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Structure, organization and evolution of the mitochondrial ribosomal RNA genes in *Chlamydomonas*

by

Eileen M. Denovan-Wright

Submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
August, 1994

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Abstract

The mitochondrial rRNAs of the green alga *Chlamydomonas eugametos* are discontinuously encoded in separate gene pieces that are scrambled in order and interspersed with protein coding genes. Southern and northern hybridization analyses indicate that the mitochondrial rRNA genes of *C. eugametos* are similar in structure and organization to those of its interfertile close relative *Chlamydomonas moewusii* but different in many respects to the previously described fragmented and scrambled mitochondrial rRNA gene pieces of *Chlamydomonas reinhardtii*. The mitochondrial rRNA genes of these three *Chlamydomonas* algae, therefore, contrast with the conventional mitochondrial rRNA genes found in land plants and other green algae. Three lines of evidence support the hypothesis that the discontinuous and scrambled gene pieces encode the functional rRNAs in *Chlamydomonas* mitochondria. First, no continuous rRNA genes exist in the mitochondrial DNA of the three *Chlamydomonas* algae. Second, the individual transcripts of these mitochondrial rRNA gene pieces have termini that are confined to previously defined variable rRNA domains and the rRNA transcripts have the potential to form standard rRNA secondary structures through inter-molecular base-pairing. Third, mitochondrial ribosomes and ribosomal subunits were identified by northern blot hybridization of fractionated *C. eugametos* cellular ribosomes. Based on a detailed comparative analysis of the mitochondrial rRNA genes in *C. eugametos* and *C. reinhardtii*, calculations of the minimal number of transpositions required to convert hypothetical ancestral rRNA gene organizations to the arrangements observed for *C. eugametos* and *C. reinhardtii* and on a limited survey of the size of mitochondrial rRNAs in other members of the genus, it appears that the last common ancestor of *Chlamydomonas* algae had fragmented mitochondrial rRNA genes that were co-linear with conventional rRNA genes and that separate processes responsible for the further division of the rRNA genes into separate coding regions and the scrambling of these coding regions have occurred since the divergence of the major lineages leading to *C. eugametos* and *C. reinhardtii*. However, because phylogenetic analysis did not reveal a specific affiliation between the mitochondrial rRNA gene sequences of *Chlamydomonas* and other green algae, land plants or bacteria, it is not possible at this time to rule out the possibility that the mitochondrial rRNA genes of *Chlamydomonas* had an independent evolutionary origin from those of other green algae and land plants.

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A, adenine

bp, base pair

C, cytosine

°C, degree Celsius

dNTP, 2'-deoxynucleoside-5'-triphosphate

ddNTP, 2',3'-dideoxynucleoside-5'-triphosphate

DNA, deoxyribonucleic acid

EDTA, ethylenediaminetetraacetate

g, gram

g, gravity

G, guanine

kb, kilobase pair

kDa, kiloDalton

mt$^+$, mating type "plus"

nt, nucleotide(s)

rpm, revolutions per minute

SDS, sodium dodecyl sulfate

T, thymine

Tris, tris(hydroxymethyl)aminomethane

UV, ultraviolet

U, uracil

vol, volume

Acknowledgements

I would like to thank my supervisor, Dr. Robert W. Lee, for his guidance during the course of my research and during the writing of this thesis. I also wish to thank the members of my supervisory committee, M.W. Gray and W.F. Doolittle, for many helpful discussions. I would like to thank R.R. Gutell for assistance modelling the secondary structures of the rRNAs and group I introns, M. Schnare for his valuable advice throughout the course of this work, D. Spencer for assistance with the phylogenetic analysis, D. Sankoff for the DERANGE analysis and E. Zouros and M. Ball for help with statistical analysis. This work was supported by a Natural Sciences and Engineering Research Council of Canada grant to R.W. Lee as well as an Isaac Walton Killam Memorial Scholarship and an NSERC post-graduate scholarship to E. Denovan-Wright.

Preface

Chapter 1 is a modified version of the following published paper:

Denovan-Wright, E.M. and R.W. Lee (1992) Comparative analysis of the mitochondrial genomes of *Chlamydomonas eugametos* and *Chlamydomonas moewusii*. Curr. Genet. 21:197-202.

Permission has been obtained to include data presented in this paper as part of this thesis.

General Introduction

The ability to faithfully translate genetic information into functional proteins is a fundamental biological process. Because all extant organisms utilize ribosomes to catalyze translation, a rudimentary form of a ribosome-based translational system was most likely established in the earliest living organisms (Woese, 1987). Prior to the emergence of cellular life, it has been hypothesized that RNA contained genetic information and carried out catalytic functions (Darnell & Doolittle, 1986). In these contexts, the study of RNA within modern ribosomes may lead to an appreciation of the structure of the most primitive cellular translational machinery and may indicate the form and structure of RNA molecules that initially evolved the ability to synthesize polypeptides.

Ribosomal RNA

Ribosomes are large macromolecular complexes composed of large and small ribosomal subunits. Each subunit has a unique ribosomal RNA (rRNA) core and numerous ribosomal proteins (r-proteins) (Spirin, 1974 and references therein). In addition to the small (SSU) and large (LSU) subunit rRNAs, a 5S rRNA has been found associated with prokaryotic and eukaryotic, cytosolic and chloroplast ribosomes (Azad & Lane, 1973; Monier, 1974; Azad & Lane, 1975). 5S rRNA genes have been identified in the mitochondrial genomes of land plants (Spencer *et al.*, 1981; Oda *et al.*, 1992) and the green alga *Prototheca wickerhamii* (Wolff *et al.*, 1994) but not in other mitochondrial genomes (Gray, 1992). The function of 5S rRNA has not been determined.

