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Ochromonas mitochondria contain a specific chloroplast protein

(small subunit of ribulose-1,5-bisphosphate carboxylase/immunoelectron microscopy/chrysophycean alga/promiscuous DNA)

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ABSTRACT **Antibody raised against the small subunit of ribulose-1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] of *Chlamydomonas reinhardtii* labeled the mitochondria as well as the chloroplast of the chrysophyte alga *Ochromonas danica* in sections prepared for immunoelectron microscopy by the protein A-gold technique. The same antibody labeled the chloroplast but not the mitochondria of *C. reinhardtii*. A quantitative study of labeling in dark-grown, greening (32 hr light), and mature green cells of *O. danica* revealed that anti-small-subunit staining in the mitochondria increased progressively in the light as it does in the plastid. Antibody to the large subunit of the enzyme did not label the mitochondria of either *O. danica* or *C. reinhardtii*. In view of the recent demonstrations of homologous DNA sequences in the mitochondrial and chloroplast genomes of higher plants, we suggest that the DNA sequence coding for the small subunit has migrated to the mitochondria from nucleus or chloroplast and is expressed within the organelle.**

Since many chloroplast and mitochondrial proteins are encoded by nuclear genes, it is implicit in the endosymbiont theory for the evolution of these organelles that DNA was able to migrate from the genome of the endosymbiont to that of the host cell. The recent discovery of chloroplast (1) and mitochondrial (2–8) homologous DNA sequences in the nucleus of a variety of organisms lends further support to this hypothesis. That DNA can also migrate between organelles is shown by the presence of chloroplast DNA sequences in the mitochondria of higher plants (9–12). However, to date, it has not been established whether these "promiscuous" genes are ever expressed in their new location. We demonstrate here by immunocytochemistry that the small subunit (SSU) of the chloroplast enzyme ribulose-1,5-bisphosphate carboxylase [RuBPCase; 3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] is present in the mitochondria of the chrysophyte alga *Ochromonas danica*. The explanation we favor for this unexpected observation is that a copy of the gene for SSU has migrated from either the chloroplast or the nucleus to the mitochondria and is transcribed and translated there.

MATERIALS AND METHODS

Cell Culture. The protist *O. danica* Pringsheim was obtained from the University of Texas Culture Collection (UTEX no. 1298). Dark-grown, greening (32 hr light), and light-grown cultures were grown in Aaronson and Baker's (13) medium at 29°C as described (14). Dark-grown and greening cells were fixed during the logarithmic phase of growth. Mature green cells were fixed at the end of the linear phase of growth. *Chlamydomonas reinhardtii* Dangeard, 137c +, was obtained from the Culture Centre of Algae and Protozoa (Cambridge, U.K.; no. 11/32a). Cells were grown at 20°C in Beijerinck's medium (15) at a light intensity of 4300 lux.

Fixation and Embedding. Cells were fixed in 1% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer for 90 min at 4°C, rinsed in buffer and blocked in 2% (wt/vol) agar. The agar blocks were dehydrated in 25% (vol/vol) ethanol at −5°C, then in 50%, 75%, and 95% ethanol at −18°C. Embedding was carried out at −18°C in Lowicryl K4M (16) according to the following schedule: 95% ethanol/resin, 1:1 (vol/vol), overnight; 95% ethanol/resin, 1:2 (vol/vol), 2 times for 2 hr each; pure resin, 2 hr and then overnight. Blocks were transferred to gelatin capsules and polymerized under UV light (365 nm) at −18°C for 24 hr, then at room temperature for 48 hr.

Immunostaining. Antibodies to the small and large subunit (LSU) of RuBPCase were raised in rabbits against sodium dodecyl sulfate-dissociated subunits of *C. reinhardtii* holoenzyme (17), and were kindly given to us by N.-H. Chua (Rockefeller University). Cross-reactivity to *Ochromonas* antigen was demonstrated by indirect immunofluorescence. Colloidal gold (particles of diameter ≈15 nm) was prepared by the method of Frens (18) and coupled to protein A (19). Pale gold sections were collected on formvar- and carbon-coated nickel grids, which were floated section-side down on drops of phosphate-buffered saline, 5 min; 0.5% (wt/vol) chicken egg albumin in phosphate-buffered saline, 15 min; antibody at 50–250 μg of IgG per ml in phosphate-buffered saline, 60–120 min; protein A-gold diluted between 1:10 and 1:20 fold in phosphate-buffered saline, 30 min. Sections were subsequently stained with uranyl acetate and lead citrate and viewed in a Philips EM 410 at 80 kV. In control experiments, the antibody was replaced with nonimmune IgG or the antibody was omitted altogether and the sections were incubated in phosphate-buffered saline alone prior to protein A-gold labeling.

