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Chloroplast genes are expressed during intracellular symbiotic association of *Vaucheria litorea* plastids with the sea slug *Elysia chlorotica*

(photosystem II reaction center/photosynthesis/chromophytic alga/ascoglossan mollusc/gene expression)

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ABSTRACT **The marine slug *Elysia chlorotica* (Gould) forms an intracellular symbiosis with photosynthetically active chloroplasts from the chromophytic alga *Vaucheria litorea* (C. Agardh). This symbiotic association was characterized over a period of 8 months during which *E. chlorotica* was deprived of *V. litorea* but provided with light and CO_2. The fine structure of the symbiotic chloroplasts remained intact in *E. chlorotica* even after 8 months of starvation as revealed by electron microscopy. Southern blot analysis of total DNA from *E. chlorotica* indicated that algal genes, i.e., *rbcL*, *rbcS*, *psaB*, *psbA*, and 16S *rRNA* are present in the animal. These genes are typically localized to the plastid genome in higher plants and algae except *rbcS*, which is nuclear-encoded in higher plants and green (chlorophyll a/b) algae. Our analysis suggests, however, that similar to the few other chromophytes (chlorophyll a/c) examined, *rbcS* is chloroplast encoded in *V. litorea*. Levels of *psbA* transcripts remained constant in *E. chlorotica* starved for 2 and 3 months and then gradually declined over the next 5 months corresponding with senescence of the animal in culture and in nature. The RNA synthesis inhibitor 6-methylpurine reduced the accumulation of *psbA* transcripts confirming active transcription. In contrast to *psbA*, levels of 16S *rRNA* transcripts remained constant throughout the starvation period. The levels of the photosystem II proteins, D1 and CP43, were high at 2 and 4 months of starvation and remained constant at a lower steady-state level after 6 months. In contrast, D2 protein levels, although high at 2 and 4 months, were very low at all other periods of starvation. At 8 months, *de novo* synthesis of several thylakoid membrane-enriched proteins, including D1, still occurred. To our knowledge, these results represent the first molecular evidence for active transcription and translation of algal chloroplast genes in an animal host and are discussed in relation to the endosymbiotic theory of eukaryote origins.**

The ascoglossan sea slug *Elysia chlorotica* (Gould) forms an intracellular symbiosis with chloroplasts of the filamentous chromophytic alga *Vaucheria litorea* (C. Agardh) (1, 2). The animal resembles a dark green leaf and is capable of photoautotrophic CO_2 fixation as a result of the high density of chloroplasts dispersed throughout its extensive digestive system (1, 3, 4). The photosynthetic sea slug survives in laboratory aquaria for 8–9 months when provided with only light and CO_2 (starved); a time period similar to its life cycle in nature. The symbiotic association is not inherited since the plastids are not transmitted in the eggs (5). Instead, chloroplast symbiosis is reestablished with each new generation of sea slugs (1, 6, 7). Acquisition of chloroplast symbionts begins immediately following metamorphosis from the veliger stage when juvenile sea slugs begin to feed on *V. litorea* cells (1, 2). Once ingested, the chloroplasts are phagocytically incorporated into the cytoplasm of one of two morphologically distinct, epithelial cells (3) and maintain their photosynthetic function (1, 3). The plastids are frequently found in direct contact with the host cytoplasm as revealed by ultrastructural studies (3). In nature, the adult animal feeds on algae only sporadically, obtaining metabolic energy from the photosynthetic activity of the intracellular chloroplasts (3).

While this type of symbiosis is fairly common in several families of ascoglossan slugs (Conchoidea, Stiligeroidea, and Elysioidea), the longevity of chloroplast symbioses varies from one species of sea slug to another. In some species, captured plastids are initially functional, but photosynthesis is greatly reduced or ceases after 12 hr to a week of starvation (8). In other species, intracellular plastids remain functional for at least a month as measured by chlorophyll retention, light/dark CO_2 fixation, and oxygen evolution in response to light (9–11). However, the *E. chlorotica*/*V. litorea* association of 8–9 months is the longest-lived chloroplast symbiosis yet reported (1, 2). For a comprehensive discussion of chloroplast symbioses in other species of *Elysia* and different organisms, see the reviews by Trench (4, 6) and Taylor (7).

The sustained activity of algal chloroplasts in the absence of the algal nucleus in *E. chlorotica* suggests several possibilities regarding the synthesis or maintenance of chloroplast proteins. (*i*) It is possible that all plastid and nuclear-encoded chloroplast proteins are stable and do not turn over in the symbiotic plastids. (*ii*) Some of the algal nuclear genes may have been transferred to the sea slug nuclear genome during the initial stages of symbiotic evolution, thereby providing all the essential nuclear encoded polypeptides needed by the symbiont. (*iii*) The symbiotic plastids may have a high degree of genetic autonomy allowing them to maintain the functionality of the chloroplast despite the absence of the algal cell nucleus and cytoplasm. Molecular analysis of the symbiotic association is, therefore, essential in addressing these possibilities.

