Sites of Synthesis of Chloroplast Ribosomal Proteins in *Chlamydomonas*

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ABSTRACT Cells of *Chlamydomonas reinhardtii* were pulse-labeled in vivo in the presence of inhibitors of cytoplasmic (anisomycin) or chloroplast (lincomycin) protein synthesis to ascertain the sites of synthesis of chloroplast ribosomal proteins. Fluorographs of the labeled proteins, resolved on two-dimensional (2-D) charge/SDS and one-dimensional (1-D) SDS-urea gradient gels, demonstrated that five to six of the large subunit proteins are products of chloroplast protein synthesis while 26 to 27 of the large subunit proteins are synthesized on cytoplasmic ribosomes. Similarly, 14 of 31 small subunit proteins are products of chloroplast protein synthesis, while the remainder are synthesized in the cytoplasm. The 20 ribosomal proteins shown to be made in the chloroplast of *Chlamydomonas* more than double the number of proteins known to be synthesized in the chloroplast of this alga.

Chloroplast ribosomes appear to be structurally and functionally similar to procaryotic ribosomes in many respects (6, 17, 34, 35). For example, chloroplast ribosomes have nearly the same sedimentation coefficients, size, conformation, and base sequence of the ribosomal RNA (rRNA) species as do *Escherichia coli* ribosomes. Several reports suggest that the number of ribosomal proteins of the respective subunits is also comparable (8, 13, 19, 25). Functional similarities include ionic requirements for activity and monomer dissociation in vitro and sensitivity to the same spectrum of antibiotics that block protein synthesis at the ribosomal level.

Such obvious similarities have led to much conjecture regarding the procaryotic origin of the chloroplast. Nevertheless, chloroplasts of higher plants and green algae are far from being genetically autonomous, and the biogenesis of the chloroplast ribosomes is emerging as but one of several examples of the division of labor between nuclear and organelle genomes in specifying particular complex organelle structures. While the chloroplast rRNAs are coded by the chloroplast genome, in vivo data obtained for *Euglena* using selective inhibitors (14) and in vitro experiments with isolated chloroplasts of pea (13) indicate that the site of synthesis of the ribosomal proteins is split between the cytoplasm and the chloroplast.

In *Chlamydomonas*, mutations conferring resistance to various antibacterial antibiotics have been mapped in both the chloroplast and the nuclear genomes. These mutations affect the same ribosomal subunits as do mutations of similar phenotype in *E. coli* (cf. reference 6). Davidson et al. (11) have shown convincingly that mutations to erythromycin resistance in the nuclear *ery-M1* gene alter a specific protein of the large subunit of the chloroplast ribosome. Chloroplast mutations to streptomycin resistance (7, 28), spectinomycin resistance (5), and erythromycin resistance (26) have also been reported to affect specific chloroplast ribosomal proteins. Collectively, these results suggest that proteins of both the small and large subunits of the chloroplast ribosome are dependent on two different genetic systems for their production.

A first approximation to determining where the genes coding for chloroplast ribosomal proteins are located is to ascertain which proteins are synthesized in the chloroplast and which are made in the cytoplasm and then imported (2). In *Chlamydomonas reinhardtii*, pulse-labeling in vivo in the presence of inhibitors specific for either chloroplast or cytoplasmic ribosomes has proven highly successful in ascertaining the sites of synthesis of the thylakoid membrane polypeptides (9). In the present study we used this approach, labeling cells of *Chlamydomonas* with ^{35}S in the presence of anisomycin (specific for cytoplasmic ribosomes) or lincomycin (specific for chloroplast ribosomes). Chloroplast ribosomal proteins from purified large and small subunits were separated by both one-dimensional (1-D) and two-dimensional (2-D) gel electrophoresis. Comparison of labeling patterns observed in fluorographs of these gels reveals that 14 of 31 small subunit proteins and five to six of 33 large subunit proteins are synthesized on chloroplast ribosomes. The remaining proteins were found to be synthesized on cytoplasmic ribosomes.

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 MAY 1983 1451-1463

MATERIALS AND METHODS

Strains and Culture Conditions: The wild type strain 137c of *C. reinhardtii* used in these studies was CC-125 (mt^+). We used the erythromycin-resistant mutant *er-u-AW-17* mt^+ (CC-229), whose chloroplast ribosomes are resistant to both erythromycin and lincomycin (3), as a control for the lincomycin treatment. Both stocks were obtained from the *Chlamydomonas* Genetics Center (c/o Dr. Elizabeth H. Harris, Department of Botany, Duke University).

For long-term growth, cells were inoculated from plates of high salt acetate (HSHA) medium (31) containing 4 g/l of Difco yeast extract (Difco Laboratories Inc., Detroit, MI) into 300 ml of the high-salt (HS) medium (32). The cells were grown phototrophically (~15,000 lux) at 25°C and aerated with 5% CO_2 in air. When these pregrowth cultures had reached a density of ~7×10^6 cells/ml, they were used to inoculate 6-liter carboys of HS medium to a cell density of 5×10^4 cells/ml. These cultures were grown for 48 h at 25°C under ~21,000 lux. Cultures were aerated with 5% CO_2 in air with continuous stirring.

Long-term growth of cells in the presence of ^{35}S (H_2SO_4) was as described above except that cells from pregrowth cultures were inoculated into two 1-liter flasks containing 500 ml of HS medium in which $MgSO_4$ was replaced with an equimolar amount of $MgCl_2$ high salt reduced sulfur medium (HSRS). To each flask was added 3.5 mCi of $^{35}SO_4$ and cells were grown for 48 h.

Preparation of Chloroplast Ribosomes: Cells were harvested by centrifugation and resuspended to a cell density of 2×10^9 cells/ml in a pH 7.8 buffer consisting of 25 mM Tris/100 mM KCl/5 mM MgAc/0.15% glutathione (TKM buffer). Cells were broken in a French press at 5,000 psi and centrifuged at 40,000 *g* for 30 min. ~200 OD_{260} units of the S40 supernatant were layered over each 37-ml, 10–30% sucrose gradient containing the same salts as in the TKM buffer (70s dissociating gradients, see reference 3). The gradients were centrifuged at 2°C in a SW 27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) for 20 h at 22,500 rpm. Chloroplast ribosomal subunits were collected by fractionation on an ISCO Model D fractionator with UV monitor. Respective subunit fractions from each gradient were pooled, diluted 1:1 with TKM buffer, layered over a 2-ml 30% sucrose cushion of TKM buffer, and pelleted at 50,000 rpm for 22 h in a 60 Ti rotor (Beckman Instruments, Inc.). Ribosomal subunits were resuspended in TKM buffer, and ~30 OD_{260} units of small subunit and 60 OD_{260} units of large subunit were layered on 38-ml gradients identical to those described above. After 20 h of centrifugation as above, fractionation of the gradients yielded large and small subunits free of cross contamination. The large and small subunit fractions, respectively, were pooled and pelleted as above. Pellets were then frozen (−20°C) until used. For running subunit proteins on 1-D SDS-urea gradient gels, pooled subunit fractions were occasionally precipitated by addition of 2 vol of cold 95% ethanol as opposed to pelleting. When 70s monomers were isolated, the above protocol was followed except that buffers and gradients consisted of TKM containing 25 mM KCl, and centrifugation of gradients was carried out for 14 h at 22,500 rpm, 2°C.

Electrophoretic Analyses of Chloroplast Ribosomal Proteins: Purified ribosomal subunits were subjected to a modification of the Kaltschmidt and Wittmann (20) acetic acid extraction procedure prior to electrophoresis of the constituent ribosomal proteins. Pelleted ribosomal subunits were resuspended in a 0.6-ml solution of 67 mM MgAc/10 mM Tris · HCl, pH 7.8. 2 vol of glacial acetic acid were slowly added and the mixture was stirred on ice for 1 h. The rRNA was pelleted by centrifugation and proteins in the supernatant were precipitated by the addition of 5 vol of acetone. Prior to electrophoretic analysis precipitated proteins were pelleted and dried *in vacuo* for 1–2 min.

For 1-D SDS-urea gradient gel electrophoresis the Laemmli (21) gel system was employed with the following changes: the separating gel was a 22-cm gradient slab 1-mm thick, consisting of 8–20% acrylamide-bisacrylamide stock (60%:0.8%)/8 M urea/0.1% SDS/0.375 M Tris · HCl, pH 8.8. To stabilize the gradient the 20% acrylamide solution was made in 9% sucrose. A sample, containing 2.5 OD_{260} units of ribosomal subunits prior to acetic acid extraction, was dissolved in sample buffer as described (9) and added to each well. A current of 17 mA was applied to the gel for ~22 h until the lowest molecular weight protein (~9,900) had run the length of the gel.

The first dimension of the 2-D gel system was that of Mets and Bogorad (27) except that a 22-cm slab gel 1-mm thick was employed in place of a tube gel, and a stacking gel was added consisting of 2.5% acrylamide-bisacrylamide stock (40%:1.0%)/8 M urea/0.114 M *bis*-Tris, with pH adjusted to 4.0 with glacial acetic acid. The stacking gel retained those few acidic ribosomal proteins which otherwise would not have entered the gel (27). In some experiments, the running gel was adjusted to pH 5.5 to enhance resolution of certain proteins. Proteins from the acetic acid extraction of 5 OD_{260} units of ribosomal subunits were dissolved in 60 μl of the sample buffer of (27) with the following modifications: a solution of 8 M urea and 10 mM dithiothreitol (DTT) was diluted 9:1 with upper buffer stock. Electrophoresis was carried out at 4°C for 10–11 h at 44 mA constant current. Prior to electrophoresis in the second dimension, 1-D strips were stained, destained, and then equilibrated 1 h in 0.057 M *bis*-Tris/10 mM DTT adjusted to the pH of the running gel with glacial acetic acid.

