Synthesis and degradation of dinoflagellate plastid-encoded psbA proteins are light-regulated, not circadian-regulated

Yunling Wang*, Lene Jensen†, Peter Højrup†, and David Morse*‡

*Department of Biological Science, University of Montreal, 4101 Sherbrooke est, Montreal, QC, Canada H1X 2B2; and †Department of Biochemistry, University of Southern Denmark, Campusvej 55, DK-5230 Odense, Denmark

Edited by J. Woodland Hastings, Harvard University, Cambridge, MA, and approved January 11, 2005 (received for review September 3, 2004)

In many dinoflagellate species, the plastid genome has been proposed to exist as a limited number of single-gene minicircles, and many genes normally found in the plastid genome are nuclear-encoded. Unlike the nuclear-encoded plastid-directed gene products whose expression is often regulated by the circadian clock, little is known about expression of minicircle genes. Furthermore, even the plastid location of the minicircles has recently been challenged. We have examined the incorporation *in vivo* of [^{35}S]methionine into the proteins of purified plastids, and we find that several plastid proteins are labeled in the presence of cycloheximide but not chloramphenicol. One of these proteins, labeled in two different dinoflagellate species, was identified as psbA by Western blot analysis. Furthermore, this psbA has the expected physiological characteristics, because both synthesis and degradation are induced by light. We find no evidence for circadian control over either synthesis or degradation of psbA, unlike the several nuclear-encoded plastid-directed proteins studied. Finally, we find that levels of psbA protein or RNA do not change over a 24-h light-dark cycle, suggesting that this protein may not be involved in mediating the circadian rhythm in oxygen evolution rates. This demonstration is the first, to our knowledge, that minicircle genes encoding plastid proteins are translated in dinoflagellate plastids, and it suggests that a proteomic approach to characterizing the dinoflagellate plastid genome is feasible.

gene expression | plastid genome | *Gonyaulax* | photosynthesis

The majority of photosynthetic dinoflagellates harbor plastids that are surrounded by three membranes (1), rather than the more typically found two or four membranes, suggesting that these plastids are derived from a secondary endosymbiotic event followed by loss of a single membrane. However, the identity of the endosymbiont that gave rise to the plastids is not firmly established and, based on the identity of nuclear-encoded plastid-directed proteins, may have had some unusual characteristics. For example, neither the unusual light-harvesting protein peridinin-chlorophyll a protein (PCP) (2) nor the allenic oxicarotenoid peridinin to which it binds is found in other organisms (3). Furthermore, the peridinin-type plastids contain a form II ribulose-1,5-bisphosphate carboxylase/oxygenase, previously observed only in some species of anaerobic proteobacteria (4, 5).

Clearly, the true dinoflagellate plastid phylogeny is more likely to be revealed by using analysis of plastid-encoded genes. However, many proteins usually encoded by the plastid genome are found as nuclear-encoded genes in dinoflagellates (6–8), suggesting that the organelle genome is highly reduced. The best candidates for the plastid-encoded genes include the 16S and 23S rRNA genes, eight photosystem components (*psaA-B*, *psbA-E*, and *psbI*), two ATP synthase subunits (*atpA-B*), two cytochrome b_6f-subunits (*petB* and *petD*), two unidentified ORFs (*ycf16* and *ycf24*), and two ribosomal proteins (*rpl28* and *rpl23*). These genes form the most reduced set of plastid genes known (with the exception of the Apicomplexan plastids). Furthermore, in a feature unique among plastid genomes, they are all encoded by small single-gene minicircles (7, 9). One question of major biological interest is how and why dinoflagellates have evolved and maintained this reduced set of plastid genes.

If the minicircles are to be considered as a novel form of a true plastid genome, a second and more fundamental question is whether the minicircle genes are indeed expressed in the plastid. To date, although transcripts derived from minicircles have been observed, there is no direct experimental evidence to demonstrate their translation. Furthermore, whereas psbA transcripts have been observed in the plastids of *Symbiodinium* by *in situ* hybridization (10), recent cell fractionation studies using the dinoflagellate *Ceratium* showed that minicircle DNAs were located in the nucleus (11). Because none of the minicircle genes sequenced to date encodes the characteristic leader sequence believed to be required for targeting to the triple membrane-bound plastids (12), a nuclear location for the minicircles would preclude them from encoding functional plastid proteins. An elegant and conclusive resolution to issues of both expression and location of the minicircles can be obtained by determining the antibiotic sensitivity of protein synthesis derived from the minicircle genes.

We show here that *psbA* is one of several plastid proteins synthesized in the presence of cycloheximide by two different dinoflagellate species, *Amphidinium* and *Gonyaulax*. We also find that, in *Gonyaulax*, the regulation of psbA synthesis and degradation is light-regulated, as has been observed in higher plants. Our results provide compelling evidence for placing the *psbA* gene in the plastids of dinoflagellates and for ruling out circadian control of psbA levels. Dinoflagellate plastid-encoded proteins may be unlike the nuclear-encoded plastid-directed gene products, whose synthesis is often clock-controlled (13–15).