Ribosomal RNA has been implicated in each stage of translation including mRNA selection during initiation, elongation, termination, ribosomal subunit association and the alignment of charged tRNA with mRNA (reviewed by Dalhberg, 1989; Noller, 1991). In addition, peptidyl transferase activity remains associated with rRNA even after 95% of the protein has been removed from ribosomes or large ribosomal subunits by treatments with proteases, ionic detergents and phenol extraction but is inactivated by treatment with ribonuclease, chelating agents or kethoxal, a chemical that modifies guanosine within the rRNA (Noller, 1991; Noller *et al.*, 1992). Several protein synthesis-inhibiting antibiotics protect specific nucleotides within rRNA from chemical modification and many antibiotic-resistant mutants have altered rRNA sequences in close proximity to the protected nucleotides (Moazed & Noller, 1987; Cundliffe, 1990; Woodcock *et al.*, 1991). Moreover, omission of individual ribosomal proteins either using genetic or ribosome reconstruction experiments impairs but does not abolish translation (Nomura & Held, 1974; Dabbs, 1979). Together, these observations support the hypothesis that rRNA, in a functional conformation, is directly involved in aligning interacting molecules and catalyzing some of the reactions that occur during translation (Noller *et al.*, 1990). This conclusion contrasts with earlier hypotheses that catalytically important ribosomal proteins were positioned on an rRNA scaffold (Spirin, 1974). Ribosomal proteins are thought to be involved in the correct positioning and stabilization of rRNA and co-factors, as well as in the induction and stabilization of conformational changes in the ribosome during translation (Noller, 1991).

Comparison of the sequence of rRNA in major evolutionary lineages has revealed that some short regions (10-20 nts) within rRNA molecules are highly conserved in primary sequence suggesting that these regions are functional (Noller, 1991). Comparative analysis has also revealed that other rRNA sequences show co-variance of potential Watson-Crick pairing and are, therefore, conserved in potential secondary and tertiary structures (Gutell *et al.*, 1986; Gutell *et al.*, 1992b). Both classes of conserved rRNA sequences are interspersed

between regions that vary in primary sequence and length (Gray *et al.*, 1984; Noller, 1984; Cedergren *et al.*, 1988; Gray, 1988; Gray *et al.*, 1989; Gray & Schnare, 1995). These observations support models in which rRNA sequences, separated within the linear rRNA molecule, participate in intra-molecular base pairing and form the minimum functional core of the rRNA structure. The variable regions are confined to peripheral regions of the secondary structure models (Noller, 1984; Gutell & Fox, 1988; Gutell *et al.*, 1990; 1992a; 1992b) and some may have a role in pre-rRNA processing and subsequent ribosome assembly (Raué *et al.*, 1990). A minimum rRNA core structure is present in all major evolutionary lineages despite differences in primary sequence and size of variable regions within rRNAs, although the mitochondrial rRNAs of animals and some trypanosomes are greatly reduced in size and lack some of the characteristic helices and loops of other rRNAs (Gray *et al.*, 1984; Gray,1988). Almost every proposed base-pair within the rRNA secondary structure models has been confirmed by at least two compensatory changes (Gutell *et al.*, 1992b). In addition, the overall structure of ribosomes is similar in archaebacteria, eubacteria and eukaryotes (Lake, 1985).

Studies on the interactions between components of the ribosome are generally consistent with the secondary structure models proposed. For example, a specific fraction of the rRNA is protected by r-proteins from nuclease digestion and the protected rRNA fragments are derived from single-stranded RNA regions (Möller *et al.*, 1969). Photo-crosslinking studies of tRNA and ribosomes indicate that the anticodon loops of tRNAs interact with single-stranded regions of the SSU rRNA. The amino-acyl terminus of tRNA interacts with three single-stranded regions of the peptidyl transferase centre that are in close proximity in secondary structure models (Noller, 1991). Moreover, rRNA cross-linking studies indicate that bases within domains V and VI of the LSU rRNA physically associate, as predicted by co-variance analysis of potential tertiary interactions. Ribosomal protein and antibiotic-binding sites on rRNA also indicate that sections of rRNA dispersed throughout the linear molecule are in close

proximity in secondary structure models (summarized in Bartlett *et al.*, 1979; Harris *et al.*, 1989; Ehresmann *et al.*, 1990; Noller *et al.*, 1990).

Discontinuous rRNAs

Although the majority of SSU and LSU rRNAs are large intact polyribonucleotide chains, there are examples of discontinuous or fragmented rRNAs which, by inter-molecular base pairing, contribute the functionally important and evolutionarily conserved core of these rRNA molecules (Gray *et al.*, 1989). Low-molecular-weight rRNA was isolated from several species of bacteria (Cahn *et al.*, 1970; Doolittle, 1973 and references therein), algae (Loening, 1968; Rawson & Stutz, 1968; Woodcock & Bogorad, 1970; Bourque *et al.*, 1971; Kochert & Sansing, 1971; Rawson *et al.*, 1971), plants (Leaver, 1973; Kössel *et al.*, 1985) and insects (Loening, 1968) in the early 1970s. During this period of time, however, it was thought that low-molecular-weight rRNA was a degradation product of large rRNA molecules. This view prompted several investigators to search for conditions that allowed for the isolation of High-molecular-weight rRNA (Rawson *et al.*, 1971; Cattolico & Jones,1972; Ishikawa & Newburgh, 1972). High molecular weight rRNA was observed only under conditions that favoured rRNA base pairing.