The 2-D running gel was 12% acrylamide-bisacrylamide (30%:0.8%)/0.1% SDS/0.42 M-Tris · HCl, pH 8.9. A 2-cm stacking gel was polymerized on top of the second-dimension running gel, yielding a 30 cm × 22 cm × 1.2 mm slab gel. The stacking gel consisted of 6% acrylamide-bisacrylamide (30%:0.8%)/0.1% SDS/0.54 M-Tris · H_2SO_4, pH 6.1. Just before placing the 1-D gel strip into position, the 3-cm space above the polymerized stacking gel was filled with stacking gel solution or hot 0.8% agarose containing stacking gel salts. The 1-D gel strip was allowed to slip between the glass plates into the stacking gel solution or agarose until it was seated on top of the polymerized stacking gel. After polymerization, cold buffer of 0.082 M Tris/0.04 M boric acid/0.1% SDS, pH 8.6, was placed in the upper reservoir. The lower reservoir buffer was 0.42 M Tris · HCl, pH 8.9. The gels were run at 18 mA constant current until the tracking dye left the gel. Both 1-D and 2-D gels were stained and dried according to (9). Fluorographs were prepared as described in reference 4.

Molecular Weight Determination: To determine the apparent molecular weight of ribosomal proteins, a protein mixture containing as molecular weight standards cytochrome *c* (12,300), β-lactoglobulin (18,400), α-chymotrypsinogen (25,700), ovalbumin (43,000), bovine serum albumin (68,000), and phosphorylase B (92,500) was run in wells 1–2 cm from the edge of a 2-D SDS gel. Care was taken to load the molecular weight standards just as the ribosomal proteins ran out of the 1-D gel strip and into the 2-D stacking gel. Conditions for running the gel were the same as described previously for 2-D gels.

A plot of the log molecular weight vs. mobility for the various standards approximated a straight line. Regression analysis was used to determine the straight line equation that best fit the data points determined for protein standards. Apparent molecular weight of ribosomal proteins was calculated from the equation.

Criteria for Establishing Which Proteins Belong to the Chloroplast Ribosome: To ascertain that our preparations of chloroplast ribosomal subunits from *C. reinhardtii* were free of contamination, we compared protein profiles of SDS-urea gradient gels of large and small subunit proteins obtained using a variety of different isolation procedures. First, prior to isolation of subunits as described above, the S40 supernatant was incubated at 37°C for 10 min in 0.5 mM puromycin to remove possible contaminating nascent peptides and/or mRNA. Second, monomers or subunits were subjected to one of three high-salt treatments in order to remove loosely associated proteins such as initiation and elongation factors. (*a*) 70s monomers were isolated initially and subsequently dissociated on TKM sucrose gradients containing 850 mM or 880 mM KCl. Fractionation and EtOH precipitation were done as described previously. The initial isolation of 70s monomers as opposed to subunits served to eliminate possible contamination by proteins or protein complexes that may have had sedimentation velocities identical to those of 54s or 41s subunits. (*b*) Subunits were isolated, pelleted, and resuspended in TKM buffer prior to being pelleted (SW 27 rotor/25,000 rpm/24 h/2°C) through a TKM solution containing 850 mM KCl and 7% sucrose. (*c*) Subunits were isolated from sucrose gradients containing TKM or TKM at 850 mM KCl and then diluted with an equal volume of TKM at 850 mM KCl followed by pelleting as described previously. Thirdly, to assure that the electrophoretic migration of ribosomal proteins was not being affected by their association with contaminating ribosomal RNA fragments, acetic acid-extracted proteins from large and small subunits were resuspended and treated with RNAse (23). To resuspend small subunit proteins, SDS was added to the reaction buffer to a concentration of 0.5%. The addition of SDS at this concentration had no effect on RNAse activity as determined by monitoring the control digest of *E. coli* tRNA.

Sites of Synthesis: Pregrowth cultures in HSRS medium were grown as described earlier and used to inoculate a 12-liter carboy of HSRS at a density of 5×10^4 cells/ml. Cultures were grown for 24 h to a density of approximately 2×10^6 cells/ml under conditions described earlier. Cells were harvested from the HSRS cultures by low-speed centrifugation (16,300 *g*, 10 min, 20°C) and resuspended in 2,200 ml of HSRS to a density of 5×10^6 cells/ml. 350-ml aliquots of cells were dispensed in six 1-liter flasks and incubated for 60 min on a rotary shaker under the conditions described previously for pregrowth cultures. Following this recovery period, two flasks were incubated with 500 μg/ml anisomycin and two with 500 μg/ml lincomycin. Two control flasks received neither inhibitor. The anisomycin stock was prepared in 95% ethanol, and ethanol was added to all flasks to the same final concentration. After 1 h, cells were harvested from all cultures as before, washed once with 50 ml of HSRS and resuspended in 350 ml of HSRS. Lincomycin (500 μg/ml) was added to cultures preincubated with anisomycin, and cells preincubated with lincomycin received anisomycin (500 μg/ml). 10 min later, [^{35}S]sulfuric acid was added for a 1-h labeling period. In a series of six independent experiments, we examined the incorporation of various amounts of label into chloroplast ribosomal proteins in the presence of lincomycin and anisomycin, by studying fluorographs of 1- and 2-D gels. Although the results of all experiments were qualitatively the same, the data presented here for the sites of synthesis of chloroplast ribosomal proteins in wild type cells are derived from one optimal experiment in which the control cultures received 1.5 mCi/flask, the lincomycin treatment received 3.0 mCi/flask, and the anisomycin

treatment received 8.0 mCi/flask. After labeling, flasks were chilled in ice immediately, and a 10-fold excess of $MgSO_4$ in H_2O was added to each flask to dilute the labeled sulfur. Pairwise treatments were pooled and the cells harvested by centrifugation. ^{35}S-labeled large and small subunits of the chloroplast ribosome were isolated as described in the Preparation of Chloroplast Ribosomes section, except that cell pellets were resuspended in 2.0 ml of TKM buffer, and each of the three S40 supernatants was divided equally among two 70s dissociating sucrose gradients.

To monitor incorporation of label, triplicate Whatman #3 paper disks were spotted with (*a*) aliquots of each culture to yield a measure of incorporation into whole cells, (*b*) aliquots of S40 supernatants to estimate incorporation into soluble proteins, and (*c*) aliquots of cleaned-up large and small subunits to give an estimate of incorporation into ribosomal proteins. Filters spotted with labeled material were processed and radioactivity was measured as in reference 10.

Materials: Lincomycin (Lincocin; 300 mg/ml sterile solution) was purchased from Upjohn Co. (Chamblee, GA). Anisomycin was the gift of Pfizer, Inc. (Groton, CT). Puromycin was obtained from Sigma Chemical Co. (St. Louis, MO). Sequanal grade SDS was obtained from Pierce Co. (Rockford, IL). Bethesda Research Laboratories, Inc. (Gaithersburg, MD) supplied protein molecular weight standards. ^{35}S-labeled H_2SO_4 in H_2O was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA).

RESULTS

Ribosomal proteins were visualized by 2-D gel electrophoresis using Coomassie Blue staining and fluorography of proteins derived from cells labeled with ^{35}S under conditions of long-term growth. In addition, we also compared the large and small subunit profiles resolved on 2-D gels in which the second dimension is a linear SDS-gel with that seen on 1-D SDS-urea gradient gels (Figs. 1 and 2). To correlate proteins in both gel systems, 1-D gel strips (of the 2-D gel system) were run on SDS-urea gradient gels containing adjacent wells loaded with acetic acid-extracted proteins from the same subunit (data not shown). This allowed a simultaneous comparison of 1- and 2-D protein profiles on SDS-urea gradient gels. The 2-D pattern obtained with SDS-urea gradient gels could then be related to our standard 2-D gel profiles. Several cases of co-migrating proteins and proteins with changes in apparent molecular weight in SDS-urea gradient gels were revealed by these comparisons. Observed variations in apparent molecular weight could be the result of intrinsic diffences in shape and/or detergent-binding properties of proteins when urea is present in the gel. Hence, our estimates of apparent molecular weight and our numbering system are based on the 2-D charge-SDS gel system. Proteins whose mobility changes in the 1-D SDS-urea system appear numbered out of sequence in those figures.

In certain instances we could also elucidate corresponding proteins on 2-D gels and SDS-urea gradient gels by comparing the relative position of proteins with their sites of synthesis on both gel systems. A few single bands appearing in SDS-urea gradient gels were confirmed to include more than one protein since they consisted of products synthesized in both the chloroplast and the cytoplasm.

Characterization of Large Subunit Chloroplast Ribosomal Proteins

The protein composition of large subunits was not affected by puromycin release, high-salt washing or RNAse treatment (data not shown). 29 of the 33 large subunit proteins, ranging in molecular weights from 10,000 to 37,500, consistently appeared on 2-D gels, SDS-urea gradient gels, and ^{35}S-labeled fluorographs (Fig. 1 *A*, *B*, and *C*). Proteins 32 and 33 were missing occasionally from stained or fluorographed 2-D gel profiles although they were always visible in the stained first-dimensional gels of this system (Fig. 1 *A*). Protein 32 does incorporate some ^{35}S in short-term pulse experiments (see section on Sites of Synthesis), whereas protein 33 does not, indicating that no sulfur groups are present in this protein. Although protein 14 labels strongly with ^{35}S in long-term growth experiments, it does not always stain well with Coomassie Blue in 2-D gel profiles (Fig. 1 *A* and *B*). We think the reason for this discrepancy is that protein 14 contains many sulfur residues and is more apparent in 2-D fluorograms than in stained gels. Protein 30 was also occasionally missing from 2-D gel profiles although it appeared stoichiometric in stained gels when present (Fig. 1 *A* and *C*) but did not label strongly with ^{35}S under long-term growth conditions (Fig. 1 *B*).

