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Evolution

Evidence that an amoeba acquired a chloroplast by retaining part of an engulfed eukaryotic alga

(chloroplast origins/endosymbiosis/*in situ* hybridization/rRNA phylogeny)

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ABSTRACT **Chlorarachniophytes are amoeboid algae with unusual chloroplasts. Instead of the usual two membranes that surround the chloroplasts of plants, green algae, and red algae, the chloroplasts of chlorarachniophytes have four bounding membranes. The extra membranes may reflect an unusual origin of chlorarachniophyte chloroplasts. Rather than inheriting the organelle directly from their ancestors, chlorarachniophytes may have adopted the chloroplast of an algal cell ingested as prey. Parts of the algal cell are postulated to remain within the amoeba as a reduced eukaryotic endosymbiont [Hibberd, D. J. & Norris, R. E. (1984) *J. Phycol.* 20, 310–330]. A small nucleus-like structure, proposed to be a vestige of the endosymbiont's nucleus, is located in a space between the second and third chloroplast membranes. We cloned and sequenced nuclear-type rRNA genes from chlorarachniophytes and found two highly divergent genes. *In situ* hybridization shows that one gene is expressed by the amoebal (host) nucleus and the other is expressed by the putative endosymbiont nucleus, suggesting that the latter is indeed a foreign genome. Transcripts from the endosymbiont gene accumulate in the small cytoplasmic compartment between the second and third chloroplast membranes, which we believe to be the remnant cytoplasm of the endosymbiont. Using the endosymbiont gene as a probe, we identified three small chromosomes belonging to the endosymbiont nucleus. This knowledge should allow a detailed molecular analysis of the role of the endosymbiont's genome and cytoplasm in the partnership.**

Photosynthesis first arose in prokaryotes ≈3500 million years ago (1) and was later acquired by eukaryotes through endosymbiosis (2, 3). The chloroplast, the eukaryotic photosynthetic organelle, arose from a photosynthetic prokaryote that was engulfed and retained by a eukaryotic cell (2, 3). The emerging similarity between chloroplasts at the molecular level suggests that all chloroplasts probably stem from a single partnership between a eukaryotic host and a prokaryotic endosymbiont (4–7).

While all chloroplasts may thus have a single origin, it does not necessarily follow that all organisms with chloroplasts represent a single (monophyletic) evolutionary lineage. Lateral transfer of chloroplasts between eukaryotic lineages, via a secondary endosymbiosis, has been postulated to explain the anomalous occurrence of chloroplasts in otherwise distantly related groups of protists (5, 6, 8, 9). Such lateral transfers are believed to have occurred when phagotrophic protozoans engulfed algal cells and retained them as endosymbionts. Several groups of algae—including dinoflagellates, euglenoids, brown algae, diatoms, golden/brown flagellates, cryptomonads, and chlorarachniophytes—are postulated to have "second-hand" chloroplasts acquired from another eukaryote in such a manner (5, 6, 8, 9).

The main line of evidence supporting such secondary endosymbioses is the occurrence of extra membranes surrounding the chloroplasts of certain algal groups. Like mitochondria, ordinary chloroplasts are surrounded by two membranes that probably represent the two bacterial membranes, the phagocytotic membrane of the host having been lost (5). In certain algae there are not two but four membranes bounding the chloroplast. The extra pair of membranes are proposed to represent the plasma membrane of the eukaryotic endosymbiont and the phagocytotic membrane of the host cell (see Fig. 1).

If chloroplasts with four membranes were acquired by endosymbiosis of a eukaryote, what has happened to the nucleus and cytoplasm of the endosymbiont? In most algae with quadruple chloroplast membranes (e.g., diatoms, brown algae, and golden-brown flagellates), there is no trace of these structures, and the hypothesis of secondary endosymbiosis remains largely untested for these organisms (8). However, in two groups, cryptomonads and chlorarachniophytes, structures resembling a eukaryotic nucleus and cytoplasm are present within the chloroplast envelopes (8). Recently, it has been unequivocally demonstrated that the cryptomonads contain a photosynthetic eukaryotic endosymbiont (9–13), but molecular analysis of the chlorarachniophytes is wanting.

