INTRODUCTION

Cryptomonad algae are postulated to be a chimera of two different eukaryotic cells: a flagellate host and a photosynthetic endosymbiont (Douglas et al., 1991a; Maier et al., 1991; McFadden, 1993). By engulfing and retaining a photosynthetic cell, the flagellate host was apparently able to acquire a chloroplast and convert from obligate heterotrophy to a principally autotrophic way of life. In addition to the chloroplast, the host cell also acquired the nucleus of the engulfed cell. Known as the nucleomorph, the endosymbiont nucleus in cryptomonads resides in a cell compartment that also contains the chloroplast (Greenwood, 1974; Greenwood et al., 1977). This compartment, termed the periplastidal space, is delimited by a membrane thought to be plasma membrane of the endosymbiont (Cavalier-Smith 1986), and the cytoplasm in the periplastidal space is postulated to be the remnants of the endosymbiont’s cytoplasm (Greenwood, 1974; Greenwood et al., 1977) (see also Fig. 1).

Nucleomorphs of certain cryptomonads can be isolated (Hansmann and Eschbach, 1990), and karyotyping by pulsed field gel electrophoresis shows that the nucleomorph of the cryptomonad Pyrenomonas salina contains three small chromosomes totalling only 660 kb (Eschbach et al., 1991). Each nucleomorph chromosome carries genes for ribosomal RNAs (Eschbach et al., 1991). The nucleomorph gene sequences are highly divergent from those of the host nucleus (about 70% positional identity), supporting the hypotheses that the nucleomorph is the remnant of a foreign nucleus (Douglas et al., 1991a; Maier et al., 1991). Because cryptomonad chloroplasts contain phycobilin pigments, the endosymbiont was initially thought to be a red algal-like organism (Gillott and Gibbs, 1980). Phylogenetic trees incorporating cryptomonad endosymbiont gene sequences ally them loosely with red algae (Douglas et al., 1991a,b; Douglas and Turner, 1991; Maerz et al., 1992), but the results are inconclusive and it is possible that the endosymbiont was an early evolutionary intermediate that pre-dates the red algae (Cavalier-Smith, 1992).

If the nucleomorph and periplastidal space are truly the nucleus and cytoplasm of an endosymbiont, then cryptomonads are essentially one eukaryotic cell inside another. The inner cell (the algal endosymbiont) would produce one set of ribosomes for the periplastidal compartment, while the outer cell (the host) would produce a different set of ribosomes for the main cytoplasm. Northern blotting analysis has demonstrated that both the nucleomorph and nuclear small subunit ribosomal RNA, in situ hybridization, pulsed field gel electrophoresis

SUMMARY

Cryptomonad algae contain a photosynthetic, eukaryotic endosymbiont. The endosymbiont is much reduced but retains a small nucleus. DNA from this endosymbiont nucleus encodes rRNAs, and it is presumed that these rRNAs are incorporated into ribosomes. Surrounding the endosymbiont nucleus is a small volume of cytoplasm proposed to be the vestigial cytoplasm of the endosymbiont. If this compartment is indeed the endosymbiont’s cytoplasm, it would be expected to contain ribosomes with components encoded by the endosymbiont nucleus. In this paper, we used in situ hybridization to localize rRNAs encoded by the endosymbiont nucleus of the cryptomonad alga, Cryptomonas Φ. Transcripts of the endosymbiont rRNA gene were observed within the endosymbiont nucleus, and in the compartment thought to represent the endosymbiont’s cytoplasm. These results indicate that the endosymbiont produces its own set of cytoplasmic translation machinery. We also localized transcripts of the host nucleus rRNA gene. These transcripts were found in the nucleolus of the host nucleus, and throughout the host cytoplasm, but never in the endosymbiont compartment. Our rRNA localizations indicate that the cryptomonad cell produces two different sets of cytoplasmic rRNAs in two separate subcellular compartments. The results suggest that there is no exchange of rRNAs between these compartments. We also used the probe specific for the endosymbiont rRNA gene to identify chromosomes from the endosymbiont nucleus in pulsed field gel electrophoresis. Like other cryptomonads, the endosymbiont nucleus of Cryptomonas Φ contains three small chromosomes.

Key words: endosymbiosis, chloroplast origin, cryptomonad, ribosomal RNA, in situ hybridization, pulsed field gel electrophoresis
ribosomal RNA (srRNA) genes are transcribed, and, because these transcripts can fold into secondary structures conforming to standard eukaryotic conformation, it is presumed that the transcripts are incorporated into ribosomes within the cell (Douglas et al., 1991; Maier et al., 1991). As yet, transcripts from the two different types of cryptomonad srRNA genes have not been localized within the cell. To examine this question, we produced a probe specific for each type of srRNA gene, and then used these probes for high resolution in situ hybridization analyses. We also mapped the two genes to total Cryptomonas Φ chromosomes resolved by pulsed field gel electrophoresis in order to identify chromosomes from the endosymbiont genome.