Proteins 1, 2, and 3 have similar molecular weights but distinct charge differences which result in their separation on 2-D gels (Fig. 1 *A*, and *B*) while they co-migrate in SDS-urea gradient gels (Fig. 1 *C*). Proteins 12, 13, 19, 28, 30, and 31 undergo changes in apparent molecular weight when resolved on SDS-urea gradient gels (Fig. 1 *C*) as revealed when the 1-D gel strips are run simultaneously with large subunit samples in SDS-urea gradient gels instead of the normal SDS second dimension. In SDS-urea gradient gels, proteins 12, 13, and 30 run more slowly, whereas 19, 28, and 31 run more quickly than they do in the 2-D gel system.

Proteins which appear frequently in gels, but which we do not believe to be true ribosomal proteins, are designated with letters. Protein "a" (Fig. 1 *B* and *C*) corresponds to protein LC1 as described by Hanson et al. (19). We have not numbered this protein since it rarely appeared in stained 2-D gels and, although present in 1-D SDS-urea gradient gels, always appeared to be nonstoichiometric. However, protein "a" was frequently visible on 1-D SDS-urea gradient gels even when large ribosomal subunits were isolated and pelleted through high-salt TKM buffer (850 mM KCl). If protein "a" is a contaminant, it is not easily removed. Protein "b" usually appeared to be nonstoichiometric when stained with Coomassie Blue on SDS-urea gradient gels (Fig. 1 *C*), and never appeared on stained 2-D gels. Protein "b" was, however, visible on fluorographs of large subunit proteins isolated from pulse-labeled cells (see section on Sites of Synthesis).

Characterization of Small Subunit Chloroplast Ribosomal Proteins

After small subunits were treated with puromycin or RNAse following isolation, 31 proteins, ranging in molecular weights from 13,000 to 87,000, were resolved by a combination of SDS-urea gradient gel and 2-D gel analyses (Fig. 2 *A–C*). When small subunits were isolated and pelleted in TKM buffer containing 850 mM KCl, proteins 1, 2, and 3 were reduced somewhat in staining intensity and proteins 9, 10, and 15–18 were completely removed (data not shown). Small subunit proteins 2 and 16 have apparent molecular weights similar to those of elongation factor EF_G and initiation factor IF_S of *E. coli*, respectively (38). Further investigation will be necessary to determine whether any of the chloroplast ribosomal proteins that wash off the small subunits are translation factors or nonspecific contaminants rather than true ribosomal proteins.

28 of the 31 proteins were readily seen in stained or fluorographed 2-D gels (Fig. 2 *A* and *B*). Proteins 1, 3, 6, and 8 appeared in stained SDS-urea gradient gels (Fig. 2 *C*) but failed to run properly in the 2-D system (Fig. 2 *B* and *C*). These proteins begin entering the 1-D gel when the pH of the upper reservoir buffer becomes more acidic late in the electrophoretic run. They thus remain embedded in the 1-D stacking gel and streak down the left margin of the 2-D SDS gel. Protein 2 is regularly observed in SDS-urea gradient gels (Fig. 2 *C*)

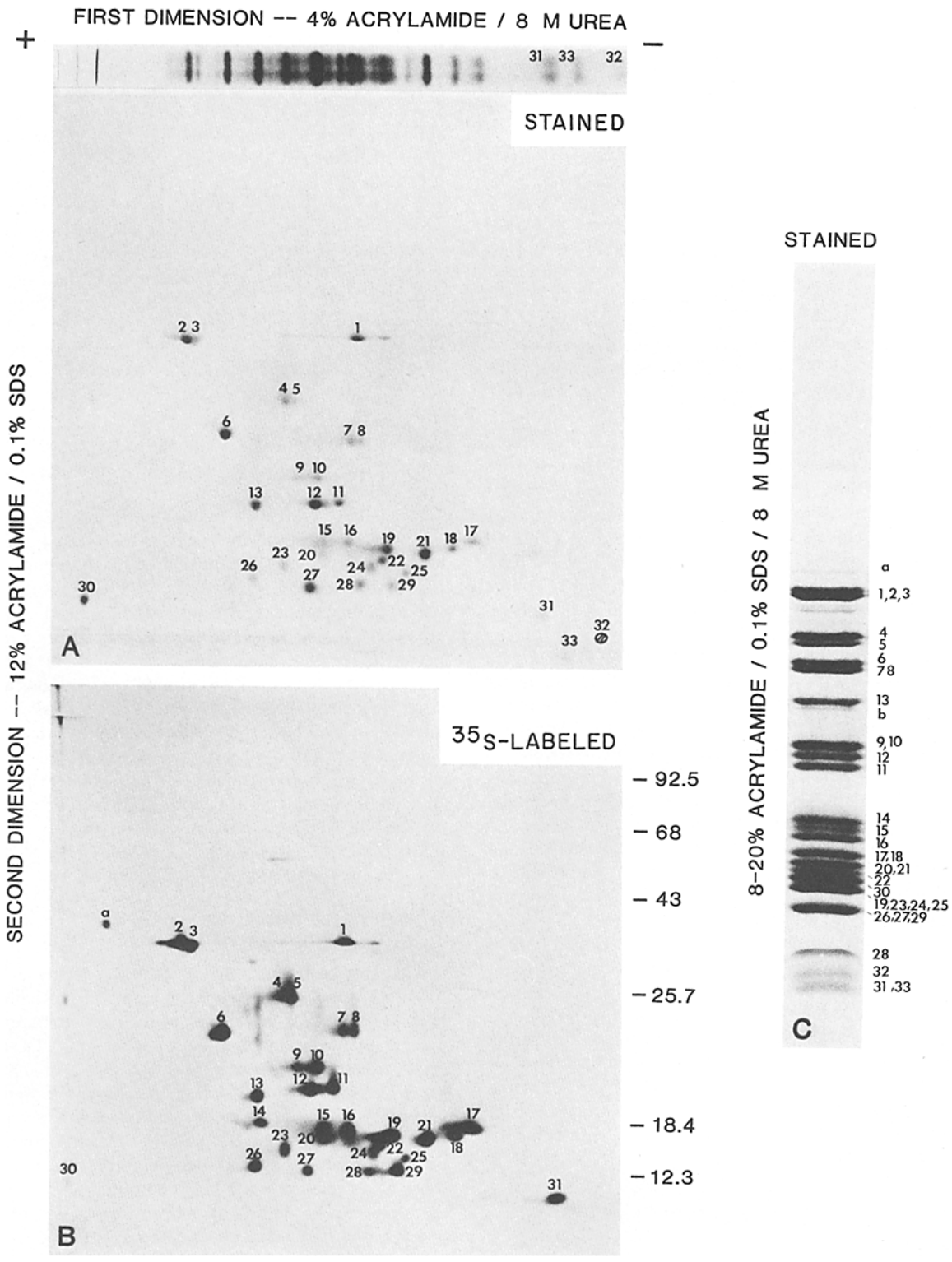

FIGURE 1 Electrophoretic profiles of proteins from the large subunit of the chloroplast ribosome of *Chlamydomonas reinhardtii*. (*A*) Coomassie Blue-stained 2-D gel with the stained 1-D strip mounted along the upper margin. Proteins are numbered from left to right in order of decreasing apparent molecular weight. Proteins 31, 32, and 33, which do not stain well after running in the second dimension, have been numbered in the 1-D gel. The location of protein 32 in the 2-D gel is indicated by the hatched circle. (*B*) Fluorograph of a separate 2-D gel of labeled ribosomal proteins from cells grown for several generations in the presence of $^{35}SO_4$. Protein 33 does not label with ^{35}S and the labeling of protein 32 is too faint to be visible here. (*C*) Coomassie Blue-stained 1-D SDS-urea gradient gel. Several of the bands can be shown each to contain two or more proteins when the 1-D and 2-D gel profiles are compared. Gel compositions and running times are described in Materials and Methods; pH of the second dimension of the 2-D gel was adjusted to 5.5. The scale on the right side of panel B shows apparent molecular weights $\times 10^{-3}$.