Materials and Methods

Cell Culture. *Amphidinium carterae* [Culture Center for Marine Phytoplankton (CCMP) no. 1314] and *Gonyaulax polyedra* (now *Lingulodinium polyedrum*; CCMP no. 1936) were obtained from the Provasoli–Guillard Culture Center for Marine Phytoplankton (Boothbay Harbor, ME) and grown in a modified seawater medium (f/2) (16) at a constant temperature (18 ± 1°C) in 12-h light/12-h dark cycles by using cool white fluorescent light at an intensity of 50 μmol of photons·m^{-2}·s^{-1}. The beginning of the light period in the light-dark (LD) cycle is defined as time 0 (LD 0), and the beginning of the dark period is defined as LD 12. Cultures were grown to a cell density of 12–14,000 cells per ml (*Gonyaulax*) or 500,000 cells per ml (*Amphidinium*) as measured

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PCP, peridinin-chlorophyll a protein; LD, light-dark; CT, circadian time; LDS, lithium dodecyl sulfate; OEE1, oxygen-evolving enhancer protein subunit 1; IEF, isoelectric focusing; LHCP, light-harvesting chlorophyll-binding protein.

‡To whom correspondence should be addressed. E-mail: david.morse@umontreal.ca.

www.pnas.org/cgi/doi/10.1073/pnas.0406522102

by counting using a hemocytometer. Both cultures were unialgal, but only *Amphidinium* is axenic.

Plastid Isolation Protocols. Cells from 4 liters of culture medium were harvested by filtration, washed extensively with seawater to remove bacterial contaminants, resuspended in 10 ml of isolation buffer (0.626 M sorbitol/0.1 M KCl/0.1 M Hepes-NaOH, pH 7.2/20 mM 2-mercaptoethanol/0.1% BSA) containing 0.05% saponin (ICN), and stirred vigorously for 15 min at room temperature. This stirred homogenate was centrifuged at 150 × *g* for 3 min to remove unbroken cells, and the supernatant was centrifuged at 1,500 × *g* for 3 min to produce a plastid-containing pellet. Plastids were purified from these crude preparations by density gradient centrifugation on a 15-ml 10–30% linear Percoll (Amersham Pharmacia Biotech, Piscataway, NJ) gradient in isolation buffer. Percoll was removed from the plastid preparation by repeated dilution with three volumes of cold isolation buffer without BSA followed by centrifugation at 1,500 × *g* for 15 min at 4°C.

Electrophoretic Analysis. Samples for 2D electrophoresis were prepared from cells broken in a bead beater with 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) by overnight precipitation at −20°C after addition of three volumes of acetone. Typically, 600 μg of protein redissolved in 250 μl of rehydration buffer [7 M urea/2 M thiourea/4% CHAPS/0.02 M DTT/0.5%, pH 4–7/immobilized pH gradient buffer (Amersham Pharmacia Biotech)] was applied to an isoelectric focusing (IEF) strip (Amersham Pharmacia Biotech) and electrophoresed at 500 V for 1 h, at 1,000 V for 1 h, and at 8,000 V for 6 h. After IEF, the strip was equilibrated for 15 min in 10 ml of SDS equilibration buffer (50 mM Tris·HCl, pH 8.8/6 M urea/30% glycerol/2% SDS/10 mg/ml DTT/Bromophenol blue) and for 15 min in 10 ml of SDS equilibration buffer containing 250 mg of iodoacetamide and electrophoresed on a standard 10% polyacrylamide gel.

The 1D lithium dodecyl sulfate (LDS) gels were run after substitution of SDS with LDS in all buffers. Plastid pellets were dissolved in two volumes of LDS sample buffer (100 mM Tris·HCl, pH 6.8/2% LDS/50 mM DTT/10% glycerol) at room temperature for 1 h, and 10 μl was electrophoresed on 10% polyacrylamide gels before electrophoretic transfer to nitrocellulose.

For Western analyses, all gels were transferred electrophoretically to nitrocellulose membranes. The protein blots were blocked with 5% powdered milk in Tris-buffered saline containing 0.05% Tween 20 before incubation with either a 1:5,000 dilution of chicken anti-psbA (Agri-Sera) or a 1:5,000 dilution of rabbit anti-PCP (17) to control for protein loading. Antibody binding was visualized by using a 1:10,000 dilution of commercial peroxidase-linked secondary antibodies and chemiluminescence (Amersham Pharmacia Biotech).