MATERIALS AND METHODS

Cells and electron microscopy

A culture of Cryptomonas Φ (CCMP 325) was obtained from the Provasoli-Guillard Center and grown in h/2 medium as recommended (Andersen et al., 1991). Cells for in situ hybridization were fixed in 2% glutaraldehyde in 0.4 M sucrose, 50 mM Pipes (pH 7.2) at 4°C for 1 hour. Fixed cells were then partially dehydrated and embedded in LR Gold resin as previously described (McFadden et al., 1988). Biotinylated sense and antisense RNA runoff transcripts were produced, and in situ hybridization performed as previously described (McFadden, 1991). Briefly, the probes were hybridized to ultrathin sections at 55°C in a buffer containing 50% formamide, and then washed in 1X SSC at 55°C. Hybrids were then detected using rabbit anti-biotin (Enzo Biochem, Inc, USA) followed by goat anti-rabbit conjugated to 15 nm colloidal gold (Amersham, UK).

Probes

The universal cytoplasmic-type rRNA probe was a 1 kb BamHI/EcoRI fragment of the srRNA gene of Pisum sativa (Jorgensen et al., 1987). The chloroplast probe was an 800 bp EcoRI fragment of the chloroplast srRNA of Chlamydomonas reinhardtii (Grant et al., 1980). The probes specific for the two different genes of Cryptomonas Φ were derived from previously described clones of srRNA genes (Douglas et al., 1991a). Using the polymerase chain reaction (PCR), Douglas et al. (1991a) were able to isolate two distinct srRNA genes from Cryptomonas Φ that differ in length by approximately 200 bp (see Fig. 1). We refer to these as the long and short genes. Because rRNA genes contain large domains of highly similar sequence, the genes cross-hybridize and a small fragment must be used to produce a gene-specific probe. Alignment of the long and short Cryptomonas Φ genes indicated that the variable region V4, corresponding to helices 22 through E23-7 in the standard eukaryotic rRNA secondary structure (DeRijk et al., 1992), shares minimal sequence identity (Douglas et al., 1991a), so we selected this region to produce gene-specific probes. The sequences and hypothetical secondary structures for the gene fragments used as probes are shown in Fig. 1. The long gene probe was a 186 bp PstI/NlaIII restriction fragment subcloned into pGEM™-blue (Promega Corp.). The short gene of Cryptomonas Φ lacks a PstI site at the equivalent position in the long gene so a site was engineered into this position by PCR site-directed mutagenesis. A portion of the short gene was amplified using a rightwards PCR primer (5’-TAAAAATGTTCG- GCAGTTAAAAGC-3’) that changed the sequence TTGCAG in the gene to CTGCAG in the PCR product. The leftwards primer was a universal srRNA gene primer (LDC) that anneals downstream of the StuI site in the Cryptomonas Φ genes (Saunders and Druehl, 1992). A 165 bp PstI/StuI fragment from the short gene amplification product was then cloned into pBluescript™ II KS+ (Stratagene Inc.).

Pulsed field gels and Southern blotting

Cells for pulsed field gel electrophoresis were embedded in low-gelling temperature agarose at a density of 1.4×10^8 cells per ml and digested as previously described (Eschbach et al., 1991). Chromosomal DNAs were electrophoresed in 1% agarose (SeaKem LE, FMC BioProducts) at 175 V with a 20 second pulse in a CHEF DRII apparatus. The plugs were removed from the gel and then DNA was transferred to Zeta Probe (Bio-Rad) using the acid depurination and alkaline transfer protocol recommended by the manufacturer. The blot was probed sequentially with the four probes described above. The universal probe and chloroplast probe were labelled by random priming to incorporate [α-32P]dCTP (Feinberg and Vogelstein, 1983). The probes for the short and long Cryptomonas Φ genes, which were considered too small to label effectively by random priming, were labelled with [α-32P]dCTP by using the Klenow fragment of DNA polymerase to extend a sequencing primer (T7) annealed to its site in the vector. To minimize incorporation of vector sequence into the probe, the plasmids were linearized downstream of the insert prior to annealment and extension of the primer. All four probes were hybridized to the blot at 42°C in standard Southern blot hybridization buffer containing 50% formamide (Maniatis et al., 1982). Post-hybridization washes were performed at 68°C in 0.1× SSC with the universal cytoplasmic RNA probe and the chloroplast probe. The probes specific for the short and long genes were washed at 55°C in 0.5× SSC. After each probing, the membrane was stripped by incubating it in two changes of 0.5% SDS (w/v), 0.1× SSC at 95°C for 20 minutes each. Absence of residual signal was verified by autoradiography prior to probing with the subsequent probe.