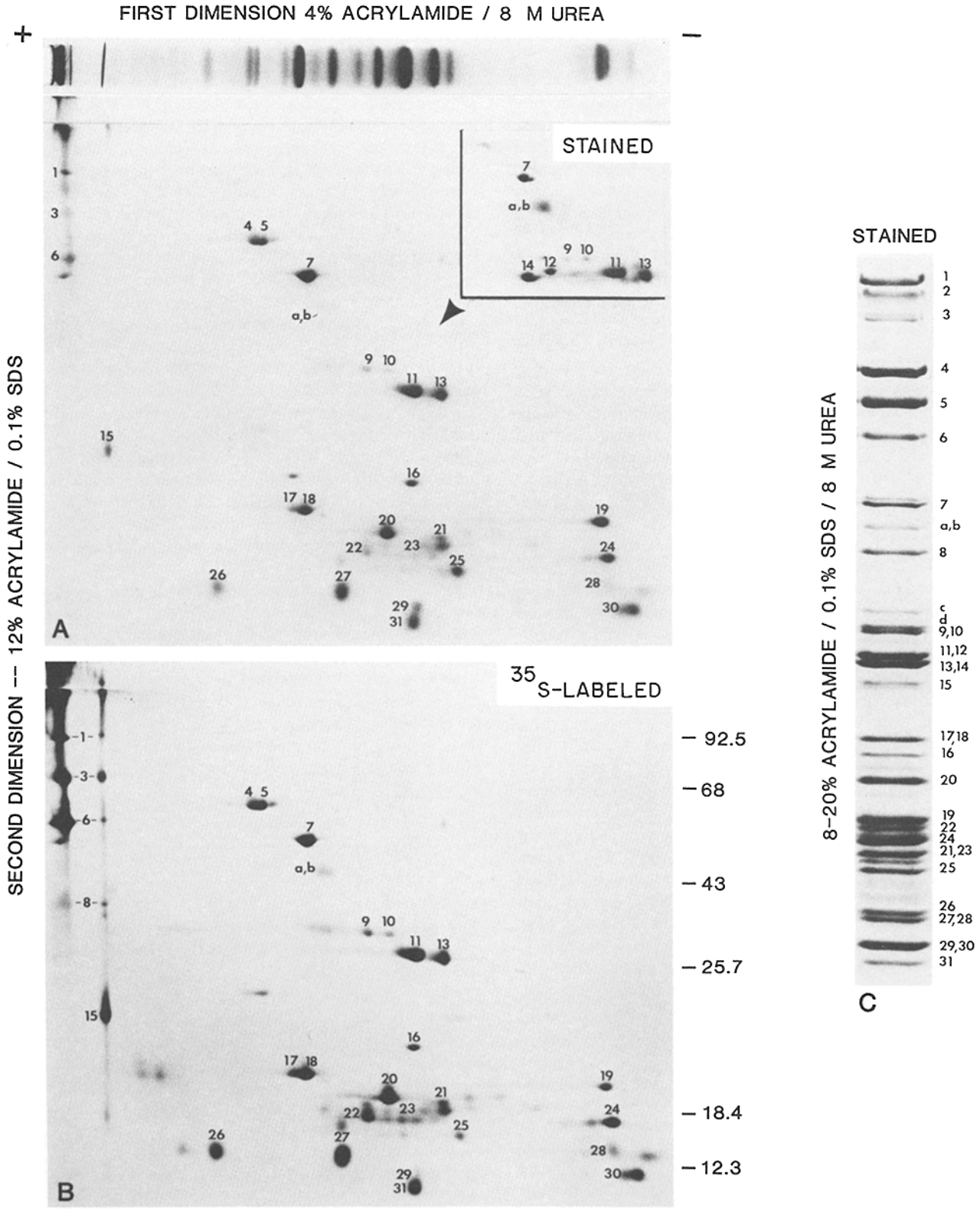

FIGURE 2 Electrophoretic profiles of proteins from the small subunit of the chloroplast ribosome of *C. reinhardtii*. (*A*) Coomassie Blue-stained 2-D gel with the stained 1-D strip mounted along the upper margin. The *inset* shows the relative location of proteins 12 and 14 in a second 2-D gel as these proteins were not distinguishable in the 2-D gel of *A*. (*B*) Fluorograph of the 2-D gel shown in *A* where the labeled ribosomal proteins were isolated from cells grown for several generations in the presence of $^{35}SO_4$. (*C*) Coomassie Blue-stained 1-D SDS-urea gradient gel. Several high molecular weight proteins are well resolved on this gel that fail to run in the first dimension of the 2-D gel above. Gel compositions and running times are as described in Materials and Methods; pH of the second dimension of the 2-D gel was adjusted to 5.5. The scale on the right side of panel B shows apparent molecular weights $\times 10^{-3}$.

but only infrequently appeared in 2-D gels above protein 4. Proteins 4 and 5 are similar in charge and apparent molecular weight in 2-D gels (Fig. 2*A*) but occur as two single bands in SDS-urea gradient gels (Fig. 2*C*).

Proteins 9 and 10 consistently appeared in SDS-urea gradient gels (Fig. 2*C*) but are frequently observed to be nonstoichiometric in 2-D gels (Fig. 2*A*). On SDS-urea gradient gels these proteins are seen in preparations of both large and small subunits that have not been purified by a second centrifugation through 70s dissociating gradients (data not shown). After purification, proteins 9 and 10 appeared stoichiometric only in small subunit gels. As mentioned previously, these proteins are removed when the subunits are treated with high-salt buffer. Collectively, this evidence suggests that proteins 9 and 10 may be loosely bound to the small subunit at its interface with the large subunit.

Proteins 12 and 14 were stoichiometric when visualized in ~50% of the 2-D gels observed (Fig. 2*A*, *inset*). In SDS-urea gradient gels, results of sites of synthesis experiments indicated that the two bands corresponding to that region of the gel were doublets (see section on Sites of Synthesis), suggesting that protein 11 was migrating with 12 and that protein 13 was migrating with 14. Often, protein 21 and 22 each appears as two spots having identical charge but slightly different molecular weights (Fig. 2*A* and *B*). Examination of the behavior of these proteins on SDS-urea gradient gels failed to resolve this enigma. Until further analyses can show that 21 and 22 are definitely composed of more than one protein each, we choose to designate them by single numbers. We assume that proteins 16, 19, 21, and 24 have different conformations and/or detergent-binding properties in SDS-urea gradient gels since their apparent molecular weights are different from those observed in the 2-D gels.

While proteins 22, 23, and 28 labeled with ^{35}S in long-term growth experiments (Fig. 2*B*) they consistently stained faintly in 2-D gels (Fig. 2*A*). In both long-term (Fig. 2*B*) and short-term (see section on Sites of Synthesis) labeling experiments, both protein 23 and 24 are resolved into two to three charge forms, with the more negatively charged species being absent from the stained gels. Protein 22 stains well in SDS-urea gradient gels (Fig. 2*C*) but one cannot determine the staining of proteins 23 and 28 in this system, since they co-migrate with proteins 21, 26, and 27, respectively. Proteins 29 and 30 have different charges but are similar in molecular weight in 2-D gels (Fig. 2*A*). They also co-migrate in SDS-urea gradient gels as evidenced by the sites of synthesis experiments (see section on Sites of Synthesis). Protein 29 is only weakly labeled with ^{35}S in long-term growth (Fig. 2*B*).

On SDS-urea gradient gels, proteins "a," "b," "c," and "d" were consistently observed but appeared nonstoichiometric (Fig. 2*C*). Proteins "a" and "b" were occasionally detected in stained 2-D gels (Fig. 2*A*) and faintly labeled with $^{35}SO_4$ in long-term growth experiments (Fig. 2*B*). Proteins "c" and "d," however, were not detected under either of these conditions, but they were observed on SDS-urea gradient profiles of small subunits when cells were pulse-labeled with $^{35}SO_4$ (see section on Sites of Synthesis). In the case of the 1-D SDS-urea gels, we occasionally see other faintly staining bands whose identity is unclear, e.g., between bands 6 and 7 and 16 and 20 of Fig. 2*C*.

Sites of Synthesis of Chloroplast Ribosomal Proteins

We examined the sites of synthesis of chloroplast ribosomal proteins in *Chlamydomonas*, in a series of six experiments, by isolating assembled ribosomes, after pulse-labeling in the presence of the cytoplasmic protein synthesis inhibitor anisomycin (ANISO) or the chloroplast protein synthesis inhibitor lincomycin (LINCO). To maximize the chances of pulse-labeled proteins being assembled into ribosomes, cells labeled in the presence of one inhibitor (e.g., ANISO) were first preincubated in the presence of the other (e.g., LINCO) to provide a pool of cold proteins made in one compartment that could be assembled with labeled proteins made in the other. To obtain sufficient counts in chloroplast ribosomal proteins labeled in the presence of inhibitors as compared with the controls, we found it necessary to increase the amount of isotope added to the inhibitor-containing flasks. In the experiment shown here, a twofold increase in the amount of isotope added to the LINCO flask yielded virtually the same whole cell incorporation rate as the control (Table I). The total incorporation of counts into the small and large subunits of the chloroplast ribosome was substantially less than that seen in the control (Table I). This result was consistent with the observation that a substantial number of chloroplast ribosomal proteins are synthesized in the chloroplast (see below). However, when the data are corrected to reflect incorporation of counts only into ribosomal proteins known to be made in the chloroplast, the incorporation rate for the small subunit is virtually the same as that of the control whereas the incorporation rate for the large subunit is 56% of the control.