Although some biochemical studies of chloroplast function and structure have been conducted previously in *E. chlorotica* (1, 2, 12), molecular studies have lagged behind mainly due to difficulties in isolating DNA and RNA. The sea slugs produce enormous quantities of mucus that, when using standard protocols, copurifies with the nucleic acids making them

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Abbreviations: PSI, photosystem I; PSII, photosystem II; ER, endoplasmic reticulum; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; LS, large subunit.

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unsuitable for molecular analysis. Recently, Rumpho *et al.* (13) developed a method for isolating *E. chlorotica* DNA free of interfering mucus, making it possible to verify the presence of algal chloroplast genes in this animal host. Herein, we report both the presence and expression of several algal photosynthetic genes in the symbiotic plastids demonstrating active transcription and translation throughout their 8-month symbiotic association with *E. chlorotica*.

MATERIALS AND METHODS

Sea Slug and Algal Cultures. *E. chlorotica* (Gould) was collected in late October 1994 from an intertidal marsh on Martha's Vineyard Island, MA. The animals were maintained in aerated aquaria on a 14-hr photoperiod at 10°C. Twenty-five to 30 sea slugs were harvested every month for 8 months. Each sea slug was blotted dry, frozen immediately using liquid nitrogen, weighed, and stored at −80°C until used. *V. litorea* (C. Agardh) was maintained in culture using a modified f/2 medium in 25% artificial sea water as described elsewhere (12).

Electron Microscopy. Algal filaments and tissues from sea slugs (starved in laboratory aquaria for 2 or 8 months) were fixed in 2% paraformaldehyde/2% glutaraldehyde mixture in 25 mM sodium phosphate (pH 7.2) containing 0.5 M sucrose for 3 hr initially under a slight vacuum (25 torr; 1 torr = 133 Pa). Postfixation was carried out overnight on ice in 1% osmium tetroxide in sodium phosphate buffer with sucrose, followed by dehydration in a series of ethyl alcohol (10–100%) and 100% propylene oxide before embedding in Epon/araldite (14). Semi-thin sections (1 μm thick) and ultrathin sections (50–70 nm thick) were prepared (Ultrotome; LKB, type 4801A). The semi-thin sections were stained with toluidine blue. The ultrathin sections were poststained with uranyl acetate in alcohol for 30 min followed by Reynolds lead citrate for 10 min (15). Sections were examined and photographed using a transmission electron microscope (Zeiss 10C) at 60 kV and Kodak electron microscope film 4489 (ESTAR thick base).

DNA Isolation and Southern Blot Analysis. DNA was isolated and purified from individual sea slugs according to the protocol developed by Rumpho *et al.* (13). Algal total and chloroplast-enriched DNA were obtained as described by Manhart *et al.* (16). Digested DNA (0.2 μg per sample) was subjected to 0.8% agarose gel electrophoresis and blotted onto Zeta-Probe membranes (Bio-Rad) as described (13). Hybridization was carried out at 42°C for 24 hr using [α-^{32}P]dCTP-labeled randomly primed DNA probe. The radioactive probe bound to the membrane was visualized by autoradiography after a final wash in 0.2× standard saline citrate (SSC)/0.1% SDS for 30 min at 60°C. The following heterologous DNA probes were used: *psaB* (barley), *psbA* (barley), *psbD* (barley), 16S *rRNA* (barley), *rbcL* (*Chlamydomonas* sp.), *rbcS* (*Olisthodiscus luteus*), *atpB* (barley), and *fcp* (*Phaeodactylum tricornutum*).

RNA Isolation. Ten frozen sea slugs (750 to 1500 mg fresh weight) were ground in liquid nitrogen and the powder (200 mg) was homogenized in 600 μl of RNA extraction buffer [0.2 M sodium acetate, pH 5.0/1% SDS/0.01 M EDTA/heparin (0.5 mg/ml) in diethyl pyrocarbonate-treated water] and extracted with an equal volume of acid-phenol/chloroform mixture, 5:1 (vol/vol) and pH 4.7 (Ambion, Austin, TX) for 20 min with vigorous mixing. The phases were separated by centrifugation for 20 min at room temperature at 13,000 × *g* using a microcentrifuge. The phenol phase was reextracted with RNA isolation buffer, mixed for 20 min, and centrifuged as above. The aqueous phases were pooled and reextracted with phenol/chloroform/isoamyl alcohol mixture, 25:24:1 (vol/vol) and pH 8.0, for 20 min. After extracting the aqueous phase a third time with chloroform/isoamyl alcohol, 24:1 (vol/vol), total RNA was precipitated overnight at 4°C by the addition of LiCl to a final concentration of 2.5 M. Total RNA was collected by centrifuging at 12,000 × *g* for 20 min at 4°C, the resultant pellet was rinsed once with 2.5 M LiCl, twice with 70% ice-cold ethanol, and dried for 15 min using a Speed-Vac. Finally, the dried pellet was resuspended in diethyl pyrocarbonate-treated water, quantified spectrophotometrically, separated as 10-μg aliquots of total RNA, and stored at −80°C until used.

