transit peptide present in the wildtype L-6 protein is not essential for import and processing and whether processing of ery L-"6" can occur with the *N*-terminal processing site deleted.

We are also currently attempting to expand our observations on precursors of chloroplast ribosomal proteins using antisera directed against small subunit proteins. This larger sample size should allow us to characterize more fully the differences between transit peptides of various chloroplast ribosomal proteins, and perhaps shed light on how synthesis of these chloroplast proteins in both cytoplasm and organelle is coordinated to achieve their 1:1 stoichiometry in the chloroplast ribosome.

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