Even when the cells were labeled with a fivefold excess of isotope in the presence of ANISO, incorporation of label into whole cell protein and chloroplast ribosomal subunits was substantially lower than seen in the case of the control or the LINCO treatment (Table I). This was also the case when the data were corrected to reflect only ribosomal proteins made in the chloroplast, where the incorporation rate for both large and small subunit proteins was 17% of the control. Increasing the isotope added to the ANISO-treated cells did not increase

TABLE I
Effect of Inhibitors

	Treatment		
	Control	Lincomycin	Anisomycin
$^{35}SO_4$ added cpm/10^6 cells	1.3×10^6	2.6×10^6	7.0×10^6
cpm incorporated into whole cell protein/10^6 cells	2.6×10^4	2.6×10^4	0.5×10^4
cpm incorporated into S40 supernatant/10^6 cells	9.2×10^3	10.9×10^3	0.7×10^3
cpm incorporated/OD_{260} of large subunit of chloroplast ribosomes	9.3×10^3	4.2×10^3	0.3×10^3
cpm/OD/kdalton of labeled large subunit protein	15.3	8.5	2.6
cpm incorporated/OD_{260} of small subunit of chloroplast ribosomes	23.5×10^3	12.0×10^3	1.8×10^3
cpm/OD/kdalton of labeled small subunit protein	23.6	22.1	4.0

Effect of inhibitors of chloroplast (lincomycin) and cytoplasmic (anisomycin) protein synthesis on the incorporation of $^{35}SO_4$ by *C. reinhardtii* cells during a 1-h pulse. Cells labeled in the presence of the respective antibiotics were pretreated with the other antibiotic for a 1-h period, pelleted, and washed prior to the treatment shown.

incorporation further (data not shown). From these data, one can conclude that labeling and assembly of ribosomal proteins in the presence of LINCO is much more extensive than it is in the presence of ANISO. This may reflect the fact that the majority of chloroplast ribosomal proteins are made in the cytoplasm and that preincubation in the presence of LINCO may not have a substantial effect on the pool size of one or many of the chloroplast ribosomal proteins made in the cytoplasm.

As a positive control to establish that LINCO was, in fact, blocking synthesis of proteins on chloroplast ribosomes specifically, we also pulse-labeled a mutant, *er-u-AW-17*, having LINCO-resistant chloroplast ribosomes (3) in the presence of both LINCO and ANISO. In the presence of ANISO, the same ribosomal proteins whose synthesis was inhibited in the control were inhibited in the mutant, but all chloroplast ribosomal proteins were synthesized normally in the presence of LINCO (data not shown). These results demonstrate that ribosomal proteins not made in the presence of LINCO are made on the LINCO-resistant ribosomes of the mutant and rule out the possibility that LINCO is inhibiting synthesis of these proteins for some secondary reason.

Large Subunit Proteins

Of the 33 proteins in the large subunit, five and possibly six proteins appear to be made in the chloroplast. Proteins 1, 13, and 17 did not incorporate label in the presence of lincomycin but clearly labeled in the presence of anisomycin (Figs. 3 and 4), indicating that they are products of chloroplast protein synthesis. Proteins 26 and 27 also did not label in the presence of lincomycin but were faintly labeled in the anisomycin treatment. This is evident for protein 27 in the first dimension of Fig. 3 *D*, but not in the second dimension. Protein 26, which is not discernible in the first dimension of this gel, because of its co-migration with protein 13, is visible as a faint spot on the original fluorogram of the 2-D gel. Proteins 26 and 27, which co-migrate in SDS-urea gradient gels, appear as a weakly labeled band in the ANISO lane (Fig. 4). Thus they also appear

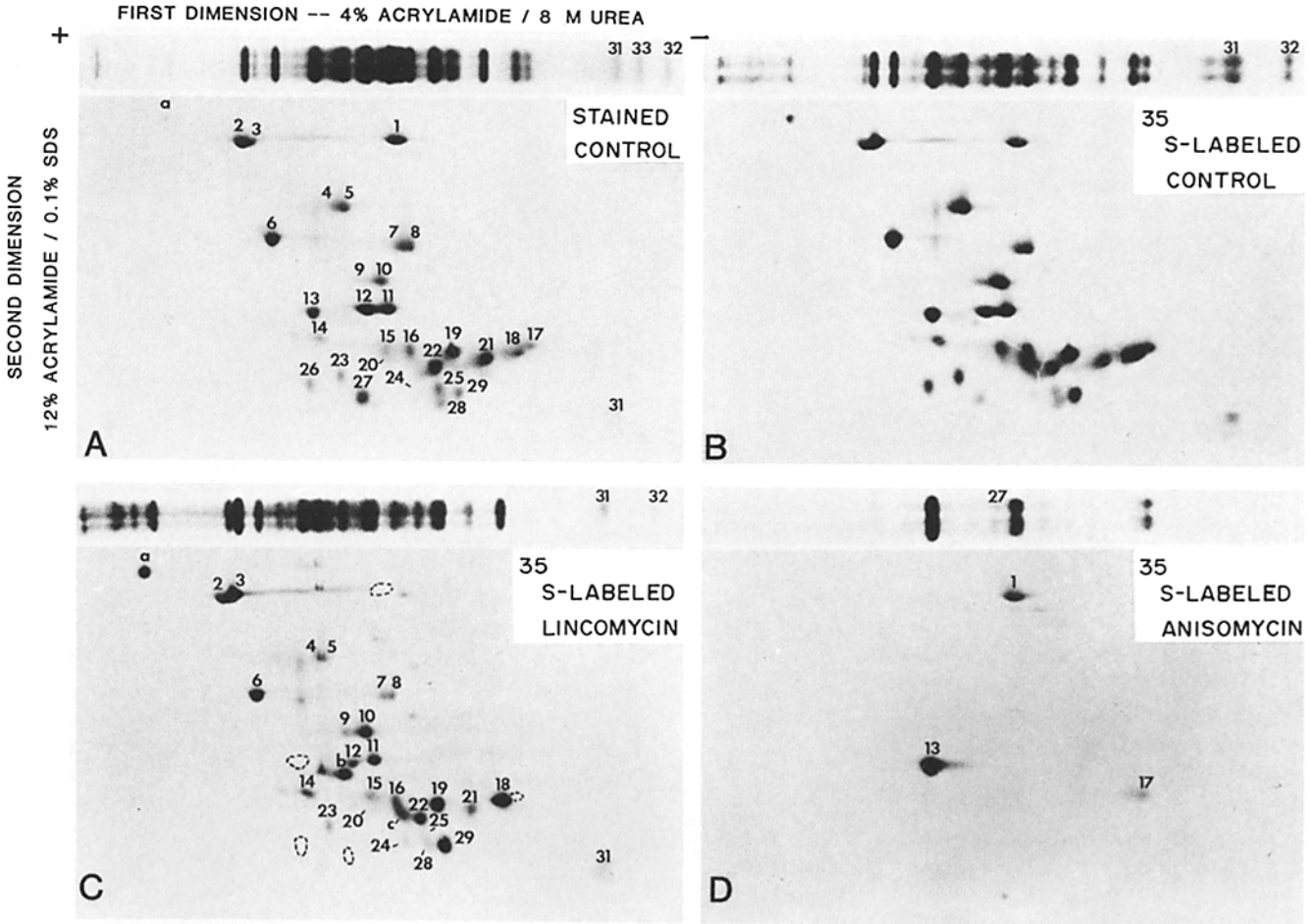

FIGURE 3 2-D gel profiles showing proteins from the large subunit of the chloroplast ribosome that were synthesized in the presence of inhibitors of chloroplast and cytoplasmic protein synthesis from one of six independent experiments. (*A*) Coomassie Blue-stained 2-D gel of ribosomal proteins from control cells used in the pulse-labeling experiment. (*B*) Fluorograph of the gel shown in *A* where the cells received a 1-h pulse of $^{35}SO_4$ in the absence of any inhibitor. (*C*) Fluorograph of ribosomal proteins labeled during a 1-h pulse in the presence of lincomycin an inhibitor of chloroplast protein synthesis. (*D*) Fluorograph of ribosomal proteins labeled during a 1-h pulse in the presence of anisomycin an inhibitor of cytoplasmic protein synthesis. The first-dimension stained gel or fluorograph is mounted above each of the 2-D gels shown above. With minor exceptions, the composite of the proteins labeled in *C* and *D* gives the labeling pattern for the untreated cells shown in *B*. The proteins labeled in the presence of anisomycin and not labeled in the presence of lincomycin are indicated in *C* by dotted circles. Protein 27 is too faintly labeled to be seen in the second dimension of *D* but is visible in the 1-D gel above that panel. Protein 33 does not label with $^{35}SO_4$ as can be seen by comparing the 1-D gel of *A* with that of *B*. See Materials and Methods for details of labeling and gel electrophoresis.

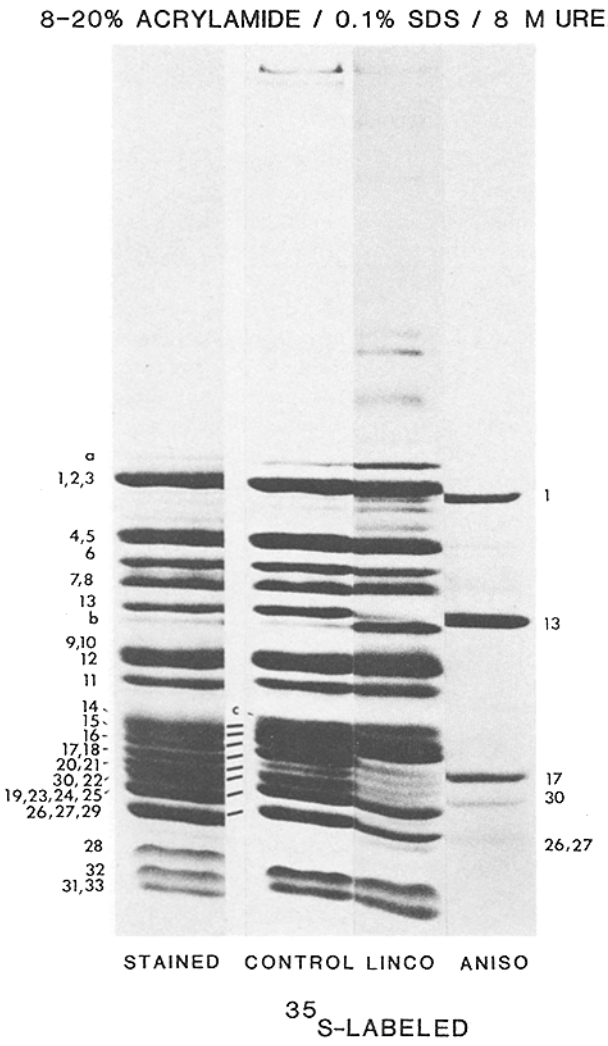

FIGURE 4 1-D SDS-urea gradient profiles of large subunit proteins synthesized in the presence of inhibitors of chloroplast and cytoplasmic protein synthesis. The lane labeled *STAINED* is the Coomassie Blue-stained profile of ribosomal proteins from control cells used in the pulse-labeling experiment and corresponds to the *CONTROL* lane of the fluorograph of ^{35}S pulse-labeled cells. The lane labeled *LINCO* is a fluorograph of ribosomal proteins labeled during a 1-h pulse in the presence of lincomycin, an inhibitor of chloroplast protein synthesis. The lane labeled *ANISO* is a fluorograph of ribosomal proteins labeled during a 1-h pulse in the presence of anisomycin, an inhibitor of cytoplasmic protein synthesis. The ribosomal proteins run on this gel are from the same experiment as those shown in Fig. 3.