Quantitative Evaluation. Since this technique labels only the antigenic sites present on the surface of the sections, it is possible to make quantitative comparisons of the amount of labeling in each cell organelle by determining the number of gold particles per μm^2 of organelle sectioned. Area determinations and gold particle counts were made using a Zeiss MOP-3.

Materials. Lowicryl K4M was purchased from J. B. EM Services (Montreal), protein A was from Pharmacia, and nonimmune rabbit IgG was from Sigma.

RESULTS

Fig. 1 shows a mature green cell of *O. danica* labeled with anti-SSU. The chloroplast is labeled (only lightly in this particular section) as are, unexpectedly, the cell's numerous mitochondria. The mitochondria are narrow and elongate and can be identified by their tubular cristae, whose lumens stand out as white dots against the dense mitochondrial ma-

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Abbreviations: RuBPCase, ribulose-1,5-bisphosphate carboxylase; SSU, small subunit of RuBPCase; LSU, large subunit of RuBPCase.

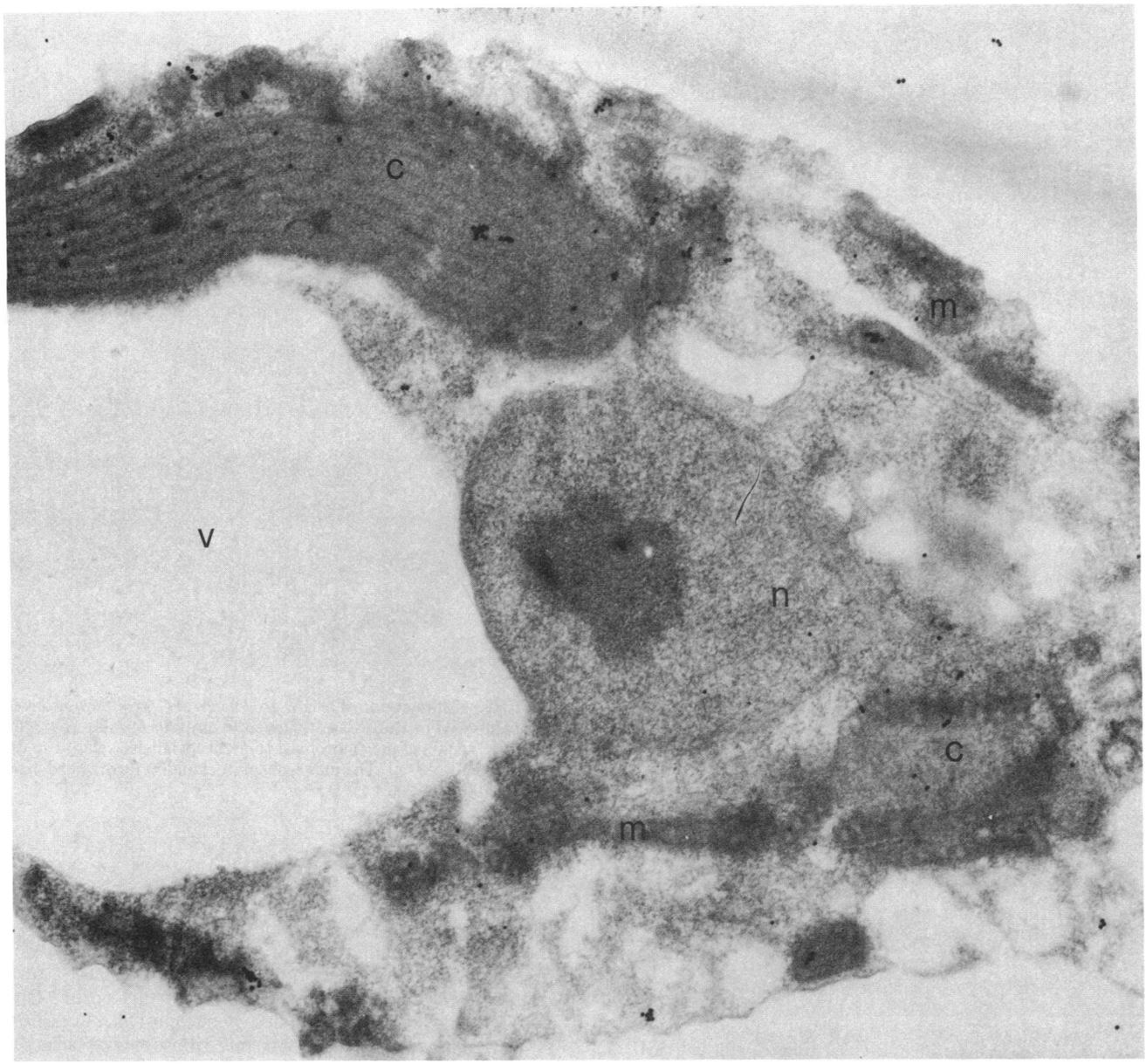

FIG. 1. Thin section of a mature green cell of *O. danica* treated with anti-SSU of RuBPCase followed by protein A-gold. Gold particles are localized over the chloroplast (c) and the mitochondria (m). The cytoplasm, vacuole (v), and nucleus (n) are almost free of label. (×33,800.)

trix. Virtually all the cytoplasmic gold particles are associated with the mitochondria. The cytoplasm proper and the nucleus have only a low level of background labeling. The large vacuole, which contains a carbohydrate storage product, is unlabeled. Fig. 2*A* shows the chloroplast and adjacent mitochondria of a mature green cell at higher magnification. The chloroplast is moderately well labeled by anti-SSU as is the elongate mitochondrion that lies appressed to the chloroplast. Fig. 2*B* shows the small proplastid and a typically enlarged mitochondrion in a dark-grown cell treated with anti-SSU. The proplastid is lightly labeled and the mitochondrion is moderately labeled.