The most common example of the sub-division of one rRNA transcript into two separate rRNA molecules is found in eukaryotic cytosolic ribosomes. The LSU rRNA in these ribosomes is typically composed of a 5.8S and a 25-28S rRNA. However, the LSU rRNA of the ancient eukaryote *Vairimorpha necatrix* (Vossbrinck & Woese, 1986) is a single LSU rRNA. The 5.8S rRNA is homologous to the 5' end of the *E. coli* LSU rRNA while the 25-28S rRNA is homologous to the 3' portion of the *E. coli* LSU rRNA (Nazar, 1980, 1982; Cox & Kelly, 1981; Jacq, 1981; Clark & Gerbi, 1982). The 5.8S and 28S rRNAs are co-transcribed in the 5' to 3' order in which they appear in the mature rRNA secondary structure models, and

the nucleotides separating the two mature rRNAs are excised post-transcriptionally after base-pairing occurs in the primary LSU rRNA transcript (Pene *et al.*, 1968; Pace *et al.*, 1977). The eukaryotic rRNA gene structure is co-linear with eubacterial and *V. necatrix* LSU rRNA genes but the mature rRNAs are discontinuous due to removal of an internal transcribed spacer (ITS). The position of the eukaryotic ITS separating the 5.8S and 28S rRNA coding regions coincides with an rRNA region that is variable in inter-specific comparisons (Gerbi, 1985; Gray & Schnare, 1990). Similarly, discontinuous land plant chloroplast LSU rRNAs are encoded by genes that are co-linear with those of eubacteria (Edwards *et al.*, 1981; Mackay, 1981). The recognition that the 5.8S and 28S rRNAs could associate without being covalently continuous, and that these and other discontinuous rRNAs duplicated the function of continuous rRNAs, led to the acceptance of the role of discontinuous rRNAs *in vivo*. Moreover, the *A. nidulans* LSU rRNA is synthesized as a large LSU rRNA and converted, *in vivo*, from a single RNA species to two individual rRNAs of 0.88 and 0.17 MDa demonstrating that these rRNAs are not degradation products (Doolittle, 1973).

The ability of discontinuous rRNAs to catalyze translation has been demonstrated experimentally *in vitro* (Cahn *et al.*, 1970; Pelham & Jackson, 1976). In *E. coli*, nuclease-treated large ribosomal subunits retain the ability to synthesize polyphenylalanine despite the disruption of the LSU rRNA. The sedimentation behaviour of these treated large ribosomal subunits does not change significantly, suggesting that the ribosomal subunit structure is unaffected by the disruption of the rRNA. Ribonuclease treatment initially cleaves the LSU rRNA into two molecules of 0.69 and 0.39 MDa (Cahn *et al.*, 1970). The position of the initial cleavage site corresponds to one of two possible variable regions and one of these variable regions is a site of discontinuity in the LSU rRNA of other organisms. To date, 25 of 46 variable regions as defined by Gray & Schnare (1995) have been found to be sites of

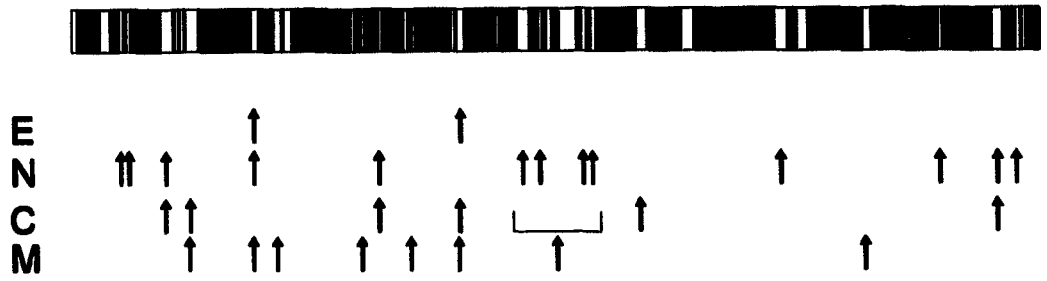


Figure 1. Sites of discontinuity (vertical arrows) in eubacterial (E), nucleocytoplasmic (N), chloroplast (C) and mitochondrial (M) LSU rRNAs relative to the continuous 2.9 kb LSU rRNA of *E. coli*. Conserved (solid) and variable (open) regions, as determined by the comparison of eukaryotic nucleocytoplasmic LSU rRNAs, are shown as they map to the linear *E. coli* LSU rRNA (redrawn from Gray & Schnare, 1990).

discontinuity in eubacterial, nucleus-encoded, mitochondrial and chloroplast rRNAs as determined by rRNA gene sequencing and transcript analysis (Fig. 1). Variable regions, therefore, can change in sequence and size or be amputated from the rest of the rRNA without, apparently, preventing ribosome biogenesis or function.

Although no specific function has been assigned to the variable domains defined by comparative analysis, there is evidence that some variable regions within rRNA are under structural and perhaps functional constraints (Engberg *et al.*, 1990) Hancock & Dover (1990) suggested that expansion of variable regions within rRNA may occur through amplification of simple sequence repeats, but noted that variable regions with complementary base-pairing may have a selective advantage. Sweeney & Yao (1989) demonstrated that certain variable rRNA regions can tolerate the addition of non-rRNA sequences, but mutants with rRNA containing the same sequence in other variable regions were not recovered. Burgin *et al.* (1990) have suggested that the expansion of the variable region within *Salmonella typhimurium* LSU rRNA is due to the acquisition of a transposable element converting a small variable region into an ITS.