to be made on chloroplast ribosomes. While protein 27 labels weakly during the 1-h pulse in both the control and the ANISO treatment, protein 26 incorporates much less label in the ANISO treatment than in the control (Fig. 3 *B* and *D*). This is in contrast to the situation observed for proteins 1, 13, and 17 and is not easily explained in terms of differences in pool sizes of these proteins. However, it might be related to problems with the assembly of protein 26 in the absence of cytoplasmic protein synthesis. In the anisomycin treatment a sixth large subunit protein (protein 30) was seen to be labeled in SDS-urea gradient gels (Fig. 4), but not in the 2-D gels.

Except for proteins 20, 32, and 33, all ribosomal proteins not labeled in the presence of anisomycin were clearly labeled in the presence of lincomycin (Figs. 3 and 4), indicating that they are products of cytoplasmic protein synthesis. The cytoplasmic site of synthesis of protein 20 was verified in 2-D gels by longer exposure of the autoradiogram (data not shown). Although protein 32 is not visualized in the second dimension of 2-D gels, it is slightly visible in the 1-D gel strips (Fig. 3 *C*) and clearly discernible as a product of cytoplasmic protein synthesis as evidenced by its appearance in the LINCO lane of the SDS-urea gradient gel (Fig. 4). A comparison of the 1-D gel strips of Fig. 3 *A* and *B* reveals that protein 33 fails to label with $^{35}SO_4$ and hence its site of synthesis could not be determined in these experiments. The spots designated "a" and "b" in the lincomycin treatment are much more strongly labeled then in the control, whereas peptide "c" is less strongly labeled than in the control (Figs. 3 and 4).

Small Subunit Proteins

14 of the 31 small subunit proteins label in the presence of anisomycin (Figs. 5 and 6) and thus appear to be synthesized in the chloroplast. Protein 3, one of the acidic, high molecular weight proteins not well resolved in the 2-D gel system can only be clearly seen to label in the SDS-urea gradient system (Fig. 6). Protein 19 labels strongly under long- but not short-term exposure to $^{35}SO_4$, suggesting that it may not turn over rapidly. While this protein can be seen only faintly in Fig. 5 *D* and the ANISO lane of Fig. 6, it is resolved upon further exposure of the fluorogram and thus appears to be made in the chloroplast. As mentioned earlier, proteins 23 and 24, each of which appears as a single, intensely labeled band in the ANISO lane of the SDS-urea gradient gel (Fig. 6), are resolved in the 2-D gels as a series of three spots, all of the same molecular weight but with the new forms having slightly more negative charge (Fig. 5 *D*). This could be explained if one assumed that the newly synthesized polypeptides were phosphorylated prior to assembly and subsequently dephosphorylated slowly in situ.

All small subunit proteins not labeled in the presence of ANISO incorporate label in the presence of LINCO, and we assume them to be products of cytoplasmic protein synthesis (Figs. 5 and 6). Proteins 2, 8, 12, and 14 are not seen in the 2-D gels of Fig. 5 (for reasons discussed previously), but the cytoplasmic origin of these three proteins is evident from their appearance in the LINCO lane of Fig. 6. Protein 29, which incorporates only low levels of ^{35}S and is thus only barely detectable in Fig. 5 *C*, is revealed as a product of cytoplasmic protein synthesis in the LINCO lane of Fig. 6. When labeling in the presence of LINCO (Fig. 5 *C*), several spots appear which do not correspond to any numbered ribosomal protein. These could represent minor cytoplasmic contaminants which do not show up in the control gels where the cells received less label (see Materials and Methods).

DISCUSSION

Characterization of the proteins present in the large and small subunits of chloroplast ribosomes from *C. reinhardtii* by 1- and 2-D gel electrophoresis has revealed that 33 proteins are found consistently in the large subunit and 31 in the small subunit (Table II). None of the large subunit proteins are removed by high-salt washing, but six of the small subunit proteins are detached by this treatment (Table II). For the moment, we are including the latter proteins as true ribosomal proteins although they may prove to be specific (e.g., initiation and elongation factors) or nonspecific contaminants upon further analysis. We have designated by letter certain proteins that are present in substoichiometric amounts which we believe to be contami-

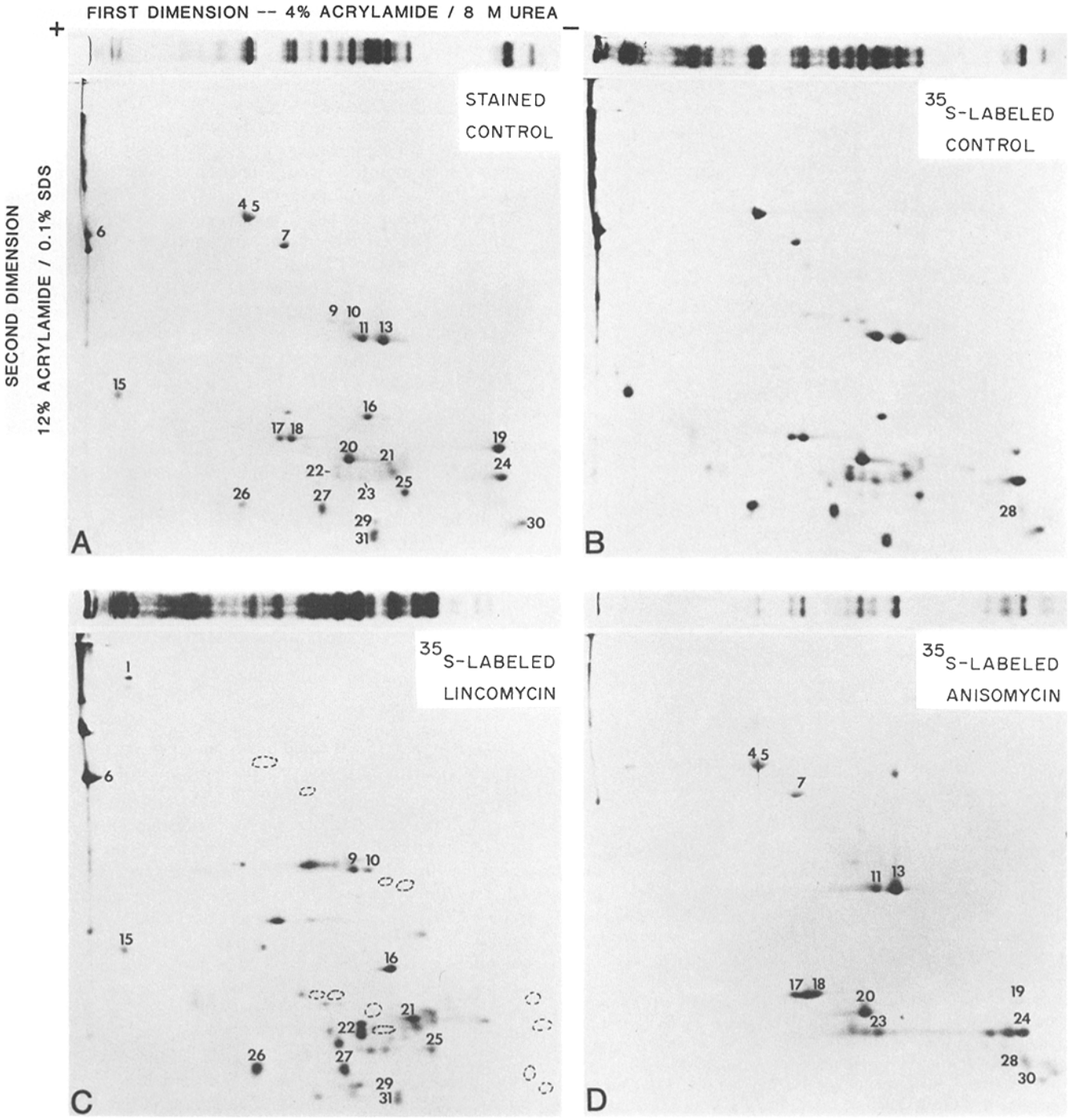

FIGURE 5 2-D gel profiles showing proteins from the small subunit of the chloroplast ribosome that were synthesized in the presence of inhibitors of chloroplast and cytoplasmic protein synthesis from the same experiment shown in Fig. 3. (*A*) Coomassie Blue-stained 2-D gel of ribosomal proteins from control cells used in the pulse-labeling experiment. (*B*) Fluorograph of the gel shown in *A* where the cells received a 1-h pulse of $^{35}SO_4$ in the absence of any inhibitor. (*C*) Fluorograph of ribosomal proteins labeled during a 1-h pulse in the presence of lincomycin, an inhibitor of chloroplast protein synthesis. (*D*) Fluorograph of ribosomal proteins labeled during a 1-h pulse in the presence of anisomycin, an inhibitor of cytoplasmic protein synthesis. The first-dimension stained gel or fluorograph is mounted above each of the 2-D gels shown above. With minor exceptions, the composite of the proteins labeled in *C* and *D* gives the labeling pattern for the untreated cells shown in *B*. The proteins labeled in the presence of anisomycin and not labeled in the presence of lincomycin are indicated in *C* by dotted circles. Proteins 1, 2, 3, 8, 12, and 14 are not visible on these gels (see text) and their labeling patterns are revealed in the 1-D SDS-urea gradient gels of Fig. 6. See Materials and Methods for details of labeling and gel electrophoresis.

nants. These include proteins "a" and "b" in the large subunit (Fig. 1 *C*) and proteins "a"–"c" in the small subunit (Fig. 2 *C*).