To establish the specificity of the labeling, the following controls were performed (values in Table 1). First, the sections were incubated with nonimmune rabbit IgG instead of with antibody. At high concentrations of IgG (>300 μg/ml), nonimmune IgG stuck nonspecifically to the sections, and numerous gold particles were seen over all cell structures. When lower concentrations of IgG were used (50–250 μg/ml), only low levels of nonspecific labeling were observed. Therefore, antibody was used at these concentrations. Second, a very low level of labeling was observed when sections were labeled with protein A-gold only, indicating that protein A does not bind to some element in the section.

Finally, since anti-SSU unexpectedly labeled the mitochondria, we wished to exclude the possibility that a mitochondrial polypeptide might have contaminated the *Chlamydomonas* antigen used to prepare the antibody. Cells of *C. reinhardtii* were fixed by the same methods and labeled with anti-SSU at the same IgG concentrations. Fig. 3 shows that anti-SSU labels only the chloroplast of *Chlamydomonas* with the label heavily concentrated over the pyrenoid. The mitochondria are unlabeled. Anti-LSU also labels only the chloroplast of *Chlamydomonas* (data not shown).

Table 1 gives the quantitative results obtained when dark-grown, greening and mature green cells of *Ochromonas* are labeled with anti-SSU and compares them with the results obtained with anti-LSU. As would be expected, the chloroplast is labeled to approximately the same extent by anti-LSU and anti-SSU, and the amount of labeling by each antibody increases progressively in the light.