Some conserved regions of rRNA must be covalently continuous

In contrast to the fragmentation of rRNA in variable regions of the molecule, there is considerable evidence that the conserved helices and single-stranded rRNA loops must maintain covalently continuous phosphodiester bonds if the ribosome is to remain functional. The SSU rRNA isolated from colicin E3-treated *E. coli* is fragmented near the 3' end of the molecule between adenosine and guanosine residues at positions 1493 and 1494, respectively (Bowman *et al.*, 1971; Senior & Holland, 1973), separating the terminal 3' end of the SSU rRNA from the remainder of the molecule; this region binds specifically to bacteriophage mRNA upstream of the initiator methionine codon (Steitz, 1969; Steitz & Jakes, 1975) and

includes the anti-Shine-Dalgarno (Shine & Dalgarno, 1974) and the translation-initiation promoting (Thanaraj & Pandit, 1989) sequences responsible for the recognition of the translation start site of mRNA in eubacteria and bacteriophage (Dalhberg, 1989). Colicin E3 cleavage inactivates the small ribosomal subunit (Bowman *et al.*, 1971). Ribosome function is also abolished by the fungal toxin α-sarcin, which catalyses phosphodiester bond cleavage at a specific guanosine residue within the nearly universal 14-nt α-sarcin loop in LSU rRNAs (Endo & Wool, 1982; Endo *et al.*, 1987). The bases within the α-sarcin loop of the *E. coli* LSU rRNA are protected from chemical modification when elongation factors EF-Tu or EF-G are bound (Moazed *et al.*, 1988). Elongation factor EF-Tu participates in the presentation of incoming tRNA during translation, while EF-G is necessary for translocation, both processes being central to translation (Moazed *et al.*, 1988). α-Sarcin can cleave de-proteinized rRNA after each purine within the sequence, suggesting that base pairing between regions of the rRNA or the binding of r-proteins limits the action of α-sarcin to a single base-specific cleavage of rRNA in ribosomes (Endo *et al.*, 1983). Finally, depurination of an adenosine residue in the α-sarcin loop by the RNA N-glycosidase, ricin, also abolishes translation (Endo *et al.*, 1987; Endo & Tsurugi, 1987).

Further evidence for the importance of maintaining the conserved rRNA sequences without interruption is provided by the study of retrotransposable elements. Retrotransposable elements R1 and R2 are found in the conserved helices of Domain IV of many insect LSU rRNA genes (Jakubczak *et* al., 1991). It has been estimated that up to 40% of the LSU rRNA genes within insect genomes contain these elements; however, the rRNA genes containing these retrotransposons are infrequently transcribed. Moreover, rRNA transcripts containing the insertion sequences are rapidly degraded during transcription (Jamrich & Miller, 1984). Therefore, rRNA with retrotransposon sequences in conserved regions is not incorporated into functional ribosomes.

Scrambled rRNA gene pieces

The majority of discontinuous rRNAs are derived from primary transcripts by the excision of ITSs and, therefore, homologous regions are co-linear with conventional rRNA genes. In contrast, there are a few examples of discontinuous rRNAs that are encoded by rRNA gene pieces that are not in the usual 5' to 3' order; to date, such scrambled rRNA gene pieces have only been found in mitochondrial genomes. The SSU and LSU mitochondrial rRNAs of *C. reinhardtii* are encoded by four and eight gene pieces, respectively, which are transcriptionally linked and processed to produce 12 discrete rRNA species in the size range 100 to 700 nt (Boer & Gray, 1991). These rRNA gene pieces are interspersed with each other and with protein-coding genes as illustrated in Figure 2 (Boer & Gray, 1988a; 1988b). The mitochondrial LSU rRNAs of *T. pyriformis* are encoded by two gene pieces and the sequence at the 3' end of the secondary rRNA structure is upstream, in the direction of transcription, from the 5' portion; these coding regions are separated by a transfer RNA gene (Heinonen *et al.*, 1987). Despite the rearrangements of the rRNA coding regions within these mitochondrial genomes, the low-molecular-weight, abundant rRNA transcripts maintain through inter-molecular base pairing the necessary sequences to form the minimum rRNA core suggested by secondary structure models, and the division of the rRNA coding regions is (and, therefore, the ends of the mature rRNA transcripts are) within variable rRNA domains. The stability of the rRNA species and the maintenance of the interacting bases within the primary rRNA sequence strongly supports their role as functional rRNAs. Within the putative mitochondrial DNAs (mtDNA) of a group of related Apicomplexan parasites, including *Plasmodium yoelii, Plasmodium falciparum* and *Theileria parva*, there are short segments of DNA that have sequence similarity to SSU and LSU rRNA coding regions (Vaidya *et al.*, 1989; Feagin *et al.*, 1992; Kairo *et al.*, 1994). Although not all of the conserved rRNA domains have been identified within these putative mtDNAs, five coding regions of the *T. parva* LSU rRNA have

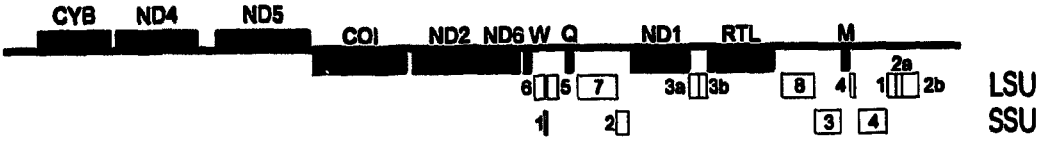


Figure 2. Organization of the linear 15.8 kb mitochondrial genome of *C. reinhardtii*. Protein-coding and tRNA genes are shown as solid boxes and include subunits 1,2,4,5 and 6 of NADH dehydrogenase (ND1,2,4,5 and 6), cytochrome oxidase subunit I (COI), apocytochrome b (CYB), reverse-transcriptase-like gene (RTL), tRNA-Met (M), tRNA-Gln (Q) and tRNA-Trp (W). The rRNA coding regions are shown as open boxes and include SSU rRNA (SSU, 1-4) and LSU rRNA (LSU, 1-8) gene pieces (redrawn from Harris, 1993).

sequence similarity to conserved domains IV and V, and secondary structure modelling duplicates the majority of the 3' half of the LSU rRNA, including the entire peptidyl transferase centre (Kairo *et al.*, 1994). The core of the SSU rRNA and a portion of the LSU rRNAs can be found in secondary structure models of the *P. falciparum* mitochondrial rRNA coding regions (Feagin *et al.*, 1992). The order of the putative mitochondrial rRNA coding regions is the same in the two species of Plasmodium but this order is highly rearranged relative to *T. parva*. The *Plasmodium* and *T. parva* rRNAs are transcribed from both strands of the putative mtDNA and, therefore, must associate post-transcriptionally.