Hanson et al. (19) reported 22 and 26 proteins in the small and large subunits, respectively, of the chloroplast ribosome of *Chlamydomonas* based on 2-D gels similar to the ones used by us. We identified seven more proteins in the large subunit than seen by Hanson et al. (19). This is due primarily to the better resolution afforded by our larger gels which allow for longer

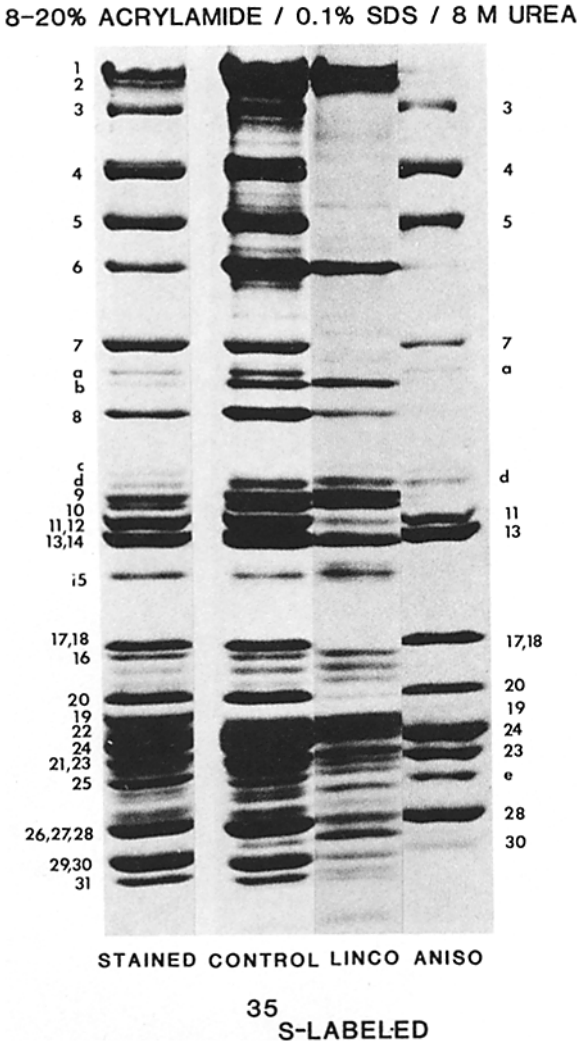

FIGURE 6 1-D SDS-urea gradient profiles of small subunit proteins synthesized in the presence of inhibitors of chloroplast and cytoplasmic protein synthesis. The lane labeled *STAINED* is the Coomassie Blue-stained profile of ribosomal proteins from control cells used in the pulse-labeling experiment and corresponds to the *CONTROL* lane of the fluorograph of ^{35}S pulse-labeled cells. The lane labeled *LINCO* is a fluorograph of ribosomal proteins labeled during a 1-h pulse in the presence of lincomycin, an inhibitor of chloroplast protein synthesis. The lane labeled *ANISO* is a fluorograph of ribosomal proteins labeled during a 1-h pulse in the presence of anisomycin, an inhibitor of cytoplasmic protein synthesis. The ribosomal proteins run on this gel are from the same experiment as those shown in Fig. 5.

electrophoretic runs in both directions. For example, LC2 of Hanson et al. (19) is resolved into proteins 2 and 3 on our gels, and LC8 of Hanson et al. (19) corresponds to our proteins 7 and 8. Likewise, their proteins LC10 and LC11 probably correspond to our proteins 11 + 12 and 9 + 10, respectively. Similarly, the poorly resolved components designated LC18 and LC23 in the 2-D gels of Hanson et al. (19) are resolved into six proteins (19, 22, 24, 25, 28, 29) in our gel system. Except for LC3 and LC7 reported by Hanson et al. (19), most of the other proteins correspond to large subunit proteins identified on our 2-D gels.

Separation of the small subunit proteins on 1-D SDS-urea gradient gels demonstrates the presence of seven high molecular weight proteins ranging from 54,000 to 87,000 which have not been previously reported by other workers using 2-D gel systems. However, these proteins were previously observed in 1-D SDS gels of chloroplast ribosomal proteins from *Chlamydomonas* by Brügger and Boschetti (7) and Gillham et al. (18).

The discrepancy of nine proteins between our estimates and those of Hanson et al. (19) can be accounted for largely by the fact that the high molecular weight proteins do not run in the first dimension of these two-dimensional gels. Thus, proteins 1, 3, and 6 fail to run in the 2-D gel system although they are clearly visible in our 1-D SDS-urea gradient system (Fig. 2 *C*). Protein 2 is seen only infrequently in 2-D gels although it is always resolved in 1-D gels (Fig. 2 *C*). Proteins 4 and 5 are resolved as separate bands in 1-D SDS-urea gradient gels. In the SDS second dimension of the 2-D gels, these proteins, which have similar charges, also have the same apparent molecular weight. These two proteins very likely correspond to a spot which lies slightly above and to the left of spot A of Hanson et al. (19) who did not regard this protein as an authentic ribosomal protein. Spot A in the scheme of Hanson et al. (19) probably corresponds to our protein 7. Protein 1, which barely enters the first dimension gels of Hanson et al. (19), could be identical with our protein 8 which does not run in the first dimension of our 2-D gels. From these comparisons alone we can account for seven of the nine small subunit proteins seen by us, but not by Hanson et al. (19). While other comparisons between our results and those obtained by Hanson et al. (19) are possible for the small subunit proteins (e.g., their proteins 10 and 11 are clearly our proteins 19 and 22, respectively), a number of discrepancies also exist. For example, we do not know how to relate protein 6 in their system to any of the proteins seen in our system.

Our estimate of the total molecular weight of proteins associated with the small subunit (992,300, Table II) is much higher than that reported by Hanson et al. (19) (451,500), because we have found these high molecular weight proteins not seen by them. Capel and Bourque (8) have reported an even lower protein mass (336,000) for the small subunit of the chloroplast ribosome from *Nicotiana*. The largest protein resolved by them in a 2-D gel system similar to the one used by us and Hanson et al. (19) had a molecular weight of 30,700. Whether high molecular weight proteins are unique to the chloroplast ribosomes of *C. reinhardtii* and are not a part of the small subunit of the *Nicotiana* chloroplast ribosome, or whether they simply were not seen because of the gel system used, remains to be determined.

On the other hand, the protein mass calculated for the large subunit of the chloroplast ribosome from *Chlamydomonas* by us (605,800) and by Hanson et al. (19) (538,000) is in fairly good agreement with the estimate for *Nicotiana* (688,000) calculated by Capel and Bourque (8). This may reflect the fact that all large subunit proteins migrate in both the first and second dimensions of the 2-D gel system used and hence were included in all the tabulations. The total mass of the chloroplast large subunit (1,700,000) calculated from our data (Table II) is about the same as that of the large subunit of the *E. coli* ribosome (37), but the chloroplast small subunit has a considerably greater mass (1,600,000) than the corresponding *E. coli* small subunit. This difference is exclusively accounted for by the difference in protein mass since the 16S rRNAs of both subunits are very similar in molecular weight (6, 17).