Chlorarachniophytes are reticulopodial amoebae (Phylum Chlorarachnida) with green chloroplasts (14). Between the second and third membranes bounding the chloroplast is a small nucleus-like organelle (15). Known as the nucleomorph because it resembles a nucleus, this structure is bounded by a double membrane with pores (15, 16), contains DNA (16), and may encode cytoplasmic-type rRNAs (17). The compartment harboring the nucleomorph, the periplastidal space, contains ribosome-like particles (15–17), and the nucleomorph and surrounding periplastidal cytoplasm are suggested to be the vestigial nucleus and cytoplasm of a chloroplast-containing eukaryotic endosymbiont (15–17).

If chlorarachniophytes truly harbor a eukaryotic endosymbiont, then they are essentially one eukaryotic cell inside another. The nucleomorph would be a foreign genome, and nucleomorph genes would be expected to have different sequences to homologous genes from the host nucleus. Moreover, the nucleomorph DNA should encode components, such as rRNAs, for maintenance of its surrounding cytoplasm in the periplastidal space. Because they are originally derived from a foreign cell, rRNAs in the periplastidal cytoplasm would be expected to have a different nucleotide sequence to rRNAs in the main cytoplasmic compartment of

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Abbreviation: srRNA, small subunit rRNA.

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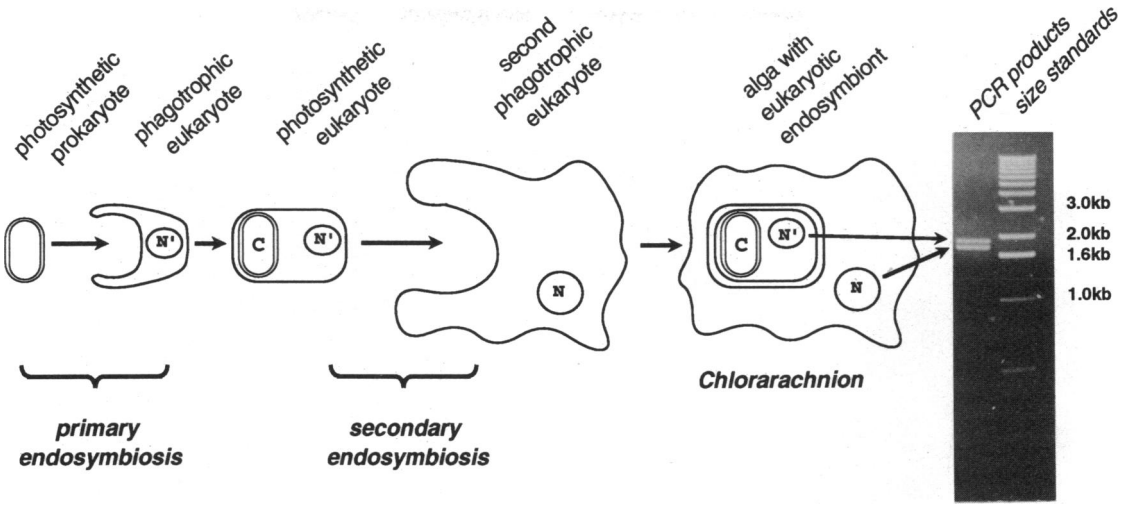

FIG. 1. Hypothetical acquisition of chloroplasts by two sequential endosymbiotic events (15). A eukaryote engulfed a photosynthetic prokaryote (primary endosymbiosis) producing a double-membrane-bound chloroplast (C), as occurs in red algae, green algae, and plants. The algal cell was then engulfed by another eukaryote (secondary endosymbiosis) producing a chloroplast with four membranes. The nucleus (N') and cytoplasm of the engulfed algal cell are located between the second and third chloroplast membranes. PCR amplification of rRNA genes from chlorarachniophytes using universal eukaryotic primers gave two products. We show by *in situ* hybridization (Fig. 2) that one gene is from the host nucleus and the other gene is from a reduced endosymbiont nucleus.

the host cell, which would be encoded by the host nucleus (Fig. 1).

We cloned cytoplasmic-type small subunit rRNA (srRNA) genes from chlorarachniophytes and identified two highly divergent srRNA genes.[§] We localized transcripts of the two genes within the cell by *in situ* hybridization and showed that one gene is expressed by the nucleomorph and the other is expressed by the putative host nucleus. To identify chromosomes belonging to the endosymbiont genome, we mapped the nucleomorph-specific gene to DNA resolved by pulsed-field gel electrophoresis.