Northern Blot Analysis and Quantitation of Hybridization Signals. Total RNA (10 μg) was separated by electrophoresis on a 1% agarose gel containing 6% formaldehyde in 50 mM Hepes/1 mM EDTA (pH 7.8), as recommended (17). Ethidium bromide (0.12 μg/μl) was added directly to each sample to verify RNA concentrations after electrophoresis (18). Separated RNA was blotted by capillary transfer onto Zeta-Probe (Bio-Rad) membrane for 18–24 hr and then UV-cross-linked for 1 min. Prehybridization, hybridization with [α-^{32}P]dCTP-labeled randomly primed DNA probe, washings, and autoradiography were performed as described (13). Heterologous DNA probes from barley were used for detecting *psbA* and 16S *rRNA* transcripts. The relative amounts of hybridization were estimated by using a Fuji Bioimaging Analyzer BAS 2000 (Fuji) and the units of radioactivity reported in units of photostimulated luminescence (PSL).

Isolation of Thylakoid Membrane-Enriched Proteins. Five frozen sea slugs (≈375 mg fresh weight) from each month's collection were combined and powdered under liquid nitrogen. Thylakoid membrane-enriched proteins were isolated essentially as described by Russell *et al.* (19). The powder (200 mg) was homogenized in a microfuge tube with ice-cold isolation buffer (0.33 M sorbitol/25 mM Hepes·KOH, pH 7.0/5 mM $MgCl_2$/10 mM NaCl/100 mM *N*-acetyl-L-cysteine/1 mM phenylmethylsulfonyl flouride), and the thylakoid membranes were centrifuged down in a microcentrifuge at 10,000 × *g* for 5 min. The pellet was washed once in isolation buffer lacking sorbitol and *N*-acetyl-L-cysteine, and the thylakoid-membrane associated proteins were then solubilized by resuspending the pellet in isolation buffer containing 1% Triton X-100 with continuous shaking for 1 hr at 4°C. Total protein was determined using the Bio-Rad protein stain with BSA as the standard.

Western Blot Analysis and Quantitation of Immunoblots. Total proteins were separated by preparative SDS/PAGE on 12.5% gels as described by Laemmli (20). After electrophoresis, the gel was stained with Coomassie brilliant blue R250 or the proteins transferred to nitrocellulose membrane (Schleicher & Schuell) by electroblotting for 20 hr at 15°C and 30 V using the methods of Towbin *et al.* (21). The nitrocellulose membrane was immunostained using the alkaline phosphatase system as recommended by the manufacturer (Promega). Polyclonal antisera raised in rabbit against the following proteins were used as the primary antibodies: D1, D2, and CP43 proteins of barley; large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco LS) of spinach; ATPase-β of *Euglena* sp.; and fucoxanthin, chlorophyll a/c protein complex (fcpc) of the chromophyte *Pavlova gyrans*. The relative amounts of D1, D2, and CP43 proteins on the immunoblots were estimated by digitizing the images on the blot using ScanJet (Hewlett–Packard) attached to an IBM personal computer and quantifying their optical density using the SIGMAGEL (Jandel, San Rafael, CA) program.

***In Vivo* Labeling with [^{35}S]Methionine.** Six sea slugs that had been starved for 3 or 8 months (≈450 mg fresh weight) were placed in 2 ml of sterile artificial sea water containing [^{35}S]methionine (50 μCi/ml; ICN, Trans[^{35}S]methionine; 1 Ci = 37 GBq) in glass scintillation vials under intense light (40-W GE soft white incandescent lamp) at 25°C for 6 hr. After incubation, the sea slugs were rinsed with artificial sea water, blotted dry, frozen immediately with liquid nitrogen, and powdered. Thylakoid membrane-enriched proteins were isolated as above and an aliquot was immunoprecipitated with D1 antibody (22). An aliquot of total proteins (10 μg) and the immunoprecipi-

tated D1 protein sample were subjected to Western blot analysis and fluorography. Alternatively, the separated proteins were fixed in the SDS/PAGE gel for 1 hr in 20% ethanol/20% methanol/10% glycerol/7% acetic acid followed by washing in 2,5-diphenyloxazole/xylene mixture for 1 hr (22). The gel was then rinsed briefly, washed twice with deionized water for 20 min, dried on Whatman paper, and subjected to fluorography at −80°C for 5 days.