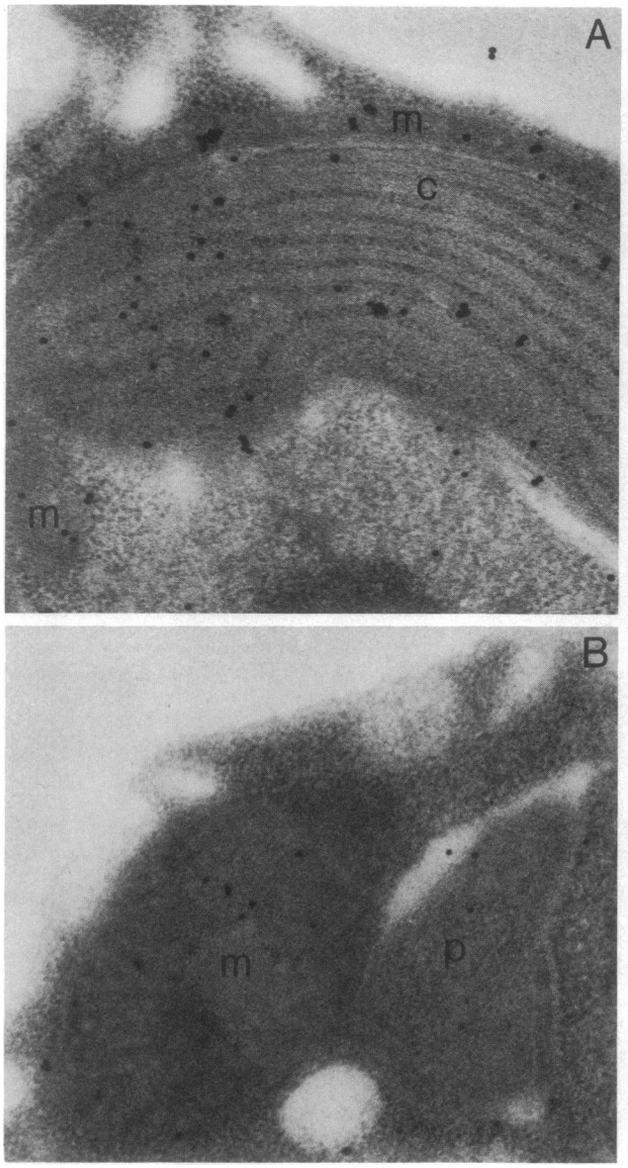

FIG. 2. Thin sections of *O. danica* treated with anti-SSU of RuBPCase followed by protein A-gold. (*A*) Mature green cell. Gold particles are localized over the chloroplast (c) and the mitochondria (m), in particular over the elongate mitochondrion that lies appressed to the outside surface of the chloroplast. (×47,400.) (*B*) Logarithmic-phase dark-grown cell. A few gold particles are present over the proplastid (p) and the large mitochondrion (m). (×43,000.)

Anti-SSU also labels the mitochondria at the same or at a slightly higher level than the plastid (Table 1). The amount of mitochondrial labeling also increases progressively in the light. However, unlike the plastid, which ultimately increases its volume 10-fold in the light (14), the mitochondrial compartment actually decreases somewhat in volume during greening. Thus, the observed increase in density of mitochondrial labeling represents a relatively small increase in the total amount of immunoreactive material present compared to the increase in the quantity of chloroplast RuBPCase that takes place during greening.

Anti-LSU does not label the mitochondria of greening or mature green cells of *Ochromonas* (Table 1). The density of grains observed over the mitochondria of mature green cells is not significantly higher than the background labeling of the nucleus ($P > 0.1$). A significant level of labeling is present over the mitochondria of dark-grown cells, but we are uncertain of the meaning of this observation.

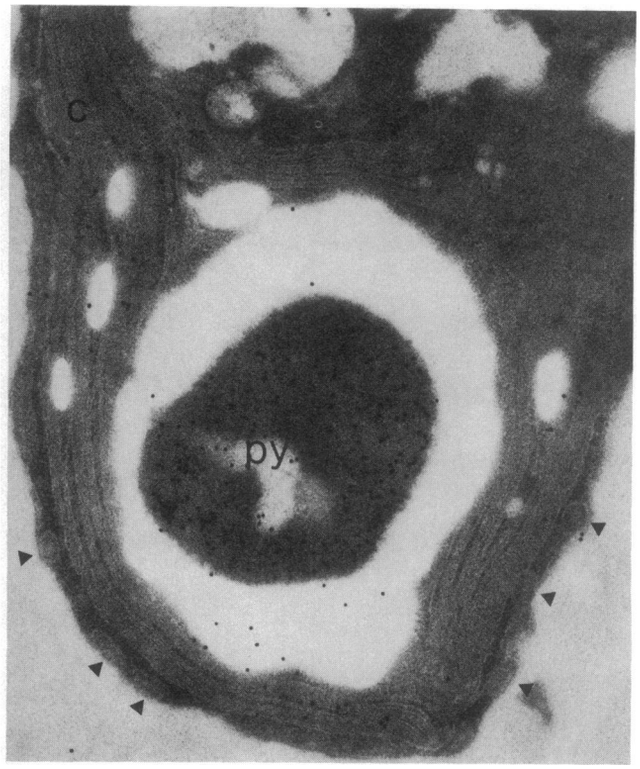

FIG. 3. Thin section of a light-grown cell of *C. reinhardtii* treated with anti-SSU of RuBPCase followed by protein A-gold. The pyrenoid (py) region of the chloroplast is intensely labeled relative to the chloroplast itself (c). The numerous mitochondria (arrowheads) are unlabeled. (×35,000.)

DISCUSSION

We conclude on the basis of the immunocytochemical data presented that the small subunit of RuBPCase, or at least a polypeptide containing some of its antigenic determinants, is present in the mitochondria of both light- and dark-grown cells of *Ochromonas danica*. How could this occur? One possibility is that SSU is encoded by nuclear DNA in *Ochromonas*, synthesized on cytoplasmic ribosomes as a larger precursor, and subsequently transported into the mitochondria. However, this is very unlikely because transport of proteins into mitochondria is highly specific (20). Also, although nuclear-encoded plastid proteins are synthesized on free cytoplasmic ribosomes in green algae and higher plants (21), this is unlikely to be the case in *Ochromonas*. The chloroplast of *Ochromonas* is completely enclosed by a cisterna of endoplasmic reticulum that has polysomes on the cytoplasmic surface of its outer membrane (22). Nuclear-encoded plastid proteins are believed to be synthesized on these bound ribosomes and to pass cotranslationally into the endoplasmic reticulum lumen and from there via vesicles to the chloroplast envelope (23). Thus, SSU, if nuclear-encoded, may never be present in the cytoplasm.