MATERIALS AND METHODS

Cells and PCR Amplification. Four chlorarachniophyte strains [*Chlorarachnion reptans* (CCMP 238), *Chlorarachnion reptans* (ref. 18), *Chlorarachnion* sp. 1 (CCMP 1408), and *Chlorarachnion* sp. 2 (CCMP 242)] were cultured as described (15), and total DNA was isolated as described (19). Nuclei were isolated as described (12). Universal cytoplasmic-type srRNA gene primers (5'-TACCTGGTGGATCCTGCCAG-3' and 5'-TGATCCTTCTGCAGGTTCACCTAC-3') were used to amplify srRNA genes by PCR (10). The PCR products were cloned into plasmid vectors and both strands were sequenced by the dideoxynucleotide termination method.

Probes. The chloroplast srRNA probe was a 0.8-kb *Eco*RI fragment from the chloroplast srRNA gene of *Chlamydomonas reinhardtii* (20). The universal cytoplasmic-type srRNA probe was a 1.0-kb *Bam*HI–*Eco*RI fragment from the srRNA gene of garden pea (21). Because rRNA genes share large regions of high similarity, they cross-hybridize and it is necessary to use small regions with minimal sequence similarity for gene-specific probes. From an alignment of the two cytoplasmic-type srRNAs isolated from *Chlorarachnion* sp. 2, we identified unique regions in each gene. A 97-bp fragment (positions 1457–1553) corresponding to helices 41 and 42 in variable domain 7 (22) was used as a probe specific for the longer gene. For the shorter gene, a 115-bp fragment (positions 615–729) from helices E21.1 through E21.2 in variable domain 4 (22) was used.

***In Situ* Hybridization.** Cells of *Chlorarachnion* sp. 2 were fixed and embedded for *in situ* hybridization as described (23). The gene-specific fragments of the long and short genes were cloned into a transcription vector and biotinylated sense and antisense RNA probes were synthesized. The probes were hybridized to ultrathin sections and bound probe was detected with anti-biotin immunogold markers as described (23).

Northern Blot Analysis. Total RNA was isolated from *Chlorarachnion* sp. 2 as described (24), electrophoresed in a denaturing agarose gel (1.5%) and then transferred to ZetaProbe nylon membrane (Bio-Rad) as recommended by the manufacturer. The blot was stripped between probings and removal of the probe was monitored by autoradiography prior to reprobing. Autoradiograms were exposed for 1 h at room temperature (see Fig. 3, lanes C and D), 20 h at −70°C with an intensifying screen (see Fig. 3, lanes E and F), and 3 h at room temperature (see Fig. 3, lanes G and H).

Pulsed-Field Gel Electrophoresis. Flagellate cells of *Chlorarachnion* sp. 1 were embedded in plugs of low-gelling-temperature agarose at 1.4×10^8 cells per ml and then digested as described (12). Chromosomal DNAs were electrophoresed in 1% agarose (SeaKem LE, FMC) in a CHEF DRII apparatus (Bio-Rad) at 170 V with a 20-sec pulse for 30 h. DNA was transferred to ZetaProbe nylon membrane (Bio-Rad) as recommended by the manufacturer. The three probes were hybridized to targets at 42°C in a buffer containing 50% (vol/vol) formamide and washed at 68°C in 0.1× standard saline citrate. Nucleomorph chromosomes were excised from gels, and DNA was purified for use as a PCR template with Prep-A-Gene matrix (Bio-Rad).

RESULTS AND DISCUSSION

PCR Amplification and Sequence Comparison. Amplification of srRNA genes from total chlorarachniophyte DNA yielded two PCR products (Fig. 1). We refer to these as the long gene and the short gene. Alignment of the long and short gene sequences revealed that the length difference is primarily attributable to an extended loop in variable domain 7 (22)

[§]The sequences reported in this paper have been deposited in the GenBank data base [accession nos. CCMP 1408 Nm, U02040; CCMP 1408 Nu, U02075; CCMP 238 Nm, U03275; CCMP 238 Nu, U03477; CCMP 242 Nm, U03478; CCMP 242 Nu, U03479; *Chlorarachnion reptans* (ref. 18) Nm, X70808; *Chlorarachnion reptans* (ref. 18) Nu, X70809].