***In Vivo* Labeling.** Exponentially growing cells were treated with 100 μM chloramphenicol or 100 μM cycloheximide for 1–12 h before the start of labeling to ensure inhibition of protein synthesis on 70S or 80S ribosomes, respectively. At a time corresponding to the start of the light period, 1 liter of cells was concentrated to ≈10 ml by filtration by using a 20-μm nylon filter and incubated with 2 mCi (1 Ci = 37 GBq) of [^{35}S]methionine (>1,000 Ci/mmol; ICN) for 40 min. After labeling, the cell suspension was repeatedly washed to remove unincorporated radiolabel, and the plastids were purified as above. Plastid proteins were then extracted and analyzed by electrophoresis and autoradiography. For labeling by using high light, cells were exposed to a sodium arc lamp light (≈2,500 μmol of photons·m^{-2}·s^{-1}) passed through 1 cm of cold flowing water to filter out the infrared wavelengths. Cell samples did not increase in temperature during the labeling period.

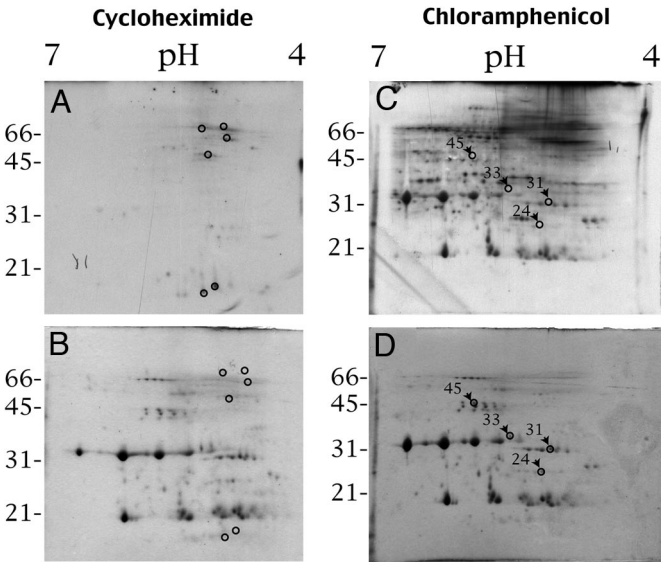

Fig. 1. The 2D electrophoresis of *in vivo*-labeled plastid proteins identifies only nuclear-encoded gene products. Proteins (0.6 mg) extracted from plastids purified after *in vivo* labeling with [^{35}S]methionine in the presence of 100 μM cycloheximide (*A* and *B*) or 100 μM chloroamphenicol (*C* and *D*) were electrophoresed on 2D gels. (*A* and *C*) Autoradiographs. (*B* and *D*) Coomassie blue-stained gels. Molecular mass markers (kDa) are shown on the left, and pH extremes (linear gradient) are shown at the top of each gel. Circles represent potential plastid-encoded proteins.

Protein Microsequence. Sequencing of tryptic fragments prepared from LDS/PAGE gel-purified proteins (Fig. 6, which is published as supporting information on the PNAS web site) was performed essentially as described (refs. 18 and 19 and *Supporting Methods*, which is published as supporting information on the PNAS web site). MS analysis was performed on an ABI Voyager Elite operated in the positive reflector mode, and MS/MS analysis was performed on a Micromass QTOF MALDI instrument.

Sequencing of tryptic fragments prepared from 2D gel-purified proteins was performed by the Harvard Microchemistry Facility (Cambridge, MA), using microcapillary reverse-phase HPLC ion-electrospray tandem MS on a Finnigan LCQ DECA XP Plus quadruple ion-trap mass spectrometer.

Nucleic Acid Analysis. PCR using oligonucleotides (5′-TCAGCTTTTATTTTGCTCCG-3′ and 5′-CCTGATGAATCAATTATGGACTG-3′), based on GenBank accession no. AB025588, were designed to amplify a psbA sequence, and our sequence was identical to that reported. The sequence was analyzed by using MACVECTOR software (Accelrys). The techniques for extraction and hybridization of nucleic acids were performed as described (20).

Results

To identify plastid-encoded proteins in *Gonyaulax*, we first examined purified plastids for proteins that incorporated [^{35}S]methionine *in vivo* in the presence of cycloheximide. A first series of experiments was designed to examine plastid proteins globally by using 2D gel analysis. However, none of the proteins incorporating radiolabeled methionine in the presence of cycloheximide (circled proteins in Fig. 1*A*) was present in levels appropriate for microsequencing (Fig. 1*B*). This finding suggested that either the plastid proteins in these prepara-

Table 1. Plastid proteins identified by microsequencing from 2D PAGE

Protein identification no.		No. of MS/MS spectra	No. of amino acids	Predicted molecular mass, kDa
P45	BSA	66	311*	41*
P33	*Heterocapsa* OEE1	10	91	33
P31	*Gonyaulax* PCP	20	164	32
P25	*Amphidinium* LHP	5	38	19

*Recovered from the C-terminal 359 amino acids.

tions did not incorporate significant levels of methionine or only nuclear-encoded proteins were extracted by using this procedure.