The sites of synthesis of all chloroplast ribosomal proteins except protein 33 of the large subunit, which does not label with sulfur, have been identified with reasonable certainty in the experiments reported here (Table II). 14 of the 31 small

TABLE II

Sites of Synthesis and Apparent Molecular Weights of Chloroplast Ribosomal Proteins

Large subunit proteins			Small subunit proteins		
Protein	Apparent molecular weight	Site of synthesis	Protein	Apparent molecular weight	Site of synthesis
L-1	37,600	Chloroplast	S-1	87,100	Cytoplasm
L-2	37,500	Cytoplasm	S-2	80,800	Cytoplasm
L-3	37,300	Cytoplasm	S-3	74,700	Chloroplast
L-4	28,700	Cytoplasm	S-4	63,900	Chloroplast
L-5	28,700	Cytoplasm	S-5	63,900	Chloroplast
L-6	24,500	Cytoplasm	S-6	59,000	Cytoplasm
L-7	24,300	Cytoplasm	S-7	54,100	Chloroplast
L-8	24,300	Cytoplasm	S-8	40,100	Cytoplasm
L-9	20,400	Cytoplasm	*S-9	35,000	Cytoplasm
L-10	20,400	Cytoplasm	*S-10	34,900	Cytoplasm
L-11	18,400	Cytoplasm	S-11	31,800	Chloroplast
L-12	18,200	Cytoplasm	S-12	31,600	Cytoplasm
L-13	17,900	Chloroplast	S-13	31,000	Chloroplast
L-14	15,900	Cytoplasm	S-14	30,700	Cytoplasm
L-15	15,300	Cytoplasm	*S-15	24,500	Cytoplasm
L-16	15,200	Cytoplasm	*S-16	21,200	Cytoplasm
L-17	15,200	Chloroplast	*S-17	19,700	Chloroplast
L-18	14,700	Cytoplasm	*S-18	19,700	Chloroplast
L-19	14,700	Cytoplasm	S-19	18,200	Chloroplast
L-20	14,500	Cytoplasm	S-20	17,400	Chloroplast
L-21	14,300	Cytoplasm	S-21	16,600	Cytoplasm
L-22	14,000	Cytoplasm	S-22	15,900	Cytoplasm
L-23	13,800	Cytoplasm	S-23	15,600	Chloroplast
L-24	13,600	Cytoplasm	S-24	15,500	Chloroplast
L-25	13,100	Cytoplasm	S-25	14,400	Cytoplasm
L-26	12,900	Chloroplast	S-26	13,300	Cytoplasm
L-27	12,500	Chloroplast	S-27	13,200	Cytoplasm
L-28	12,500	Cytoplasm	S-28	13,200	Chloroplast
L-29	12,400	Cytoplasm	S-29	12,100	Cytoplasm
L-30	11,700	Chloroplast?	S-30	11,900	Chloroplast
L-31	10,900	Cytoplasm	S-31	11,300	Cytoplasm
L-32	10,500	Cytoplasm			
L-33	9,900	Not determined			
				992,300 total protein mol wt	
				560,000 16s rRNA mol wt	
	605,800 total protein mol wt				
	1,100,000 3s, 5s, 7s, and 23s rRNA mol wt				
				1,552,300 total subunit mol wt	
	1,705,800 total subunit mol wt				

Sites of synthesis of chloroplast ribosomal proteins from *C. reinhardtii* and estimates of the apparent molecular weights of these proteins from 2-D charge-SDS slab gels. Estimates of rRNA molecular weights are from (6, 17, 30). Proteins designated with an asterisk are removed completely when subunits are isolated and pelleted in TKM buffer containing 850 mM KCl. Apparent molecular weights are the average of at least three determinations. For proteins with molecular weights >55,000, deviations are <2,100; and for proteins with molecular weights <55,000, the deviations are <1,000.

subunit proteins are products of chloroplast protein synthesis, with the remainder being made in the cytoplasm (Table II). Of the 32 proteins of the large subunit whose sites of synthesis can be determined, five and possibly six are made in the chloroplast and the rest in the cytoplasm. This means that roughly a third of the chloroplast ribosomal proteins in *C. reinhardtii* are chloroplast gene products, and that these make up about one-half of the total proteins known to be synthesized on chloroplast ribosomes (cf. references 2, 17). Furthermore, knowledge of the sites of synthesis of chloroplast ribosomal proteins in *Chlamydomonas* will allow us to search more effectively for protein differences in chloroplast and nuclear mutations known to affect chloroplast ribosomes in this alga (cf. reference 6). For example, protein LC6 (protein 6) of the large subunit, shown by Davidson et al. (11) to be affected by nuclear mutations at the *ery-M1* locus, has proven to be a product of cytoplasmic protein synthesis. Likewise, protein LC4 reported by Mets and Bogorad (26) to be affected in the chloroplast mutation *ery-Ula* corresponds in our gel system to protein 1 of the large subunit which is synthesized in the chloroplast. Presumably, other chloroplast mutations will affect the primary structure of those proteins synthesized on chloroplast ribosomes. Other nuclear mutations could affect either the primary structure of those proteins which are made in the cytoplasm or cause secondary modifications of chloroplast ribosomal proteins synthesized in either compartment.

The sites of synthesis of chloroplast ribosomal proteins have previously been investigated in *Euglena* (14) and in pea (13). Freyssinet (14) pulse-labeled chloroplast ribosomal proteins of *Euglena* in the presence of cycloheximide or lincomycin. Total

chloroplast ribosomal proteins were displayed on 2-D gels, after which the stained spots were cut out and counted. Of the 39 proteins resolved on these gels, the synthesis of 12 was blocked by cycloheximide and of nine by lincomycin. The effect of inhibitors on the sites of synthesis of the remaining proteins could not be determined. While Freyssinet's experiments (14) established that a substantial number of chloroplast ribosomal proteins are synthesized on chloroplast ribosomes, his results did not determine which protein belonged to which ribosomal subunit. Furthermore, they were ambiguous with respect to the sites of synthesis of almost half of the proteins visualized. Eneas-Filho et al. (13) examined the sites of synthesis of ribosomal proteins in isolated chloroplasts of pea using the light-driven system of Ellis and Hartley (12). They reported that six of 24 small subunit and five of 32 large subunit proteins were made in the chloroplast. By nature of their design, these in vitro experiments only establish which proteins are made within the chloroplast, and there is no way of ascertaining which chloroplast ribosomal proteins are made in the cytoplasm. Thus, the proteins not made in isolated pea chloroplasts could either be synthesized in the cytoplasm or made in the chloroplast but not assembled. The advantage of in vivo experiments is that they also yield a positive identification of those chloroplast ribosomal proteins which are made in the cytoplasm. Despite differences in experimental design, the major conclusion from sites of synthesis experiments in *Chlamydomonas*, *Euglena*, and pea is that numerous chloroplast ribosomal proteins are synthesized in the chloroplast and hence very likely are coded by the chloroplast genome.

In contrast, only a single mitochondrial ribosomal protein has been identified as a product of mitochondrial protein synthesis. This protein belongs to the small subunit and has so far been reported only in yeast (33) and *Neurospora* (22, 23). No ribosomal proteins have yet been identified as gene products of the mammalian mitochondrial genome which has been sequenced completely for human beings and bovines (1) and mice (36). However, eight unidentified reading frames remain in the mammalian mitochondrial genome for which gene products have not yet been determined.

All experiments done so far to determine the sites of synthesis of chloroplast ribosomal proteins have involved pulse-labeling these proteins either in vitro in isolated chloroplasts or in vivo in the presence of inhibitors, following which assembled ribosomes are isolated and the labeled proteins determined. This experimental design can only succeed if there is a pool of cold ribosomal proteins and rRNA with which the labeled proteins can assemble during the pulse. Since the experimental design works, neither transcriptional nor translational control of chloroplast ribosomal protein synthesis can be so stringent as to preclude the existence of a small pool of free ribosomal proteins. The inhibitor experiments in particular show that these pools must exist for chloroplast ribosomal proteins made in both the chloroplast and cytoplasmic compartments. While we have made no direct determination of pool sizes of free chloroplast ribosomal proteins in our experiments, our results suggest that the pool of some or all of the cytoplasmically synthesized chloroplast ribosomal proteins is probably smaller than the corresponding pool of ribosomal proteins made in the chloroplast. When cells are pulse-labeled with equal amounts of isotope in the presence of LINCO, the counts incorporated into chloroplast ribosomes (normalized for the molecular weight of the proteins synthesized) are approximately three times greater than when cells are pulse-labeled in the presence of ANISO (data not shown). Estimates of free pool sizes for ribosomal proteins in *E. coli* are in the neighborhood of 2% of the total ribosomal proteins (15).

Finally, strong evidence now exists that regulation of the synthesis of ribosomal proteins in both *E. coli* (cf. reference 24) and *Saccharomyces* (29) occurs not only at the transcriptional but also at the translational level. The initial demonstration of translational control in *E. coli* relied on the observation that strains merodiploid for ribosomal protein genes showed a dose-dependent increase in rates of ribosomal protein mRNA synthesis, but no proportionate increase in the synthesis of ribosomal proteins themselves (cf. reference 24). Since then, direct inhibition of ribosomal mRNA translation by ribosomal proteins has been demonstrated. In *Saccharomyces*, Pearson et al. (29) have shown that a plasmid containing the gene coding for ribosomal protein L3 is maintained at a level of five to ten copies per cell and that these cells transcribe 7.5 times as much L3 mRNA as control cells, maintain 3.5 times as much L3 mRNA, but synthesize only 1.2 times as much L3 protein as normal cells. In *Chlamydomonas*, the nuclear genome is composed of unique sequences (39), while the chloroplast genome is amplified 80-fold (cf. reference 16). Barring the unlikely possibility that nuclear genes coding for chloroplast ribosomal proteins are amplified 80-fold, there must be regulation at the transcriptional or translational level or both to produce chloroplast ribosomal proteins stoichiometrically. Either transcription rates of nuclear genes coding for chloroplast ribosomal proteins must be high relative to chloroplast genes coding for these proteins or translation of ribosomal protein messages must be much less efficient in the chloroplast. Investigation of these questions is a logical extension of the experiments reported here and studies designed to answer them are already underway in our laboratory.

This work was supported by National Institutes of Health research grant GM-19427. During the initial phases Mr. Schmidt was supported by a Cell and Molecular Biology Training Grant (GM-07184) awarded to Duke University.

Received for publication 4 November 1982, and in revised form 21 January 1983.

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