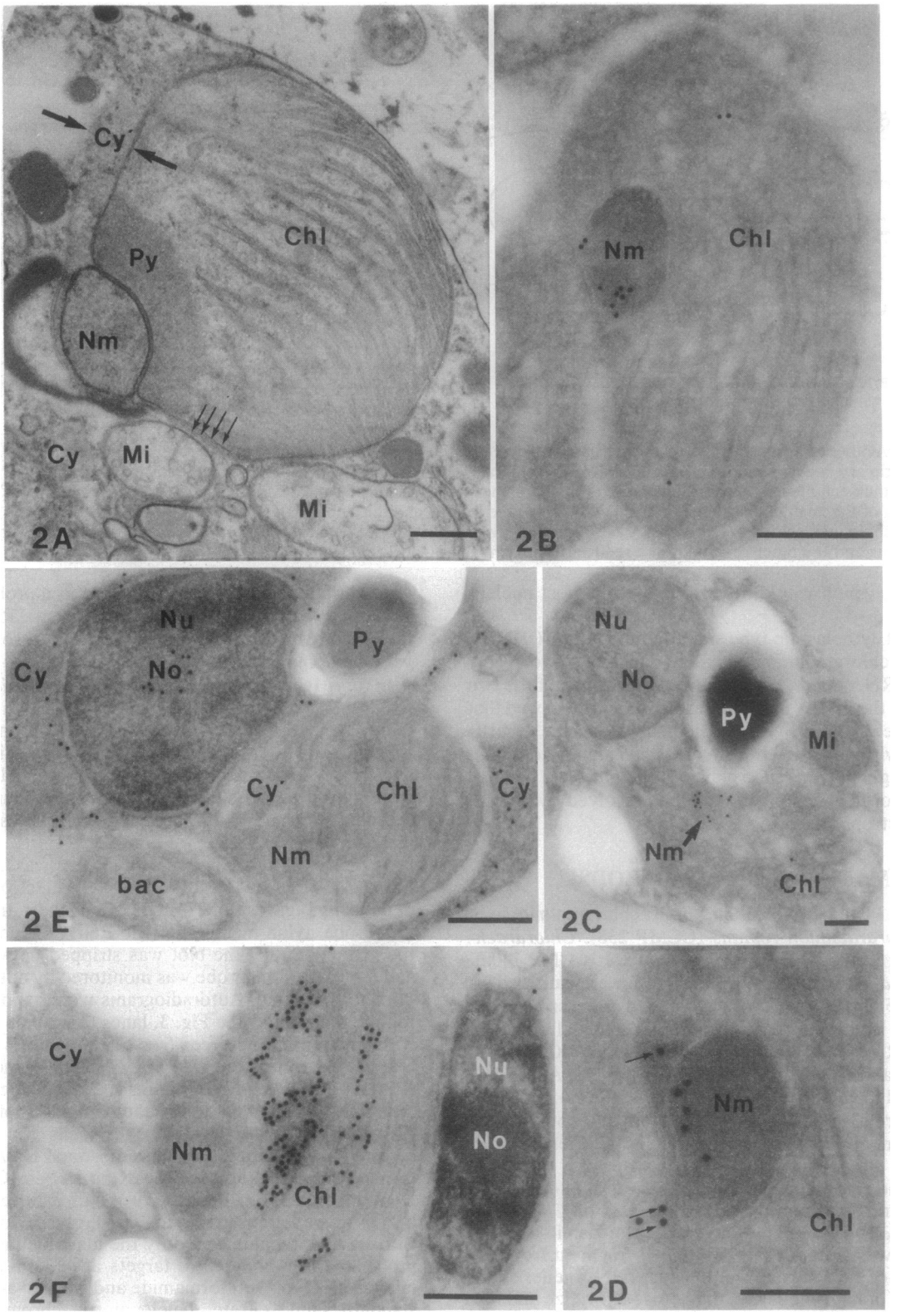

FIG. 2: Subcellular localization of srRNA gene transcripts in *Chlorarachnion* sp. 2 by *in situ* hybridization. (*A*) Standard electron micrograph of chlorarachniophyte chloroplast (Chl) showing the four surrounding membranes (arrows). Between the second and third membranes are the nucleomorph (Nm) and ribosome-like particles in the periplastidal space (Cy'). The chloroplast contains thylakoids organized loosely into pairs, stroma, and a pyrenoid (Py). Mitochondria (Mi) with tubular cristae are present in the cytoplasm (Cy) of the amoeba. (*B*) Localization of long gene transcripts to nucleomorph (Nm). (*C*) Localization of long gene transcripts to nucleomorph (Nm). (Bar = 400 nm.) (*D*) Localization of long gene transcripts to nucleomorph (Nm) and periplastidal space (arrows). (*E*) Localization of the short gene transcripts to the nucleolus (No) of the main nucleus (Nu) and to the cytoplasm (Cy) of the amoeba. No signal is present in the nucleomorph (Nm), periplastidal space (Cy'), the chloroplast (Chl), pyrenoid (Py), or a bacterium (bac) in the food vacuole. (*F*) Localization of eubacterial-like srRNA transcripts in the chloroplast (Chl). No signal is present in the nucleus (Nu), nucleolus (No), nucleomorph (Nm), or host cytoplasm (Cy). (Bars: *A–C*, *E*, and *F*, 400 nm; *D*, 200 nm.)

of the longer gene. The long and short genes share only 65% positional identity, which is consistent with chlorarachniophytes containing the srRNA genes of two eukaryotes.

Ultrastructure and *in Situ* Hybridization. The chloroplast of chlorarachniophytes is surrounded by four membranes (Fig. 2*A*). Between the second and third membranes is a double-membrane-bound structure known as the nucleomorph. Surrounding the nucleomorph is the periplastidal space, which contains ribosome-like particles. *In situ* hybridization using the probe specific for the longer gene localized transcripts to the nucleomorph (Fig. 2 *B* and *C*) and periplastidal space (Fig. 2*D*). The nucleomorph/periplastidal space region was labeled with an average of 114 gold particles per μm^2 (n = 17, range 96–185 particles per μm^2). Labeling density outside the nucleomorph/periplastidal space averaged 0.6 gold particle per μm^2 (n = 17, range 0.16–0.9 particle per μm^2). We conclude that the longer gene is located in the nucleomorph and encodes srRNAs for ribosomes in the periplastidal space.