RESULTS

Ultrastructure of Chloroplasts in *V. litorea* and *E. chlorotica*. Cultured *V. litorea*, a coenocytic moderately branching yellowish-green alga (23), is shown in Fig. 1*A*. The ultrastructure of chloroplasts from laboratory grown algae is characterized by thylakoids arranged in parallel groups of three, large pyrenoids, the absence of starch granules, and the presence of numerous plastoglobuli and large lipid deposits (Fig. 1*D*). Moreover, at least two layers of ER usually surround the chloroplasts (chloroplast ER), a characteristic found in several chromophytic alga (23). Adult *E. chlorotica* appear bright green (Fig. 1*B*) due to the presence of *V. litorea* chloroplasts in the cells of the digestive diverticula (1, 3). At least 2 months after acquisition, the incorporated chloroplasts appear ultrastructurally intact (Fig. 1*E*). Pyrenoids are present in the chloroplasts as well as several small plastoglobuli and large lipid deposits (Fig. 1*E*). However, the chloroplast ER present in the algal cell was absent from the chloroplasts of the molluscan cytoplasm (Fig. 1*E*). By 8 months of starvation, *E. chlorotica* was less green and more yellowish (Fig. 1*C*), yet the majority of the chloroplasts appeared intact and maintained their fine structure (Fig. 1*F*). The trilayered thylakoids still traversed large pyrenoids and small plastoglobuli were present.

Algal Chloroplast Genes are Present in *E. chlorotica*. Southern blot analysis using heterologous probes indicated the presence of genes for the B subunit of photosystem I (PSI) reaction center (*psaB*), the D1 protein of photosystem II (PSII) reaction center (*psbA*), the chloroplast 16S ribosomal RNA (16S *rRNA*), and the small subunit of Rubisco (*rbcS*) in DNA isolated from *E. chlorotica*. The restriction enzyme digestion patterns of the four genes were identical when DNA isolated from *E. chlorotica* was compared with that of chloroplast-enriched DNA from *V. litorea* (Fig. 2).

The *rbcL* gene, which encodes Rubisco LS, was also detected by Southern blot hybridization of *E. chlorotica* DNA. The DNA patterns after digestion with a number of restriction enzymes are shown in Fig. 3*A*. Western blot analysis indicated the presence of the Rubisco LS protein (55 kDa) in the sea slug after a 2-month starvation (Fig. 3*B*). Southern blot hybridization using heterologous probes to the chloroplast gene, *atpB* (barley) or the nuclear gene *fcp* (diatom) yielded no hybridization signals with either *E. chlorotica* or *V. litorea* total DNA most likely due to the low homology of the heterologous probes (data not shown). However, immunoblot analysis indicated the presence of a 51-kDa and a 19-kDa polypeptide using antisera to barley ATPase-β and diatom fcpc, respectively (Fig. 3 *C* and *D*).

Expression of PSII Reaction Center and 16S *rRNA* Genes During Symbiosis. Northern blot analysis indicated that the steady-state levels of *psbA* transcripts were high in sea slugs kept from 2 to 4 months of starvation and began to decline after 4 months of starvation (Fig. 4*A*). In contrast, the steady-state levels of 16S *rRNA* remained relatively constant throughout the entire starvation period (Fig. 4*B*). Transcription of *psbA* could be inhibited by incubating sea slugs (starved for 2 months) with 3 mM 6-methylpurine for 12 hr. Transcript levels of *psbA* decreased to about 50% (data not shown) indicating active transcription of this chloroplast gene.

The steady-state levels of D1, the protein product encoded by *psbA*, were high at 2 and 4 months and slowly declined after 6 months. The drop at 3 months was consistently observed, but its cause is unknown (Fig. 5*A*). Over the entire time course, the D1 protein levels were maintained at a relatively higher level than the transcripts (Fig. 4*A*). Unlike D1, the steady-state levels of D2, the protein product encoded by *psbD*, were high in sea slugs at 2 and 4 months of starvation but were low in samples starved for 3 and 6–8 months (Fig. 5*B*). The immunoblot pattern for CP43, the protein product encoded by *psbC*, resembled that of D1, indicating high levels up to 4 months before declining to a lower steady-state level from 6 to 8