RESULTS

Cryptomonas Φ ultrastructure

The general morphology of Cryptomonas Φ is reported elsewhere (Gillott and Gibbs, 1980; Gibbs, 1983). Cells contain a large nucleus with typical nucleolus (Figs 1 and 2A).

Fig. 1. Diagrammatic representation of cryptomonad evolution by eukaryote/eukaryote endosymbiosis. The endosymbiont, a eukaryotic alga with a double-membrane-bound chloroplast, was engulfed by a eukaryote phagotroph (host). The phagocytic vacuole is suggested to have fused with the rough endoplasmic reticulum (rer), effectively situating the endosymbiont in the lumen of the ER (Cavalier-Smith, 1986). The endosymbiosis results in a chloroplast bounded by four membranes: two from the original chloroplast envelope, the plasma membrane of the endosymbiont, and a membrane homologous to the rer now termed the periplastid rough endoplasmic reticulum (rer). The cytoplasm (cy′) and nucleus (nm) of the endosymbiont persist. When PCR is used to amplify cytoplasmic-type rRNA genes from total cryptomonad DNA, two different sized genes are recovered (Douglas et al., 1991a). An ethidium bromide-stained agarose gel presented here shows the long and short srRNA genes of Cryptomonas Φ. Size standards in kilobase pairs are shown in the right hand lane. We used a portion of each gene, the sequences and structures of which are shown, as probes to localize transcripts of each gene within the cell. The in situ hybridization results demonstrate that the short gene is transcribed in the nucleolus (nu) of the host cell nucleus (nu). These nuclear-encoded transcripts accumulate in the host cytoplasm (cy). Transcripts of the longer gene were localized to the electron-dense zone of the endosymbiont nucleus (nm) and also to the cytoplasm deriving from the endosymbiont (cy′). The cryptomonad is thus a true chimera with two radically divergent nucleocytoplasmic compartments, one inside the other.
The chloroplast is bounded by four membranes: two chloroplast envelopes, a membrane believed to represent the plasma membrane of the endosymbiont and termed the periplastid membrane, and an outer membrane known as the periplastid rough endoplasmic reticulum (PRER) (Cavalier-Smith, 1989). The PRER membrane is continuous with the nuclear envelope and bears ribosomes on the cytoplasmic side (Fig 1, 2A,C), and is therefore equivalent to the rough endoplasmic reticulum (Cavalier-Smith, 1986). Between the two outer membranes (PRER and periplastid membrane) and the two chloroplast
envelope membranes is a compartment known as the periplastidal space, which contains the nucleomorph and surrounding ribosome-like particles (Figs 1 and 2). The nucleomorph has a double membrane with pores and an electron-dense region (Fig. 2B,C) perhaps analogous to a nucleolus.

In situ hybridization
The universal cytoplasmic-type srRNA probe hybridized to the nucleolus of the host nucleus, the host cytoplasm and the periplastidal space (Fig. 3A). The chloroplast probe hybridized to the stroma of the chloroplast but no other structures (Fig.
The probe for the short gene hybridized to the nucleolus of the main nucleus and the main cell cytoplasm (Figs 3C, 4C). No short gene transcripts were detected in the nucleoplasm, mitochondria, chloroplast, periplastidal space, or nucleomorph (Figs 3C, 4C). The probe for the longer gene only hybridized to the periplastidal space (Figs 3D and 4A) and to the electron-dense zone of the nucleomorph (Fig. 4B).

The sense strand probes, which were used as negative controls, did not label any structures (not shown).

**Mapping of long and short genes to chromosomes**

Ethidium bromide staining of chromosomal DNAs resolved by pulsed field gel electrophoresis detected four small chromosomes (130 kb, 175 kb, 180 kb, 195 kb) plus a cluster of unresolved chromosomes (Fig. 5, lane B). The electrophoretic conditions we used do not resolve DNAs larger than 300 kb, and these molecules migrated as a single group (Fig. 5, lanes A and B). Chromosomes carrying cytoplasmic-type (eukaryotic) srRNA genes were first identified with the universal probe. This universal probe hybridized with three of the small chromosomes (175 kb, 180 kb, 195 kb), and to one or more chromosomes present in the unresolved cluster of larger chromosomes (Fig. 5, lane C). The probe for the shorter gene hybridized to the cluster of larger unresolved chromosomes but not to any of the small chromosomes (Fig. 5, lane D). The probe for the longer gene hybridized to the three small (175 kb, 180 kb, 195 kb) chromosomes (Fig. 5, lane E). The chloroplast probe hybridized to the 130 kb chromosome (Fig. 5, lane F). This 130 kb was markedly brighter with ethidium bromide staining than the three other small chromosomes (175 kb, 180 kb, 195 kb), which were of equivalent brightness to each other (Fig. 5, lane B). The chloroplast probe also hybridized to the well into which the plug was loaded (Fig. 5, lane F).