To address the possibility that labeling of dinoflagellate plastid proteins was inefficient *in vivo*, a complementary series of experiments was performed to identify proteins whose synthesis was blocked by chloramphenicol. The majority of plastid proteins observed on the gels did incorporate radiolabel in the presence of chloramphenicol and act as a complement to the cycloheximide inhibition (Fig. 1 *C* and *D*). However, the four circled proteins were not labeled during this experiment. These were isolated from preparative gels and identified by microsequencing (Table 1). The protein at 45 kDa was identified as BSA and presumably corresponds to a proteolytic fragment of the protein added to stabilize the plastids during the purification, because all of the sequences obtained were derived from the C-terminal two-thirds of the protein. The other three proteins were identified as oxygen-evolving enhancer protein subunit 1 (OEE1) (p33, similar to OEE1 of the dinoflagellate *Heterocapsa*), PCP p31, and light-harvesting polyprotein (LHP) precursor (p24, similar to LHP from the dinoflagellate *Amphidinium*). The molecular masses of the proteins on SDS/PAGE agree with those predicted from the sequence except for LHP, which is larger than the 19 kDa predicted from the *Amphidinium* sequence.

PCP and OEE1 have been previously identified in a screen for proteins whose synthesis is under circadian control in *Gonyaulax* (13, 21). It thus seemed possible that little radiolabel was incorporated into these proteins because the time of *in vivo* labeling was inappropriate, rather than because their synthesis was inhibited by chloramphenicol. In agreement with this hypothesis, when the *in vivo* labeling protocol was repeated at midday, PCP, OEE1, and light-harvesting chlorophyll-binding protein (LHCP) were observed to incorporate radiolabel (data not shown and Fig. 3). These proteins are thus indeed nuclear-encoded.

The absence of minicircle-encoded proteins in this global screen was surprising. However, because many of the minicircle genes encoding plastid proteins should produce hydrophobic thylakoid membrane components, it was possible their extraction in the buffers used for isoelectric focusing was inefficient. We therefore tested a protocol employing room temperature LDS extractions that can be used for electrophoresis of hydrophobic proteins on 1D gels. Under these conditions, several proteins in the purified plastids were observed to incorporate radiolabel in the presence of cycloheximide (Fig. 2*A*). In particular, a band at 34 kDa is particularly notable (arrow) for the amount of label incorporated. Of all of the thylakoid proteins, the psbA is expected to have the highest turnover rate (22), suggesting that the highly labeled protein at 34 kDa might be psbA. To test this hypothesis, a commercial anti-psbA was used to determine the electrophoretic mobility of psbA in our conditions. This antibody identifies psbA as a protein of 34 kDa in whole-cell extracts of

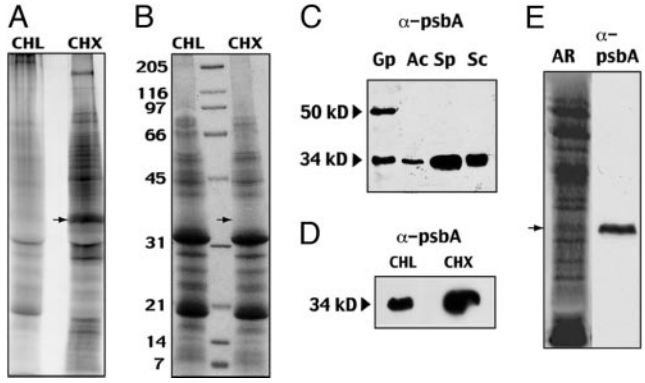

Fig. 2. *In vivo* labeling of psbA is sensitive to chloramphenicol. Autoradiograph (*A*) and Coomassie blue staining (*B*) of purified plastid proteins (0.1 mg) electrophoresed on 1D LDS gels after *in vivo* labeling with [^{35}S]methionine in the presence of either 100 μM chloramphenicol (CHL) or 100 μM cycloheximide (CHX) are shown. Molecular mass markers (kDa) are shown on the left of *B*. (*C*) Western blot analysis with anti-psbA and chemiluminescence detection of total protein extracts (50 μg) from the dinoflagellates *G. polyedra* (Gp) and *A. carterae* (Ac), and two higher plants, *Spinacia oleracea* (spinach, Sp) and *Solanum chacoense* (wild potato, Sc). A band at 34 kDa in *Gonyaulax* whole-cell extracts corresponds to the psbA gene product. (*D*) Western blot analysis of the labeled proteins in *A* by using anti-psbA identifies the position of psbA (arrow). (*E*) Autoradiography (AR) and western analysis of whole-cell extract (50 μg) from *A. carterae* labeled *in vivo* with [^{35}S]methionine in the presence of 100 μM cycloheximide also shows labeled psbA (arrow).

two dinoflagellates and two higher plants (Fig. 2*C*). An additional band at 50 kDa in the *Gonyaulax* extract is not present in extracts from purified plastids (data not shown).