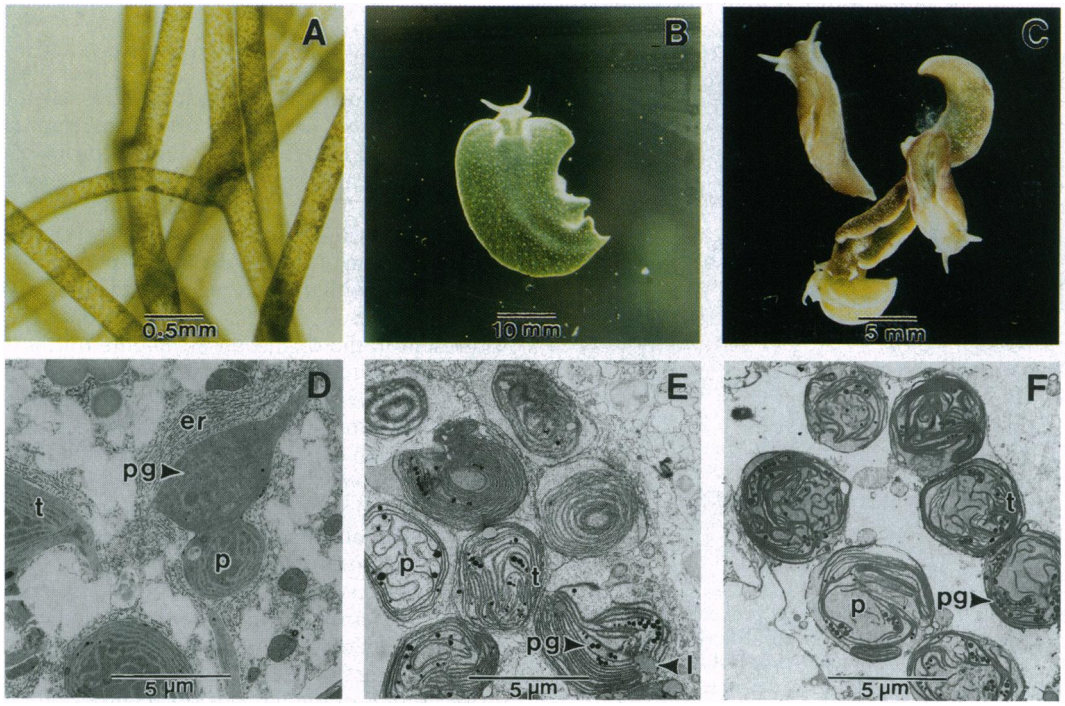

FIG. 1. Representative specimens of *V. litorea* (*A*) and *E. chlorotica* starved of algal food source for 2 months (*B*) and 8 months (*C*) and electron micrographs of their corresponding chloroplasts (*D–F*). er, Endoplasmic reticulum (ER); l, lipid deposit; p, pyrenoid; pg, plastoglobuli; t, thylakoid.

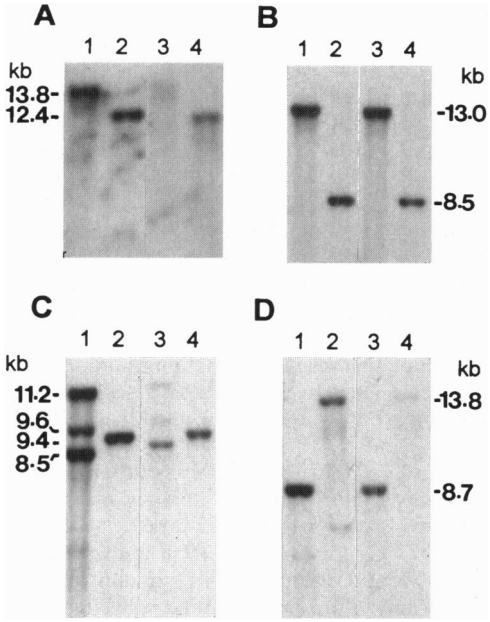

FIG. 2. Southern blot of *Eco*RV (lanes 1 and 3)- and *Bgl*II (lanes 2 and 4)-digested DNA from *E. chlorotica* (lanes 1 and 2) and chloroplast-enriched DNA from *V. litorea* (lanes 3 and 4) probed for *psaB* (*A*), *psbA* (*B*), and 16S *rRNA* (*C*) and *rbc*S (*D*). Each lane contained 200 ng of DNA. Fragment sizes in kb were determined by coelectrophoresis of phage λ DNA fragments produced by digestion with *Hin*dIII (Life Technologies, Gaithersburg, MD).

months (Fig. 5*C*). In contrast to D1, the level of CP43 protein at 3 months did not decline.

Synthesis of Thylakoid Membrane-Enriched Proteins in *E. chlorotica*. The profiles of *de novo*-synthesized thylakoid membrane-enriched proteins isolated from 3- and 8-month-starved sea slugs after labeling with [^{35}S]methionine are presented in Fig. 6*A* and *B*, respectively. In 3-month-starved sea slugs, three major proteins with molecular masses of about 110, 82, and 48 kDa (indicated by arrows in Fig. 6*A*) were synthesized. In contrast, only one major polypeptide of about 68 kDa was synthesized in 8-month-starved sea slugs (Fig. 6*B*). Several minor polypeptides in the molecular mass range of 19–217 kDa were synthesized at both stages of sea slug development.