Internal transcribed spacers and introns

Transcribed spacers and introns are fundamentally different types of intervening sequence found within functional primary rRNA transcripts. Transcribed spacers are positioned between continuous SSU and LSU rRNAs (Gerbi, 1985) and within discontinuous rRNA coding regions (Gray & Schnare, 1990), and are associated with variable regions of primary rRNA transcripts. Unlike transcribed spacers, all rRNA intron sequences are localized in highly conserved regions of the rRNA genes (see Gray & Schnare, 1990; 1995; Turmel *et al.*, 1993a). The post-transcriptional removal of transcribed spacers from the primary rRNA transcript generates rRNA termini (Pace & Burgin, 1990). Intron sequences, however, are precisely excised and the exon sequences covalently re-ligated during the splicing reaction, thereby maintaining the integrity of the polyribonucleotide chain (Cech, 1986; 1988; 1990). While comparisons between closely related organisms show that the transcribed spacers between SSU and LSU coding regions appear to have changed rapidly in sequence and length (Goldman *et al.*, 1983; Tague & Gerbi, 1984), introns contain a conserved structural core necessary for accurate splicing (Michel & Dujon, 1983; Cech & Bass, 1986; Cech, 1988; Doudna & Szostak, 1989; Williamson *et al.*, 1989; Michel & Westhof, 1990; Dávila-Aponte *et*

al., 1991). Several rRNA introns are self-splicing *in vitro* in the presence of Mg^{2+} and guanosine triphosphate (Cech *et al.*, 1981; Cech & Bass, 1986; Sogin & Edman, 1989; Cech, 1990; Kück *et al.*, 1990; Dürrenberger *et al.*, 1991). Some introns contain open reading frames that encode maturases (Lazowska *et al.*, 1980), recombinases (Manna *et al.*, 1991) or double-stranded DNA endonucleases (Jacquier & Dujon, 1985; Gauthier *et al.*, 1991; Marshall & Lemieux, 1991; 1992; Ma *et al.*, 1992a; Thompson *et al.*, 1992). No open reading frames or conserved secondary structures have been observed in ITSs associated with the internal division of rRNA. Moreover, transcribed spacers show an evolutionary pattern of vertical transmission (Gerbi, 1985; Engeberg *et al.*, 1990), while some introns are mobile genetic elements that have appeared sporadically in homologous genes in closely related organisms (Sogin *et al.*, 1986; Dujon, 1989; Lambowitz, 1989; Scazzocchio, 1989; Jacquier, 1990; Belfort, 1991; Van Oppen *et al.*, 1993).

Processing of discontinuous rRNAs

Little is known about the processing of the ITSs found in discontinuous rRNA. The *Drosophila melanogaster* cytosolic LSU rRNA is produced by the post-transcriptional removal of a 60-nt ITS separating two LSU rRNA species (Fujiwara & Ishikawa, 1986). A separate processing event removes the terminal 30 nt from the 3' end of the 5.8S rRNA to generate a 2S and 5.8S-like rRNA (Pavlakis *et al.*, 1979; Hancock *et al.*, 1988). Comparison of the position, size and sequence surrounding the ITS in these and other insect rRNA genes led to the suggestion that the sequence UAAU, within a single-stranded loop at the end of an AU-rich helix formed by the ITS sequence, is recognized specifically as the substrate by an endonuclease in these insects (Fujiwara & Ishikawa, 1986). However, Hancock *et al.* (1988) determined that this sequence occurs randomly throughout the coding region of the LSU rRNA in both single- and double-stranded regions and concluded that it was not a specific processing

signal. The division of the LSU rRNA occurs in the nucleus, while the subdivision of the 5.8S rRNA is a cytoplasmic event (Hancock *et al.*, 1988); therefore, these cleavages result from enzymes resident in different sub-cellular compartments. The cytosolic LSU rRNA of several species of trypanosome is composed of two large and five small rRNAs that are produced by the removal of ITSs (Campbell *et al.*, 1987). Although no consensus sequence was observed in the ITSs in trypanosomes, several polyadenosine and polyuridine tracts were observed in close proximity to the processing sites. Imperfect helices could be generated by computer analysis, suggesting that the processing signal in trypanosome fragmented rRNA is a helix separated from the functional rRNA segment of the pre-rRNA by a single-stranded region of the precursor (Campbell *et al.*, 1987). The ITS that is removed from the eubacterial LSU rRNA in *Salmonella* can also be modelled as an imperfect helix, and Burgin *et al.* (1990) suggested that this helix is recognized by ribonuclease III. However, the internal discontinuity within the mitochondrial LSU rRNA of *P. primaurelia* is located within RNA sequence devoid of base-pairing (Seilhamer *et al.*, 1984). The presence of a helix within a variable rRNA domain ., therefore, not the only requirement for internal division of the rRNA.