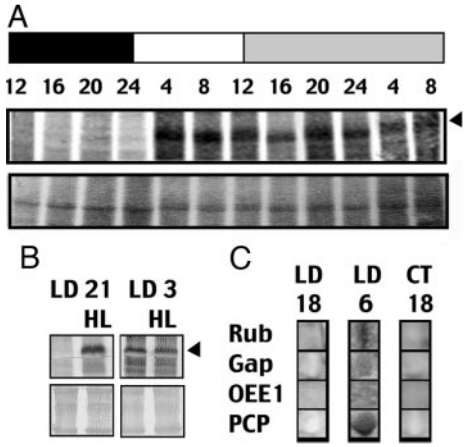

Fig. 3. Light induces labeling of psbA *in vivo*. (*A*) Cells taken at various times from an LD (white and black bars) or a light-light (gray bar) light regime were labeled with [^{35}S]methionine in the presence of 100 μM cycloheximide. Total protein extracts ($\approx$100 μg) were prepared by using LDS, electrophoresed on LDS gels, and transferred to nitrocellulose. (*Middle* and *Bottom*) Shown are autoradiograms and Ponceau-stained membranes, respectively. Arrowhead indicates position of psbA. (*B*) Cells taken at the indicated times from an LD cycle were labeled with [^{35}S]methionine in the presence of 100 μM cycloheximide in darkness (LD21, left lane), normal culture room lights (LD3, left lane; 50 μmol of photons$\cdot$m$^{-2}\cdot$s^{-1}), or exposed to high-light intensity (HL; right lanes $\approx$ 2,500 μmol of photons$\cdot$m$^{-2}\cdot$s^{-1}). (*Upper* and *Lower*) Shown are show autoradiograms and Ponceau-stained membranes, respectively. (*C*) Cells taken at LD 18, LD 6, and circadian time (CT) 18 were labeled with [^{35}S]methionine in the presence of 100 μM chloramphenicol, and the radiolabeled nuclear-encoded proteins were resolved by 2D electrophoresis as a control for the light induction of psbA synthesis. The spots corresponding to ribulose bisphosphase carboxylase/oxygenase (Rub; 55 kDa), glyceraldehyde-3-phosphate dehydrogenase (Gap, 45 kDa), OEE1 (33 kDa), and PCP (32 kDa) are shown.

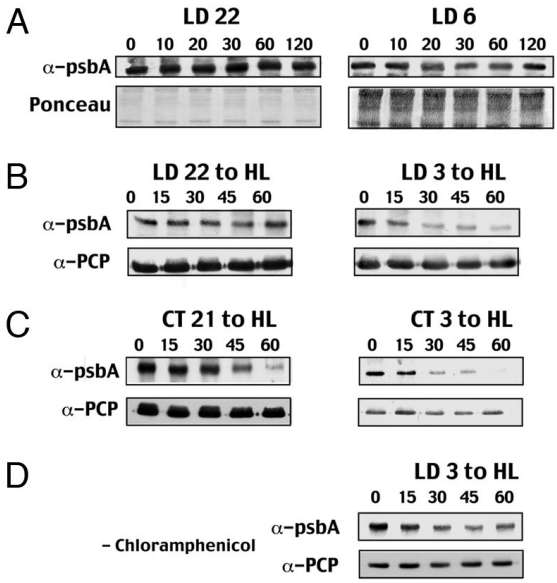

Fig. 4. Degradation of psbA can be induced by high-light intensity in cells previously exposed to light. (*A*) Cells in either late-night phase (LD 22) or midday phase (LD 6) were treated with 100 μM chloramphenicol to block new psbA synthesis, and cell extracts were prepared at various times thereafter (time in minutes after chloramphenicol addition) by using normal lighting. Cell extracts were electrophoresed on LDS, transferred to nitrocellulose and psbA, visualized by using anti-psbA and chemiluminescence. (*B*) Cells in either late-night phase (LD 22) or early day phase (LD 3) were treated with 100 μM chloramphenicol and exposed to high-light intensity ($\approx$2,500 μmol of photons$\cdot m^{-2}\cdot s^{-1}$), and cell extracts were examined for psbA as above. Membranes were stripped and reprobed with anti-PCP as a control for protein load. (*C*) Cells in either late subjective night phase (CT 21) or early subjective day phase (CT 3) were treated with 100 μM chloramphenicol and exposed to high-light intensity ($\approx$2,500 μmol of photons$\cdot m^{-2}\cdot s^{-1}$), and cell extracts were examined for psbA and PCP as above. (*D*) Cells in early day phase (LD 3) were exposed to high-light intensity ($\approx$2,500 μmol of photons$\cdot m^{-2}\cdot s^{-1}$) in the absence of chloramphenicol treatment, and cell extracts were examined for psbA and PCP as above.