Whether SSU is nuclear-encoded in *Ochromonas* is not known. In green algae and higher plants, SSU is encoded by nuclear DNA and LSU is encoded by chloroplast DNA (24). However, recently Heinhorst and Shively (25) have shown that in the cyanelles of *Cyanophora* [cyanelles are believed (26) to be evolutionary intermediates between cyanobacteria and chloroplasts], the genes for SSU and LSU are located close to each other on cyanelle DNA. Other evidence suggests that SSU is encoded by chloroplast DNA in red algae (27) and the raphidophyte alga *Olisthodiscus luteus* (R. A. Cattolico, personal communication). *Ochromonas* is in the

Table 1. Density of labeling over various cell compartments in *O. danica*

Cells	Gold particles per μm^2				
	Mitochondria	Chloroplast	Vacuole	Nucleus	Cytoplasm
Mature green					
SSU	29.6 ± 3.9	19.8 ± 1.4	2.9 ± 1.3	6.2 ± 0.9	5.6 ± 0.8
LSU	5.3 ± 1.0	20.4 ± 3.2	2.2 ± 0.5	4.4 ± 1.7	3.8 ± 0.6
Greening					
SSU	16.8 ± 2.6	15.0 ± 3.1	0.9 ± 0.5	3.1 ± 0.9	1.8 ± 0.4
LSU	4.3 ± 0.7	9.6 ± 1.0	1.5 ± 0.3	1.7 ± 0.3	1.9 ± 0.3
Dark-grown					
SSU	9.9 ± 1.0	6.9 ± 0.9	1.1 ± 0.2	2.6 ± 0.3	2.4 ± 0.3
LSU	8.5 ± 1.3	8.3 ± 0.8	1.2 ± 0.3	2.2 ± 0.4	1.6 ± 0.3
Control					
Nonimmune IgG	2.7 ± 0.7	1.5 ± 0.4	1.0 ± 0.5	1.3 ± 0.5	1.1 ± 0.3
Phosphate-buffered saline	1.4 ± 0.8	0.7 ± 0.2	0.5 ± 0.2	0.8 ± 0.4	0.4 ± 0.1

Results are means ± SEM. For anti-SSU, 33 pictures of mature cells were used, 7 of greening cells, and 20 of dark-grown cells; for anti-LSU, we used 10 pictures of mature cells, 18 of greening cells, and 26 of dark-grown cells. Densities were compared in each series by Student's *t* test. Values for *C. reinhardtii* labeled with anti-SSU are as follows: chloroplast, 46.2 ± 7.1 gold particles per μm^2; mitochondria, 4.2 ± 1.3; remainder of cell, 4.0 ± 1.0.

same chlorophyll *c* line of evolution as *Olisthodiscus*, so it is possible that the primary gene for SSU is located on its chloroplast genome. If SSU is chloroplast-encoded in *Ochromonas*, it would be synthesized within the chloroplast and would have to cross six membranes to reach the mitochondrial matrix.

A more plausible explanation for the presence of SSU in the mitochondria is that a copy of the gene for SSU has migrated from either the chloroplast or the nuclear genome to the mitochondrial genome and that the mitochondrial gene is transcribed and translated within the mitochondrion. Lonsdale *et al.* (11) have shown that the mitochondria of maize contain a copy of the chloroplast LSU gene and, furthermore, that the mitochondrial sequence can be partially expressed in an *Escherichia coli in vitro* transcription–translation system. They did not determine whether the mitochondrial LSU gene might also be expressed in the plant. Jacobs *et al.* (5) have suggested that "promiscuous DNA" may be expressed in sea urchins. In the nuclear copy of the mitochondrial gene for cytochrome oxidase subunit I, the codon TGA, which would terminate a nuclear-encoded protein, has been changed to TGG, encoding tryptophan as TGA does in the mitochondrion.

An alternative route by which the mitochondria could have obtained the gene for SSU is if it were present in the symbiotic bacteria from which the mitochondria evolved and had anomalously not been lost during the long evolution of mitochondria. However, the migration of DNA sequences between cell organelles is proving to be a rampant process, especially in higher plants (10, 12, 28), so it is much more likely that the presence of SSU in the mitochondria of *Ochromonas* is due to the transcription and translation of such a migratory DNA sequence.

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