The scrambled mitochondrial rRNAs in *C. reinhardtii* are not produced by the removal of ITSs. Detailed transcriptional mapping of *C. reinhardtii* mtDNA, involving S1 nuclease protection and primer extension experiments (Gray & Boer, 1988), revealed extensive co-transcription of gene clusters including SSU and LSU rRNA gene pieces and interspersed tRNA and protein-coding genes. Boer & Gray (1991) found palindromic dispersed repetitive elements in close proximity to some, but not all, of the termini of the scrambled rRNA gene pieces. In addition, Boer & Gray (1988b) reported a consensus sequence of ACAA imperfectly aligned with the termini of the rRNAs. It has not been demonstrated that the dispersed palindromes or the ACAA consensus sequences are the substrate for processing enzymes in the mitochondria of *C. reinhardtii*.

Whether or not internal transcribed spacers are recognized and removed by a single endonuclease acting at each spacer within a discontinuous rRNA or whether the processing signals in ITSs are common to different organisms and sub-cellular compartments is not known. However, the vast number of proteins and small nuclear ribonucleoprotein particles that have been implicated in processing of nucleus-encoded and prokaryotic rRNA suggests that processing of internal rRNA discontinuities is unlikely to result from the action of a single enzyme (Hadjiolov & Nikolaev, 1976; Perry, 1976; Dalhberg *et al.*, 1978; Bowman *et al.*, 1981; Bowman *et al.*, 1983; King *et al.*, 1984; Nomura *et al.*, 1984; Mishima *et al.*, 1985; Vance, 1985; Kass *et al.*, 1987; Shumard & Eichler, 1988; Hannon *et al.*, 1989; Kuhn & Grummit, 1989; Raziuddin *et al.*, 1989; Shumard *et al.*, 1990; Srivastava & Schlessinger, 1990; Garrett *et al.*, 1991) .

Evolution of discontinuous rRNA

The discovery of catalytic RNA (Cech *et al.*, 1981; Kruger *et al.*, 1982; Guerrier-Takada *et al.*, 1983; Cech & Bass, 1986; Piccirilli *et al.*, 1992) supports earlier suggestions that the most primitive translational apparatus may have consisted exclusively of RNA (Crick, 1968; Orgel, 1968; Woese, 1970). However, it seems unlikely that rRNA molecules, several thousand nucleotides in length, were the original protein-synthesizing core. Moreover, the conservation in sequence, structure and genomic organization of rRNA and the sporadic appearance of discontinuous rRNAs among continuous rRNAs in phylogenetic trees strongly supports the hypothesis that discontinuous rRNAs have been derived from conventional rRNA genes (Gray & Schnare, 1995). The protogenote (Benner & Ellington, 1990), or last common ancestor of all extant cellular life, apparently possessed a large and complex translational machinery, with covalently continuous SSU and LSU rRNA molecules encoded by gene regions that were transcriptionally linked (Clark, 1987; Gray & Schnare, 1995). It has been

hypothesized that a collection of small interacting rRNAs or ribonucleoprotein particles (RNP) functioned co-operatively in earlier and more primitive translational systems. Over time, the complexity of these early ribosomes was increased as new functional rRNA domains were added that had the ability to interact with the most primitive translational core (Gray & Schnare, 1995). Clark (1987) suggested that small interacting rRNAs became fused by the interaction of RNA linking regions before the entrapment of rRNA information in DNA. Gray & Schnare (1995), however, proposed that fusion of the rRNA functional domains occurred by assembling coding modules in a primitive genome in an ancestor of the protogenote. Discontinuous rRNAs are thought to mimic the state of the evolving ribosome.

It may be possible to illuminate the evolutionary history of discontinuous rRNA genes by studying the structure and organization of mitochondrial rRNA genes in green algae of the genus *Chlamydomonas*. The description of the highly fragmented and scrambled mitochondrial rRNA gene pieces in *C. reinhardtii* indicates that this species and possibly other *Chlamydomonas* species have a fundamentally different structure than other chlorophycean algae and land plants (see Wolff *et al.*, 1994). There are a large number of described species within the genus that are maintained in culture collections and the phylogenetic relationships between these species are being determined by the study of the morphological characteristics and sequence analysis of nuclear and chloroplast rRNA genes (Buchheim *et al.*, 1990; Buchheim & Chapman, 1991; Turmel *et* al., 1990; 1993a). Therefore, comparisons between mitochondrial rRNA gene structure can be performed for both closely and distantly related algal pairs. Moreover, *Chlamydomonas* can be readily grown under defined conditions in the laboratory (Harris, 1989).

Statement of purpose

This dissertation has several objectives that will hopefully contribute to our appreciation of the evolution of discontinuous rRNA genes. Specifically, I wish to test whether the highly

fragmented and scrambled mitochondrial rRNA gene structure observed in *C. reinhardtii* is an ancestral characteristic of the mitochondrial genomes of *Chlamydomonas* algae or whether this unusual rRNA gene structure was derived after the divergence of the *Chlamydomonas* algae from other chlorophycean green algae. The first objective (Chapter 1) is to compare the mitochondrial genome organization, especially with regards to the organization of rRNA coding regions, of two closely related *Chlamydomonas* species, *Chlamydomonas eugametos* and *Chlamydomonas moewusii*. The second objective (Chapter 2) is to compare the genomic organization of the mitochondrial rRNA genes and their transcripts of *C. eugametos/C. moewusii* to the modular, interspersed mitochondrial rRNA gene pieces of *Chlamydomonas reinhardtii* (Boer & Gray, 1988a). *C. eugametos* and *C. reinhardtii* represent the two major lineages within the genus *Chlamydomonas* and, therefore, mitochondrial rRNA gene features common to both algae may be ancestral characteristics of mitochondrial genomes of this genus. The third objective of this thesis (Chapter 3) is to determine whether the small rRNA transcripts encoded by the mitochondrial rRNA gene pieces in *Chlamydomonas* are in fact associated with ribosomal subunits and monosomes. Such evidence would support the hypothesis that these rRNA fragments function in *Chlamydomonas* mitochondrial ribosomes. The fourth objective (Chapter 4) is to determine the phylogenetic relationship between the mitochondrial rRNA gene sequences of *C. eugametos* and *C. reinhardtii* and the relationship between the mitochondrial rRNA gene sequences of *Chlamydomonas* algae and other green algae, plants and bacteria. Phylogenetic analyses reported prior to the onset of this work (Gray *et al.*,1989; Cedergren *et al.*, 1988) did not reveal a clear affiliation of the mitochondrial rRNA gene sequences of *C. reinhardtii* with plants. It was considered possible that the addition of the *C. eugametos* mitochondrial rRNA gene sequences to the data base used in phylogenetic analysis would cause *Chlamydomonas* mitochondrial rRNA genes to affiliate with those of plants as well as that of the green alga, *P. wickerhamii*. The last objective of this dissertation (Chapter 5) is to examine the mitochondrial rRNA of several species of