Synthesis of the D1 protein was investigated from both 3- and 8-month-starved sea slugs. A labeled D1 protein was immunoprecipitated from 3-month-starved sea slugs as indicated in the autoradiograph after SDS/PAGE (Fig. 7*A*).

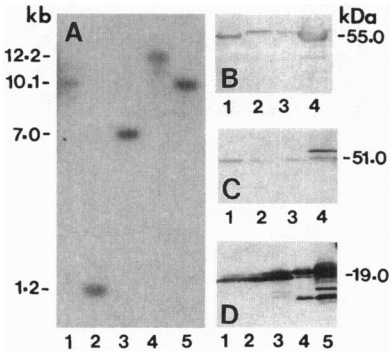

FIG. 3. Digestion patterns of DNA (100 ng) probed for *rbcL* after incubation with *Sac*I (lane 1), *Pst*I (lane 2), *Hin*dIII (lane 3), *Eco*RI (lane 4), and *Cla*I (lane 5) (*A*) and immunoblots of Rubisco LS (*B*) and ATPase-β (*C*) from *V. litorea* (lane 1), *E. chlorotica* (lane 2), *E. chlorotica* chloroplasts (lane 3), and spinach (lane 4); and fcpc polypeptide (*D*) from *E. chlorotica* (lanes 1 to 3), *E. chlorotica* chloroplasts (lane 4), and *V. litorea* thylakoids (lane 5).

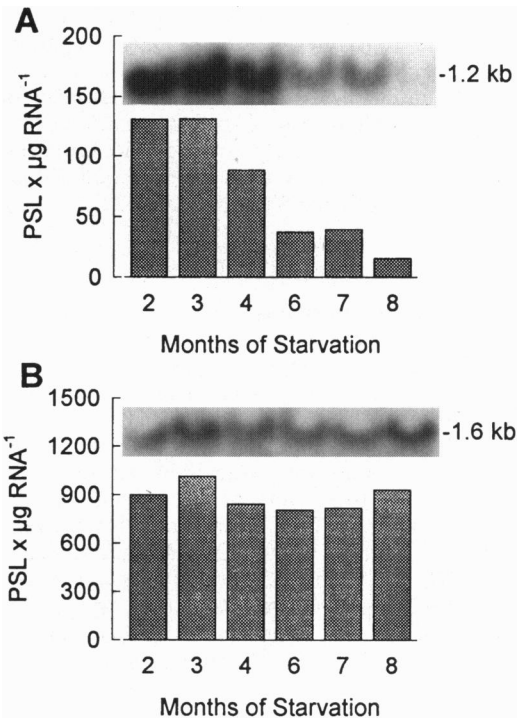

FIG. 4. Steady-state levels of *psbA* (*A*) and 16S *rRNA* (*B*) transcripts in *E. chlorotica* during the 8-month symbiotic association. Total RNA (10 µg) was subjected to formaldehyde/agarose gel electrophoresis and Northern blot analysis. The relative amounts of hybridization were estimated using a Fuji Bioimaging Analyzer BAS 2000. PSL, photostimulated luminescence.

However, synthesis of the D1 polypeptide may be very low in 8-month-starved sea slugs since no autoradiographic signal was detected from this stage. Western blot analysis of the immunoprecipitated sample and an aliquot of the crude extract indicated the presence of only unlabeled D1 proteins (Fig. 7 *B* and *C*). A low rate of D1 protein synthesis was expected since the level of *psbA* transcripts was lowest at this stage.

DISCUSSION

We have found direct molecular evidence for the presence (i.e., *psaB*, *psbA*, 16S *rRNA*, *rbcL*, and *rbcS*) and expression (i.e., *psbA* and 16S *rRNA*) of several chloroplast-encoded genes in symbiotic chloroplasts of *E. chlorotica* after several months of starvation. The protein products of some of these chloroplast genes, including D1, D2, and CP43 were detected over the entire duration of the intracellular symbiosis. This time period corresponds with the life span of *E. chlorotica* in nature and in the laboratory (1, 3). Thus, an operational genetic apparatus is present in the organellar symbionts within the animal cell. The ability of the symbiotic plastids to carry out transcriptional and translational functions helps explain their capacity for maintaining long-term photosynthetic activity. For instance, the expression of *psbA* and the synthesis of D1 (12), D2, CP43, and several other thylakoid membrane proteins ensures the successful reassembly and functionality of the PSII reaction center particularly in the earlier (1–4 months) stages of the symbiotic association.