The fragment specific for the shorter gene hybridized to the nucleolus of the main nucleus and main cytoplasm of the amoeba (Fig. 2*E*). Labeling intensity on nucleoli with the short gene probe averaged 107 gold particles per μm^2 (n = 8, range 51–157 particles per μm^2), which is comparable with the labeling of the nucleomorph by using the probe specific for the long gene. Since nucleoli are the transcription site for rRNA genes (25), we believe the short gene is being transcribed in the nucleolus of the main nucleus and that the transcripts are incorporated into ribosomes in the main cytoplasm of the amoeba. No transcripts of the short gene were localized to the periplastidal space or nucleomorph (Fig. 2*E*), indicating that the outer pair of chloroplast membranes prevents exchange of rRNAs between the main cytoplasm and the periplastidal space.

A probe for eubacterial-like srRNAs of chloroplasts labeled ribosomes in the chloroplast stroma (Fig. 2*F*). This result is consistent with the chloroplast being fundamentally prokaryotic in origin (see Fig. 1).

Northern Blot Analysis. Further confirmation that both genes are transcribed was obtained by probing a Northern blot of total chlorarachniophyte RNA with the probes for the long and short genes. The srRNA transcripts were first identified with the universal probe. The blot was then stripped and reprobed with the nucleomorph-specific probe and then stripped again and reprobed with the nucleus-specific probe. The nucleus-specific probe hybridized with a 1.8-kb transcript (Fig. 3, lane G). The nucleomorph-specific probe hybridized to a slightly larger transcript (Fig. 3, lane E). Based on the signal intensity, the nuclear gene transcript was far more abundant than the nucleomorph gene transcript, which is consistent with the relative volumes of the compartments that the transcripts occupy (15). Neither probe hybridized to the srRNAs of an unrelated alga (Fig. 3, lanes F and H).