Chlamydomonas and other algae within the Chlorophyta to determine whether fragmented mitochondrial rRNAs are a general feature of this group of algae.

Chapter 1

Comparative analysis of the mitochondrial genomes of *Chlamydomonas eugametos* and *Chlamydomonas moewusii*.

Introduction

Molecular studies on the unicellular green algae *Chlamydomonas reinhardtii* and *Chlamydomonas moewusii* are consistent with earlier morphological, physiological and cell-wall studies on these algae supporting their placement in phylogenetically distant lineages of the genus *Chlamydomonas* (reviewed by Gowans, 1976; Trainor & Cain, 1986; Buchheim *et al.*, 1990; Turmel *et al.*, 1990; 1993a). The chloroplast genomes of *C. moewusii* and *C. reinhardtii*, although having a similar complement of coding functions, are completely rearranged with respect to the order of these genes (Lemieux & Lemieux, 1985; Lemieux *et al.*, 1985); moreover, their chloroplast SSU and LSU rRNA genes display a level of sequence divergence greater than that of all land plants (Durocher *et al.*, 1989; Turmel *et al.*, 1991). Similarly, sequence data on nuclear SSU rRNA genes of *C. moewusii* and *C. reinhardtii* reveal differences equivalent to that of widely divergent land plant lineages (Jupe *et al.*, 1988; Buchheim *et al.*, 1990). In addition, the mitochondrial genome of *C. moewusii* has a circular map with a size of 22 kb (Lee *et al.*, 1991) while that of *C. reinhardtii* is linear and 6 kb smaller (Gray & Boer, 1988a; Michaelis *et al.*, 1990). Although the two mitochondrial genomes have a similar complement of genes, extensive rearrangements in the relative order of these genes have occurred between the two algae. The only other *Chlamydomonas* mitochondrial genome characterized in detail is that of *Chlamydomonas smithii* (Boynton *et al.*, 1987), an alga which is morphologically distinct from, but interfertile with, *C. reinhardtii* (Hoshaw & Ettl, 1966). The *C. reinhardtii* and *C. smithii* mitochondrial genomes show little difference except for a mobile intron in the cytochrome oxidase b (*cob*) gene of *C. smithii* (Boynton *et al.*, 1987; Matagne *et al.*, 1988; Colleaux *et al.*, 1990).

In order to study mitochondrial genome evolution in the genus *Chlamydomonas*, especially in regard to the structure of mitochondrial rRNA genes, I chose to characterize the mtDNA of *C. eugametos*, an alga which is closely related and interfertile with *C. moewusii*

(Gowans, 1963), and to compare this mtDNA with that of *C. moewusii*. Although *C. eugametos* and *C. moewusii* are morphologically identical, physiological differences exist between the two, and hybrid progeny show substantial lethality (Gowans, 1963; Cain, 1979), thereby suggesting that *C. eugametos* and *C. moewusii* are less closely related than are *C. smithii* and *C. reinhardtii*, which show no genetic incompatibilities (Bell & Cain, 1983). In addition, comparisons of the *C. eugametos* and *C. moewusii* mtDNAs will complement previous studies on the chloroplast (ctDNA) restriction fragment length polymorphisms of these species (Turmel *et al.*, 1987). Restriction fragment length polymorphisms in ctDNAs have been useful in genetic studies with *C. eugametos* and *C. moewusii* and have led to the identification and characterization of optional intron differences and other physical differences between their ctDNAs (Lemieux *et al.*, 1981, 1984a, 1984b, 1985, 1988; Lemieux & Lee, 1987; Gauthier *et al.*, 1991).

Restriction fragments of the *C. eugametos* mtDNA have been identified (Boer *et al.*, 1985; Lee *et al.*, 1990). Southern blot analysis of *C. eugametos* and *C. moewusii* total cellular DNA with a *C. reinhardtii cox1* probe identified single large *Eco*RI and *Sal* I fragments in both preparations which, based on their electrophoretic mobilities, were estimated to be around 20 kb in size; the fragments identified in the *C. eugametos* preparation were a few kb larger than those identified in the *C. moewusii* preparation (Boer *et al.*, 1985). Using the same probe, Lee *et al.* (1990) identified single-hybridizing *Ava*I fragments of 5.0 and 3.5 kb, in the total cellular DNA of *C. eugametos* and *C. moewusii*, respectively. These markers were used to demonstrate the uniparental transmission of the affected locus in reciprocal hybrid backcrosses. The *Eco*RI, *Ava*I and *Sal* I fragments of *C. moewusii* DNA identified with the *C. reinhardtii cox1* probe have now been localized on the physical map of the *C. moewusii* mtDNA (Lee *et al.*, 1991). The identification of additional differences in the mtDNAs of *C. eugametos* and *C. moewusii*, aside from their possible evolutionary significance, would permit more extensive studies on mtDNA transmission genetics in interspecific crosses with these

algae and thereby parallel the work mentioned previously with their ctDNAs. The goal of the work described here, therefore, is to clone and characterize the mitochondrial genome of *C. eugametos* and to perform a comparative study of its overall sequence structure relative to that of the *C. moewusii* mitochondrial genome.