It is interesting to note that the most prominent newly synthesized proteins in 8-month-starved sea slugs have a molecular mass of about 68 kDa. Preliminary Western blot analysis suggests that these proteins may be the products of *psaA* or *psaB* of the PSI reaction center (data not shown) and that PSI may be the predominant photosystem in older slugs (>4 months). This assumption is also supported by data

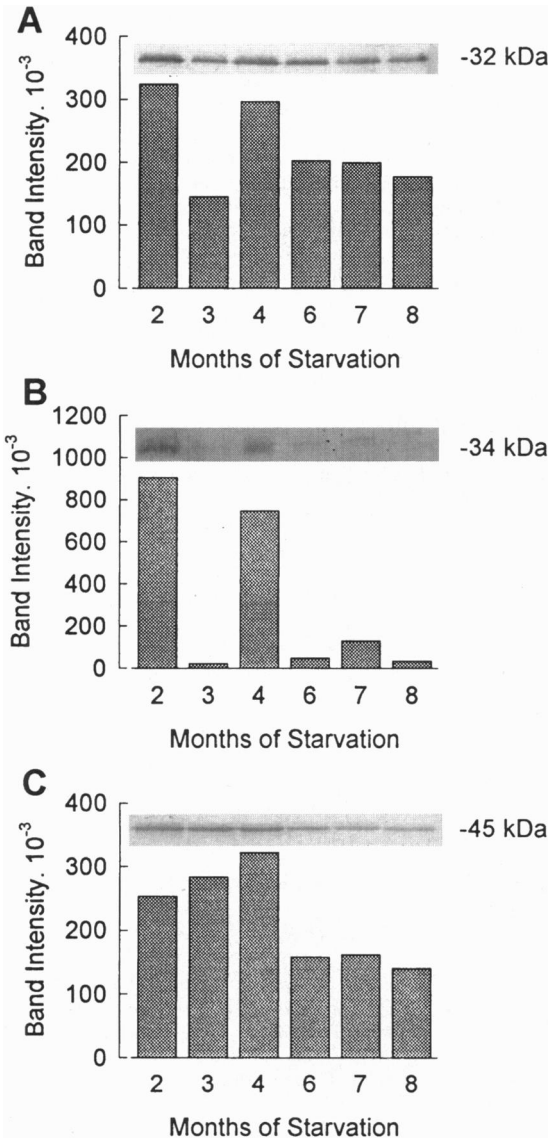

FIG. 5. Immunoblots of D1 (*A*), D2 (*B*), and CP43 (*C*) PSII reaction center proteins from *E. chlorotica* during the 8-month symbiotic association. Thylakoid membrane-enriched proteins (20 μg of total protein) were subjected to SDS/PAGE and Western blot analysis. Images on each immunoblot were digitized using the ScanJet-IBM personal computer and the relative amounts of each protein were estimated using the SIGMAGEL program.

presented herein showing the levels of PSII protein, D2 are very low beyond 4 months of starvation, suggesting an inefficient or nonfunctional PSII with age. If this is the case, then cyclic phosphorylation, which uses PSI and generates only ATP and no NADPH, may be a major source of metabolic energy at the later stages of chloroplast symbiosis. Alternatively, the activity of either PSI or PSII may also be regulated depending on the need for ATP or NADPH.

The genes encoding the two subunits of the major carboxylating enzyme Rubisco were also detected by Southern blot hybridization of *E. chlorotica* total DNA. The gene for the large subunit (*rbcL*) had never been found outside the plastid genome in any eukaryotic photosynthetic organism until the recently reported nuclear-encoded form II of Rubisco in dinoflagellates (24, 25). In all of the land plants and green algae examined, *rbcS* has only been detected in the nuclear genome (26). Our results suggest that *rbcS* is plastid localized in *V. litorea*, consistent with its location in other nongreen algae (27–29). Among several rhodophytes and chromophytes, sequence data of the plastid genomes have indicated that *rbcS* is plastid-encoded in these algal groups (29).

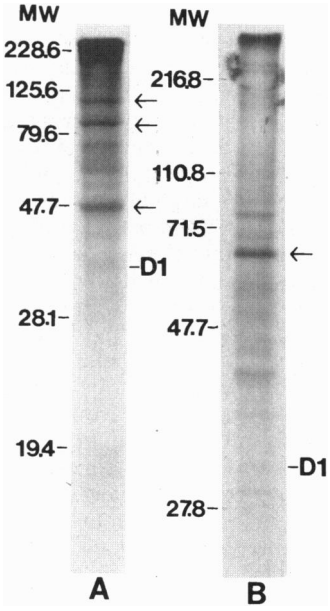

FIG. 6. SDS/PAGE profiles of newly synthesized thylakoid membrane-enriched proteins from 3-month (*A*) and 8-month (*B*) starved *E. chlorotica* labeled with [^{35}S]methionine for 6 hr. Major proteins are indicated by arrows. MW, molecular mass in kDa of standard proteins used (Life Technologies).