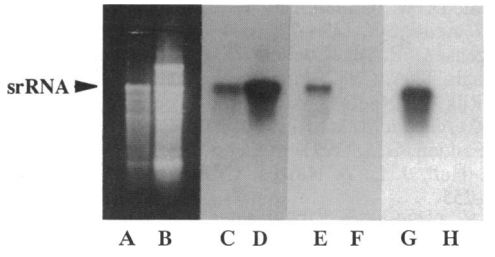

FIG. 3. Northern blot analysis of nuclear and nucleomorph gene transcripts. Total RNAs of *Chlorarachnion* sp. 2 (lane A) and the cryptomonad *Rhodomonas salina* (lane B) were probed with a universal cytoplasmic-type srRNA probe (lanes C and D), the chlorarachniophyte nucleomorph-specific fragment (lanes E and F), and the chlorarachniophyte nucleus-specific fragment (lanes G and H).

Mapping of srRNA Genes to Chromosomes. A preliminary investigation of the nuclear and nucleomorph karyotypes was undertaken by mapping the two srRNA genes to the chromosomes of whole chlorarachniophyte cells resolved by pulsed-field gel electrophoresis (Fig. 4, lane B). Chromosomes containing cytoplasmic-type srRNA genes were identified using the universal probe that hybridized to three small chromosomes (145 kb, 140 kb, and 95 kb) and an unresolved cluster of larger chromosomes (Fig. 4, lane C). The probe specific for the nucleomorph srRNA gene only hybridized to the three small chromosomes of 145 kb, 140 kb, and 95 kb (Fig. 4, lane D). The remnant endosymbiont nuclear genome thus contains at least three chromosomes totaling 380 kb. It is not yet known whether the three chromosomes contain unique DNA sequences or whether they are merely homologues that differ in size. Additional chromosomes not carrying rRNA genes could also be present in the nucleomorph and it will be necessary to isolate the nucleomorph genome for complete karyotyping.

The fragment specific for the nuclear srRNA gene hybridized to an unresolved cluster of larger chromosomes (Fig. 4, lane E). Further investigation of this chromosome cluster, using electrophoretic conditions more suited to this size range, revealed at least 16 chromosomes ranging from 460 kb to ≈2.5 megabases in size (data not shown).

Amplification of srRNA Genes from Purified Nuclear or Nucleomorph Templates. PCR amplification of srRNA genes from the four strains of chlorarachniophyte using universal eukaryotic primers always yielded two different-sized products (Fig. 5). The shorter (nuclear) gene is consistent in size (PCR product, ≈1780 bp) but the longer (nucleomorph) gene varies in size (PCR products from 1860 bp to 1907 bp) depending on the strain. The length variability of the nucle-

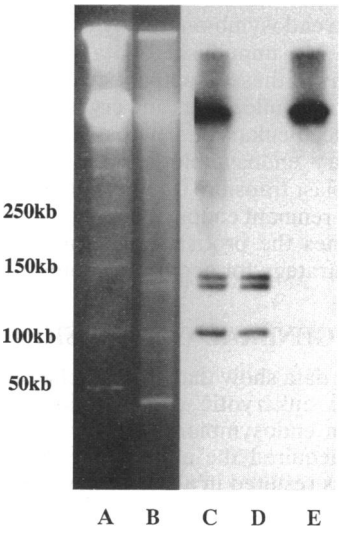

FIG. 4. Mapping of nuclear and nucleomorph srRNA genes to chromosomes of *Chlorarachnion* sp. 1 resolved by pulsed-field gel electrophoresis. Lanes: A, ladder of λ DNA (Bio-Rad); B, five small bands (145 kb, 140 kb, 95 kb, 80 kb, and 40 kb) plus an unresolved cluster of larger chromosomes visualized by ethidium bromide staining; C, probing of chromosomes with universal srRNA gene probe showing that three small chromosomes (145 kb, 140 kb, and 95 kb) plus one or more chromosomes in the unresolved cluster contain cytoplasmic-type srRNA genes; D, probing with nucleomorph (Nm)-specific fragment showing that the nucleomorph gene is carried by three small chromosomes (145 kb, 140 kb, and 95 kb); E, probing with nucleus (Nu)-specific fragment showing that nuclear srRNA genes are carried by one or more chromosomes in the unresolved cluster of larger chromosomes.