**DISCUSSION**

**The chloroplast is fundamentally prokaryotic in origin**

In situ hybridization with the chloroplast probe demonstrates that the *Cryptomonas* Φ chloroplast contains bacterial-like rRNAs in the stroma that we believe to be incorporated into chloroplast ribosomes. The chloroplast probe is also known to recognise rRNAs in eubacteria (McFadden, 1990a), which is consistent with the cryptomonad chloroplast originally being of prokaryotic origin via a primary endosymbiosis. The circular *Cryptomonas* Φ chloroplast chromosome contains two copies of a bacterial-like srRNA gene (Douglas, 1988), which presumably encode the chloroplast srRNAs detected here.

**The short gene is the host gene**

The in situ hybridization analyses demonstrate that transcripts of the short gene are present in the nucleolus of the main nucleus. This structure usually contains the nucleocytoplasmic rRNA genes (Reeder, 1990), and we believe that the short gene probe is detecting nascent srRNAs at their transcription site. Since transcripts of the short gene were also localized in the main cytoplasmic compartment of the cell, we conclude that the short gene encodes srRNAs that are incorporated into ribosomes in the host cytoplasm. Since no transcripts of the short gene could be detected within the periplastidal space, we believe that this compartment does not receive srRNAs from the host nucleus. The results provide firm proof that the short gene is nuclear, confirming that Douglas et al. (1991a), correctly assigned this gene to the host nucleus.

**The long gene resides in the nucleomorph**

Within the nucleomorph are three main regions: a background matrix that contains DNA (Hansmann et al., 1986), a collection of rod-shaped electron dense bodies of unknown composition and function (Gillott and Gibbs, 1980), and a medium-density, DNA-containing region postulated to be equivalent to a nucleolus (Gillott and Gibbs, 1980; Hansmann et al., 1986). Probing with RNase/gold has demonstrated that the nucleolus-like zone contains RNA (Hansmann, 1988), and in situ hybridization analysis using a universal probe demonstrated the presence of eukaryotic type rRNAs within the region (McFadden, 1990a, 1993). The in situ hybridization analysis reported here demonstrates that transcripts of the long gene are present in this nucleolus-like region of the nucleomorph confirming the assumption of Douglas et al. (1991a) that the long gene derives from the nucleomorph. We believe that the long srRNA genes are located and transcribed in the fibrillogranular part of the nucleomorph, further suggesting that this region is a nucleolus.

**The nucleomorph encodes rRNAs for periplastidal ribosomes**

An hypothesis proposing that the periplastidal space is a remnant of the endosymbiont’s cytoplasm was based on an assumption that this compartment contains eukaryotic ribosomes, and that the ribosome components are encoded by an endosymbiont genome (nucleomorph) (Ludwig and Gibbs, 1985, 1987; McKerracher and Gibbs, 1982). The periplastidal space contains 23nm particles bearing close resemblance to eukaryotic ribosomes (Sepenswol, 1973; Greenwood, 1974; Gillott and Gibbs, 1980), and histochemistry using ribonuclease conjugated to colloidal gold markers has localized RNA in the periplastidal space (Hansmann, 1988). Confirmation that the periplastidal space is fundamentally a eukaryotic compartment was provided by a previous in situ hybridization study that localized eukaryotic rRNAs within the periplastidal space (McFadden, 1990a). However, because a universal probe was used, it could not be determined whether the srRNAs in the periplastidal compartment had a different sequence to the srRNAs in the main cytoplasm (McFadden, 1990a). The results described here, using the probe specific for the long gene, clearly demonstrate that long gene transcripts from the nucleomorph accumulate in the periplastidal space, and we conclude that the nucleomorph encodes rRNAs for ribosomes in the periplastidal space. Transcripts of the nucleomorph (long) gene are not detectable in the main cytoplasm, suggesting that the physical isolation of the two compartments by the periplastid and PRER membranes prevents exchange of rRNAs.