The endosymbiotic theory of organellar origins suggests that chloroplasts arose from an endosymbiosis of once free-living photosynthetic bacteria with a subsequent incorporation of a considerable part of the bacterial genome into the host's nuclear genome (30, 31). The degree of gene transfer or loss of genetic material from the endosymbiont varies considerably, especially when comparing land plants and green algae to the nongreen algae (28). However, in all cases, compartmentation of the genetic material has resulted in a tightly coordinated interaction between chloroplasts and the nucleocytoplasm to regulate gene expression and biochemical activity (32). This interaction is essential because several chloroplast proteins are encoded by the nuclear genome and subsequently imported posttranslationally into the chloroplast (33). In addition, the synthesis of many proteins encoded by the chloroplast genome is controlled by regulatory factors translated in the cytosol in response to an external cue. Such nuclear-encoded regulatory signals are transported into chloroplasts where they act to control the transcription and translation of several chloroplast encoded light-harvesting components, including D1 and D2 proteins (32). This highly regulated coordination between the plant nucleocytoplasm and the chloroplasts is obviously disrupted when *V. litorea* chloroplasts are incorporated into the cytosol of *E. chlorotica*. Nevertheless, *E. chlorotica* continues

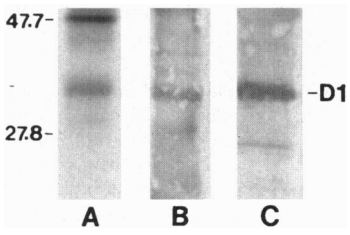

FIG. 7. Autoradiograph (*A*) and Western blot (*B*) of immunoprecipitated D1 proteins from 3- and 8-month-starved sea slugs, respectively, following labeling with [^{35}S]methionine for 6 hr. (*C*) Immunoblot of D1 protein from crude extract of 8-month-starved and [^{35}S]methionine-labeled sea slugs (control).

to photosynthesize for several months in the absence of the algal nucleus and cytoplasm (1, 3). Further, *de novo* transcription and translation of the light harvesting components in the symbiotic chloroplasts occur over a period of 8 months. Thus, in the absence of the regulatory signals from the plant cell, the synthesis and targeting of cytosolic proteins to the chloroplast and the regulation of chloroplast gene expression must either be taken over by the sea slug nucleus and cytosol or totally contained within the symbiotic plastid genome.

It has been estimated that between 80 and 90% of proteins specific to mitochondria and plastids, respectively, are encoded in the nucleus of the plant cell (34). In this respect, the algal cell is genetically different from the host cell (i.e., during intracellular symbiosis) since the genes essential to the function of the organelles are absent in the nucleus of the animal cell. Whether this genetic difference is true for long-term chloroplast symbiosis found in *E. chlorotica* should be further investigated. It is possible that in a more advanced level of intracellular symbiosis, essential algal genes encoding organellar proteins have found residence in the animal host genome. Their presence results in the formation of a new organism equipped with novel metabolic functions, such as the ability to carry out photosynthesis and to utilize the photosynthate for its own survival. This scenario may have occurred early in the establishment of chloroplast symbiosis, when algal nuclear genes encoding organellar proteins were directly integrated into the animal nuclear genome after direct feeding on the alga by the animal. After these gene transfer events, the successful accomodation and survival of the once-foreign plastid was eventually tolerated inside the animal cell in the absence of the algal cell nucleus and cytoplasm. If these events have taken place, it should be possible to detect the presence of algal nuclear genes encoding chloroplast proteins in the sea slug nuclear genome.

Alternatively, since it is now generally accepted that chromophytic plastids are products of two successive symbioses (35, 36), a functional nucleocytoplasm could exist between the chloroplast envelope and chloroplast ER. Although considerably reduced, this vestigial nucleocytoplasm could provide all the factors required for maintaining long-term survival of the chromophytic chloroplasts, especially during the absence of the algal cell nucleus and cytoplasm. However, our ultrastructural observations indicate that the chloroplast ER is not retained in the host cytosol and no evidence of a vestigial nucleus or nucleomorph-type structure has been observed in sections of *V. litorea* or *E. chlorotica*. More likely is the possibility that *V. litorea* plastids have attained an unusual degree of genetic autonomy. This could be brought about by a greater gene-coding capacity of the plastid genome and the presence of a set of genes essential for maintaining control of plastid gene expression independent of the control of the nuclear genome. These are primitive characteristics relative to the present day chloroplast genomes of land plants but have been reported for the red alga *Porphyrum purpurea* (28). Further mapping and sequencing studies of the *V. litorea* chloroplast genome should determine if unique genes are present, thereby leading to a higher degree of genetic autonomy of the symbiotic chloroplasts.

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