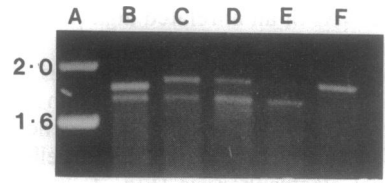

FIG. 5. PCR amplification of cytoplasmic-type srRNA genes from different species of chlorarachniophytes demonstrating the presence of two genes [data for *Chlorarachnion reptans* (18) not shown]. Lanes: A, molecular size markers in kb; B, *Chlorarachnion reptans* (CCMP 238); C, *Chlorarachnion* sp. 2 (CCMP 242); D, *Chlorarachnion* sp. 1 (CCMP 1408); E, nuclear DNA from *Chlorarachnion* sp. 1 (CCMP 1408) produced only the short gene; F, isolated nucleomorph chromosomes from *Chlorarachnion* sp. 1 (CCMP 1408) (see Fig. 2) produced only the long gene.

omorph genes is due primarily to differences in variable domain 7 (22).

Confirmation that the short gene is nuclear was obtained by using DNA prepared from isolated chlorarachniophyte nuclei in PCR experiments. When nuclear DNA from *Chlorarachnion* sp. 1 was used as a template with the universal srRNA gene primers, only the short gene could be amplified by PCR (Fig. 5, lane E).

Confirmation that only three small chromosomes (145 kb, 140 kb, and 95 kb) carry the nucleomorph srRNA gene was obtained in PCR experiments in which only the longer (nucleomorph) srRNA gene could be amplified using pulsed-field-gel-purified nucleomorph chromosomes from *Chlorarachnion* sp. 1 as template (Fig. 5, lane F).

Evolutionary Affinities of the Endosymbiotic Partners. On the basis of chloroplast morphology and pigment data, it has been proposed that the chlorarachniophyte endosymbiont is a green alga (15, 26), a euglenoid (27), or an extinct early eukaryotic alga (5). Phylogenetic trees incorporating the endosymbiont nuclear srRNA sequences determined here did not ally the endosymbiont to any extant group of eukaryote algae (G.I.M., unpublished data), so we are unable to corroborate any of these hypotheses with our sequence data. Whether chlorarachniophytes and cryptomonads, which also contain a reduced eukaryotic endosymbiont, arose from the same secondary endosymbiotic event (5) or two separate lateral chloroplast transfers (8, 9) is not yet clear. Molecular analysis of the remnant endosymbiont genomes should tell us how many times the protists have employed this unusual evolutionary strategy for acquiring photosynthetic capacity.

GENERAL CONCLUSIONS

Our molecular data show that chlorarachniophytes contain a photosynthetic eukaryotic endosymbiont. By retaining a prey cell as an endosymbiont, a phagotrophic amoeba has permanently acquired the ability to photosynthesize. The partnership has resulted in a lateral transfer of a chloroplast from one eukaryotic lineage into another, thereby creating a new phylum of algae.

Our *in situ* hybridization analysis shows that in addition to the chloroplast, the amoeba also retains vestiges of the endosymbiont's nucleus and cytoplasm. To acquire the chloroplast permanently, the amoeba probably needed that portion of the endosymbiont's nuclear genome essential for chloroplast biogenesis and function. Some sea slugs temporarily use the chloroplasts of ingested algae for photosynthesis, but because they lack genes for chloroplast proteins encoded by the algal nucleus, the slugs are apparently unable to maintain the organelle longer than 2–3 months (28). It could be that the only purpose of the endosymbiont nucleus and cytoplasm in chlorarachniophytes is to provide chloroplast proteins. To provide these proteins the endosymbiont compartment may have to create a set of translation machinery, as well as transcription and DNA replication components. The endosymbiont nucleus could thus represent a drastically pared-down version of the eukaryotic genome, retaining little more than those elements essential for protein synthesis and self-replication. Such a streamlined system would be valuable for investigating the molecular biology of core cell functions in eukaryotes. Our identification of three chromosomes from the endosymbiont nucleus should allow examination of the potential of this system and to establish the *raison d'être* of the endosymbiont genome in this unusual partnership.

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