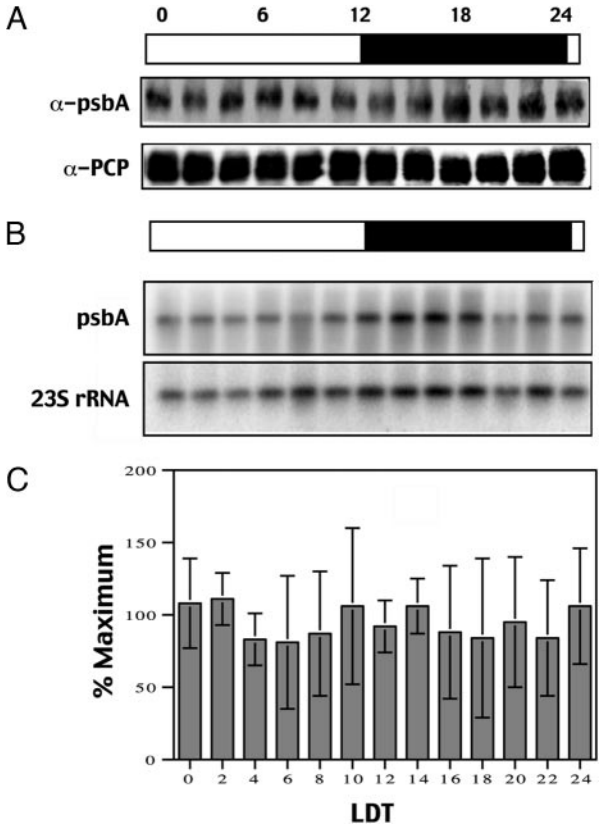

Fig. 5. psbA transcripts and protein levels are constant under LD. (*A*) The psbA in total protein extracts prepared by using LDS from cells at the indicated times was determined by using Western blot analysis. (*B*) Samples of total RNA extracted from cells at the indicated times were transferred to nylon membranes and were first probed with psbA, and then with a 23S rRNA. (*C*) The signals obtained by using psbA and 23S rRNA probes were quantitated using a PhosphorImager and averaged for three experiments over the 24-h period. LDT, light-dark time.

Western blot analyses of the labeled plastid proteins shows that the labeled protein at 34 kDa comigrates with psbA (Fig. 2*D*). Furthermore, one of the labeled proteins in whole-cell extracts from the dinoflagellate *Amphidinium* comigrates with a protein reacting with the anti-psbA (arrow in Fig. 2*E*). Taken together, these results suggest that the 34-kDa protein incorporating label in the presence of cycloheximide is psbA.

To confirm our identification of the 34-kDa band as psbA, we examined the synthesis and degradation rates of the protein under different conditions. In higher plants and green algae, psbA translation is light-regulated (23). We find that psbA synthesis is also light-regulated in *Gonyaulax*, because the amount of radiolabel incorporated into the protein is $\approx$20-fold greater during the light phase than during the dark phase, as determined by PhosphorImager quantification of the amount of radiolabel incorporated (Fig. 3*A*). This behavior is light-dependent rather than circadian, because synthesis is observed during the subjective night phase of cells kept in constant dim light. Furthermore, radiolabel incorporation into psbA in night-phase cells can be induced by brief exposure to light (Fig. 3*B*). This behavior is clearly different from that of nuclear-encoded plastid proteins, whose synthesis is circadian and not directly light-responsive (Fig. 3*C*).

In higher plants, psbA degradation is also light-dependent (24). To measure degradation rates, we took advantage of the ability of chloramphenicol to block psbA synthesis. Under normal cell culture lighting ($\approx$50 μmol of photons$\cdot m^{-2}\cdot s^{-1}$), the degradation rate is not measurable in a 2-h period, and night- and day-phase cells respond in a similar manner (Fig. 4*A*). However, psbA in day-phase cells is rapidly degraded if the cells are exposed to light intensities similar to that of bright sunlight ($\approx$2,500 μmol photons$\cdot m^{-2}\cdot s^{-}$) (Fig. 4*B*). Interestingly, the psbA in cells taken from the dark phase appears much more resistant to this light-induced degradation. Once again, this behavior is not circadian, because the rate of light-induced degradation is high in the subjective night of cells kept in constant dim light (Fig. 4*C*). Finally, psbA is degraded more slowly in the absence of chloramphenicol, suggesting that synthesis rates largely counterbalance the degradation rates under these conditions (Fig. 4*D*). Taken together, the physiological responses to light in terms of induced synthesis and degradation all support the identification of the labeled protein as psbA.

The balance between light-induced synthesis and degradation suggested that psbA would not show a significant variation in the level of either immunoreactive psbA. Indeed, this finding agrees well with measurements of psbA taken over the course of a daily LD cycle (Fig. 5*A*). We also find no change in levels of psbA mRNA (Fig. 5 *B* and *C*) over the course of the daily cycle. These results are also observed when samples are taken from cells kept in constant dim light (data not shown). We conclude that psbA does not represent a probable regulatory site for the $\approx$3-fold changes in circadian O_2 evolution rates (17).