**Mapping of srRNA genes to *Cryptomonas* Φ chromosomes**

Probing of the pulsed field gels demonstrates that the long and short srRNA genes occur on different chromosomes, which is consistent with the two genes residing in separate genomes. The nuclear (short) gene is located on one or more chromosomes larger than 300 kb.
Pulsed field gel electrophoresis of DNAs from isolated nucleomorphs of the cryptomonad Pyrenomonas salina has revealed that the nucleomorph of this species contains three chromosomes 190 kb, 195 kb, and 220 kb (Eschbach et al., 1991). We mapped the nucleomorph (long) gene to chromo-
Fig. 3. Localization of rRNAs within Cryptomonas Φ using four different probes. Bars, 0.3 μm. (A) Localization of all cytoplasmic-type rRNAs using the universal probe. Transcripts are present in the main cytoplasm (cy), the nucleolus (no) of the main nucleus (nu), and in the periplastidal space (cy'). No transcripts were observed in the chloroplast (chl). The nucleomorph is not present in this section. (B) Localization of bacterial-like rRNAs using a chloroplast-specific probe. Transcripts are present in the stroma of the chloroplast (chl) between the thylakoids. No transcripts were localized in the main cytoplasm (cy), main nucleus (nu), periplastidal space (cy'), or nucleomorph (nm). (C) Localization of short gene transcripts using the probe specific for the shorter gene (see Fig.1). Transcripts are present in the nucleolus (no) of the main nucleus (nu) and throughout the main cytoplasm (cy). No short gene transcripts were localized in the periplastidal cytoplasm (cy'). The nucleomorph is not present in this section. The few gold particles in the chloroplast (chl) are believed to be background noise. Mitochondrion, mi; pyrenoid, py. (D) Localization of long gene transcripts. Transcripts are present in the periplastidal space (cy'). No long gene transcripts are observed within the nucleolus (no) of the main nucleus (nu), the main cytoplasm (cy), the pyrenoid (py) or the chloroplast proper (chl). The nucleomorph is not present in this section.

Fig. 4. Localization of long gene transcripts and short gene transcripts. (A) Transcripts of the long gene are exclusively located within the periplastidal space (cy'), and are not detected in the chloroplast (chl), mitochondrion (mi), main cytoplasm (cy), or the nucleolus (no) of the main nucleus (nu). Pyrenoid, py. Bar, 0.4 μm. (B) Localization of long gene transcripts within the nucleolus-like zone of the nucleomorph (nm) and the periplastidal space (cy'). Chloroplast, chl; pyrenoid, py; host cytoplasm; cy. Bar, 0.4 μm. (C) Overview of short gene transcript localization in a whole cell. The short gene transcripts are present throughout the main cytoplasm (cy) but are not detected in the pyrenoid (py), nucleolus (nu), and periplastidal space (cy') or the nucleomorph (nm) which contains several electron-dense globules. The few gold particles in the mitochondrion (mi) and chloroplast (chl) are believed to be background noise. Bar, 1.0 μm.
The 130 kb band probably represents a small proportion of chloroplast chromosomes that became linearized, which would explain why this band was of a different intensity to the three nucleomorph chromosomes. The chloroplast probe also hybridized to the plug loading wells which contained circular chloroplast chromosomes that did not migrate into the gel. Because the plugs were removed prior to blotting, the signal is restricted to the plug well perimeter.

**Conclusions**

The data presented here confirm that cryptomonads are indeed a cell within a cell and provide compelling evidence for the hypothesis that these algae obtained chloroplasts from a eukaryotic endosymbiont. The endosymbiont nucleus persists and apparently produces its own set of translation machinery. The role of this translation machinery is not known, but it has been suggested that the nucleomorph may encode chloroplast proteins (see McFadden, 1993, for a summary). The chloroplast genome of cryptomonads is similar in size to land plant chloroplast genomes, which are estimated to encode only 10-20% of chloroplast proteins (Palmer, 1991). The remaining 80-90% of land plant chloroplast proteins are presumed to be encoded by nuclear DNA, and synthesized on cytoplasmic ribosomes prior to chloroplast import. By analogy, the cryptomonad nucleomorph, which represents the nucleus of the photosynthetic endosymbiont, could be expected to encode chloroplast proteins that would be synthesized on ribosomes in the periplastidal space and translocated into the chloroplast across the inner pair of chloroplast membranes. Chloroplast proteins could also be encoded by DNA in the main nucleus and synthesized in the main cytoplasm. Such proteins would need to pass through the PRER, the periplastid membrane, and the two chloroplast envelopes to reach the stroma. The identification of nucleomorph chromosomes in pulsed field gels opens the way to investigate the function of the endosymbiont genome and cytoplasm in the symbiotic partnership.

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