Our data unambiguously place psbA synthesis in the plastid. It was thus of interest to determine whether other plastid-

EVOLUTION

Table 2. Plastid proteins identified by microsequencing from LDS/PAGE

Protein identification no.		No. of peptides identified/peptides sequenced	No. of amino acid identities	Predicted molecular mass, kDa
P70	ATP synthase β-chain	3/6	41/46	59.1
P45	ATP synthase α-chain	2/5	14/19	49.4
P31	Photosystem II D2 protein	2/6	18/25	39.6

encoded genes could potentially be identified by protein microsequencing of radiolabeled bands. Although these analyses are complicated by the limited resolution of proteins separated by only 1D PAGE, 4 bands from a sample of 10 excised from LDS/PAGE gels at positions corresponding to plastid-encoded proteins were found to contain peptide sequences similar to those encoded by the known minicircle genes *atpA*, *atpB* (found twice), and *psbD* from *Amphidinium* (Table 2 and Table 3, which is published as supporting information on the PNAS web site). Of the remaining samples, two were not identifiable, and four contained peptides from an abundant nuclear-encoded LHCP. Clearly, although difficult, proteomic analysis of plastid-encoded proteins using this procedure seems feasible.

Discussion

To our knowledge, this is the first demonstration of protein synthesis in dinoflagellate plastids. The observation that plastid proteins incorporate radiolabel in the presence of cycloheximide provides strong support for a plastid location of the *Gonyaulax psbA* gene and several others. There is still a formal possibility that mRNA synthesized in the cytoplasm is relocated to the plastid, as are tRNAs in some mitochondria (25), but this possibility seems unlikely because there is no precedent for mRNA translocation into the plastid.

Our experiments using 2D electrophoresis to identify plastid gene product synthesis were unsuccessful, and two factors may have contributed to this result. First, plastids from *Gonyaulax* are remarkably fragile, and an analysis of the purified plastid preparations indicates that they contain primarily thylakoid membranes (Y.W. and D.M., unpublished work). This finding suggests that most of the stromal proteins normally located in the plastid may have been lost during the purification. Second, the hydrophobic photosystem components found encoded as minicircle genes might have been resistant to extraction by the usual 2D gel electrophoresis buffers. Indeed, when the extractions protocols were changed to include LDS, a number of radioactive bands could be detected in purified plastid preparations from cells labeled *in vivo*.

We have identified a 34-kDa protein, labeled in the presence of cycloheximide but not chloramphenicol, as psbA by using several criteria. First, the labeled protein comigrated with immunoreactive psbA on LDS/PAGE, as determined by Western blot analysis. Second, the synthesis rate of the protein is light-induced, as is psbA from higher plants. Last, exposure to high light intensities increases the degradation rate of the protein when synthesis is blocked by chloramphenicol. These results, taken together, indicate that psbA is indeed synthesized in the plastid. We observed psbA synthesis in the presence of cycloheximide in both *Gonyaulax* and *Amphidinium*, strongly supporting a general plastid location for this gene in dinoflagellates.

The degradation rates measured for the *Gonyaulax* psbA in response to high light intensities are similar to those described for the psbA of higher plants (24). It seems initially surprising that the light-dependent degradation of psbA is not observed in dark-phase cells but requires prior exposure to light. In part, this result is due to a slow response of the system to changes between dark and light phases. When cell extracts were examined only 1 h after lights on or lights off, intermediate degradation rates were observed (data not shown), suggesting that adaptation to either light or dark phase requires several hours of exposure to the new conditions. This result could then explain why dark-phase cells exposed to high light for only 1 h (the measurement period) did not degrade psbA efficiently as observed (Fig. 4*B*). The mechanism underlying the reduced susceptibility of psbA to degradation in dark-phase cells is also unlikely to involve a redox-based degradation mechanism, because the redox state might be the same in low- and high-light intensity. Instead, it seems possible that a different mechanism may influence this process. It is known that degradation of psbA in higher plants is controlled by reversible phosphorylation (26) and that the circadian-regulated phosphorylation in higher plants can take several hours to reach completion (27). We propose that a slow, light-induced psbA protein phosphorylation in *Gonyaulax* might account for our observations.

The induction of both protein synthesis and degradation by light suggests that little diurnal variation in psbA levels should be expected, which is in agreement with what was observed experimentally (Fig. 5*A*). However, degradation rates increase as a function of light intensity (Fig. 4), whereas synthesis rates appear to be related to only to the presence or absence of light (Fig. 3). Whereas our cells are difficult to grow for long periods under the high-intensity laboratory lights, preliminary experiments suggest that steady-state levels of psbA RNA may increase during high light (data not shown). Thus, cells under natural light intensities may balance degradation with synthesis because higher psbA RNA levels allow for a greater rate of translational initiation.

The lack of diurnal or circadian variation in psbA was surprising, however. There are numerous examples of circadian regulation of nuclear-encoded plastid-directed protein synthesis in *Gonyaulax* (13–15), and the ability of nuclear-encoded gene products to regulate plastid gene expression is well established in other systems (28). Clearly, more extensive studies will be required to determine whether nuclear contribution to regulation of plastid-encoded message translation will be reduced in dinoflagellates. In any event, it seems unlikely that psbA will be involved in mediating clock control over the circadian rhythm of oxygen evolution.

The demonstration that several plastid proteins can be synthesized in the presence of cycloheximide is an important step that may allow a more complete characterization of the plastid proteome. It is interesting in this regard that only a limited number of proteins in crude extracts of both *Amphidinium* and *Gonyaulax* incorporate radiolabel in the presence of cycloheximide, suggesting that the dinoflagellate plastid genome may indeed encode only a small number of genes. Our initial data suggest that it will be possible to identify plastid-encoded proteins by combining our *in vivo* labeling protocol with mass spectrometric microsequencing analysis (Table 2), although these analyses may be limited by the resolution of proteins on 1D LDS/PAGE.

We thank all members of the D.M. and Cappadocia laboratories for contributing helpful suggestions during development of the plastid purification protocol. We also thank Drs. J. Rivoal (Université de Montréal), B. Green (University of British Columbia, Vancouver), and U. Maier (Universitat Marburg, Marburg, Germany) for their critical analysis of our initial experiments and helpful suggestions for further work. This work has been supported by a grant from the National Science and Engineering Research Council of Canada.

1. Gibbs, S. (1981) *Ann. N.Y. Acad. Sci.* **361,** 193–208.
2. Hofmann, E., Wrench, P. M., Sharples, F. P., Hiller, R. G., Welte, W. & Diederichs, K. (1996) *Science* **272,** 1788–1791.
3. Prezelin, B. & Haxo, F. (1976) *Planta* **130,** 251–256.
4. Morse, D., Salois, P., Markovic, P. & Hastings, J. W. (1995) *Science* **268,** 1622–1624.
5. Whitney, S. & Yellowlees, D. (1995) *J. Phycol.* **31,** 138–146.
6. Hackett, J. D., Yoon, H. S., Soares, M. B., Bonaldo, M. F., Casavant, T. L., Scheetz, T. E., Nosenko, T. & Bhattacharya, D. (2004) *Curr. Biol.* **14,** 213–218.
7. Koumandou, V. L., Nisbet, R. E., Barbrook, A. C. & Howe, C. J. (2004) *Trends Genet.* **20,** 261–267.
8. Bachvaroff, T. R., Concepcion, G. T., Rogers, C. R., Herman, E. M. & Delwiche, C. F. (2004) *Protist* **155,** 65–78.
9. Zhang, Z., Green, B. R. & Cavalier-Smith, T. (1999) *Nature* **400,** 155–159.
10. Takashita, K., Ishikura, M., Koike, K. & Maruyame, T. (2003) *Phycologia* **42,** 285–291.
11. Laatsch, T., Zauner, S., Stoebe-Maier, B., Kowallik, K. V. & Maier, U. G. (2004) *Mol. Biol. Evol.* **21,** 1318–1322.
12. Nassoury, N., Cappadocia, M. & Morse, D. (2003) *J. Cell Sci.* **116,** 2867–2874.
13. Markovic, P., Roenneberg, T. & Morse, D. (1996) *J. Biol. Rhythms* **11,** 57–67.
14. Le, Q., Jovine, R., Markovic, P. & Morse, D. (2001) *Biol. Rhythm Res.* **32,** 579–594.
15. Fagan, T., Morse, D. & Hastings, J. (1999) *Biochemistry* **38,** 7689–7695.
16. Guillard, R. R. L. & Ryther, J. H. (1962) *Can. J. Microbiol.* **8,** 229–239.
17. Nassoury, N., Fritz, L. & Morse, D. (2001) *Plant Cell* **13,** 923–934.
18. Schevchenko, A., Wilm, M., Vorm, O. & Mann, M. (1996) *Anal. Chem.* **68,** 850–858.
19. Gevaert, K., Demol, H., Martens, L., Hoorelbeke, B., Puype, M., Goethals, M., Damme, J. V., Boeck, S. D. & Vandekerckhove, J. (2001) *Electrophoresis* **22,** 1645–1651.
20. Sambrook, J., Fritsch, E. & Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual* (Cold Spring Harbor Lab. Press, Woodbury, NY).
21. Le, Q. H., Markovic, P., Jovine, R., Hastings, J. & Morse, D. (1997) *Mol. Gen. Genet.* **255,** 595–604.
22. Mattoo, A. K., Marder, J. B. & Edelman, M. (1989) *Cell* **56,** 241–246.
23. Danon, A. & Mayfield, S. P. (1994) *Science* **266,** 1717–1719.
24. Tyystjarvi, E. & Aro, E. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 2213–2218.
25. Schneider, A. & Marechal-Drouard, L. (2000) *Trends Cell Biol.* **10,** 509–513.
26. Koivuniemi, A., Aro, E. M. & Andersson, B. (1995) *Biochemistry* **34,** 16022–16029.
27. Booij-James, I. S., Swegle, W. M., Edelman, M. & Mattoo, A. K. (2002) *Plant Physiol.* **130,** 2069–2075.
28. Zerges, W. (2002) *Trends Plant Sci.* **7,** 175–182.