The *C. eugametos* mtDNA has a 24 kb circular map and is thus 2 kb larger than the 22 kb circular mitochondrial genome of *C. moewusii*. Restriction mapping and heterologous fragment hybridization experiments indicate that the *C. eugametos* and *C. moewusii* mtDNAs are co-linear. Hybridization experiments with gene probes derived from protein-coding and rRNA-coding regions of wheat and *C. reinhardtii* mtDNAs support the view that the SSU and LSU rRNA-coding regions of the *C. eugametos* and *C. moewusii* mtDNAs are interrupted and interspersed with each other and with protein-coding regions, as are the rRNA-coding regions of *C. reinhardtii* mtDNA; however, the common arrangement of these coding elements in the *C. eugametos* and *C. moewusii* mtDNAs appears different from that of *C. reinhardtii*.

Materials and Methods

DNA isolation and restriction analysis

The major satellite or β-DNA fraction of the wild-type, mating-type "plus", strains of *C. eugametos* (UTEX 9) and *C. moewusii* (UTEX 97) were collected after equilibrium CsCl density gradient centrifugation of the total cellular DNA of each alga. The β-DNA fraction of *C. eugametos* and *C. moewusii* is predominantly ctDNA (Lemieux *et al.*, 1980) but it is also enriched for mtDNA (Lee *et al.*, 1991; and results presented here). The conditions employed in preparing the β-DNA fraction have been described by Lemieux *et al.* (1980). Restriction endonuclease digestions were performed using buffers and conditions recommended by the supplier. Restriction fragments of the *C. eugametos* and *C. moewusii* mtDNA clones, or the β-DNA fractions, were electrophoretically separated in 1% (w/v) agarose, using 1 x TBE, by standard techniques (Maniatis *et al.*, 1982)

Cloning

Prior to the onset of this work, R.W. Lee had recovered five *C. eugametos* mtDNA clones from a clone bank of *C. eugametos* β-DNA using colony hybridization with probes derived from the mtDNA clones of *C. moewusii*. These five clones represented five of the six *Hin*dIII fragments of the *C. eugametos* mtDNA. The remaining 0.5 kb *Hin*dIII fragment was not recovered despite repeated screening attempts. Each of the five *Hin*dIII *C. eugametos* mtDNA fragments was recovered using glass matrix (Geneclean, Bio101) following *Hin*dIII restriction digestion of isolated plasmids and fractionation of the restriction digests by agarose gel electrophoresis. The purified *Hin*dIII fragments were ligated into *Hin*dIII-digested and dephosphorylated pBluescript SK$^+$ vector; *E. coli* DH5α-F'IQ competent cells were transformed

with the ligated mixture and recombinant clones were identified by growth on plates containing LB medium and 100 μg/ml ampicillin and 10 μg/ml kanamycin, and which had been spread with IPTG and X-Gal. A second clone bank was generated using size-selected *Pvu*II β-DNA restriction fragments that had been purified from an agarose gel using glass matrix (Geneclean, Bio101). The DNA fragments were ligated into dephosphorylated, *Sma*I-digested pBluescript SK$^+$ and transformed into competent DH5α-F'IQ cells. A clone with a 1.2 kb *Pvu*II insert, containing the missing 0.5 kb *Hin*dIII fragment, was identified by colony hybridization using *C. eugametos* mtDNA clones flanking the missing fragment.

Hybridization

Electrophoretically separated restriction fragments were transferred from agarose gels to Hybond-N (Amersham) nylon filters according to Southern (1975). Inserts from the *C. eugametos* and *C. moewusii* mtDNA clones, used as hybridization probes, were isolated by electroelution (Maniatis *et al.*, 1982) after cleavage from their vector with *Hin*dIII and *Bam*HI respectively. The description and origin of the wheat and *C. reinhardtii* mitochondrial gene probes employed have been published (Lee *et al.*, 1991). Hybridization probes were radio-labelled to high specific activity (10^9cpm/μg) using random hexanucleotide priming (Feinberg & Vogelstein, 1983, 1984). When employing *C. eugametos* or *C. moewusii* mtDNA probes, hybridization was performed at high stringency (65°C for 16 h in 5 X SSPE, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1% Ficoll, 0.5% SDS and 100 μg/ml sonicated, denatured salmon sperm DNA). When probes were derived from *C. reinhardtii* or wheat mtDNA, hybridization was performed under less stringent conditions (50°C for 36 h) to allow the annealing of sequences with reduced sequence similarity. Following high-stringency hybridization, filters were washed twice in 2 X SSPE for 10 min at room temperature, twice in 2 X SSPE, 0.1% SDS at 65°C for 30 min and once in 0.1 X SSPE, 0.1% SDS at 65°C for 30

min. Filters probed with heterologous DNA and hybridized at lower stringency were washed in the same manner but at a temperature of 50°C and with the omission of the last low-salt wash.

Results

Cloning and mapping of the *C. eugametos* mitochondrial genome

Southern blot analysis of *Hin*dIII digests of the *C. eugametos* β-DNA fraction with six overlapping *Sau*3A-I partial-digest clones spanning 93% of the *C. moewusii* mitochondrial genome (Lee *et al.*, 1991) identified six *Hin*dIII fragments of *C. eugametos* mtDNA (Table 1) and ordered these into a circular 24 kb map (Fig. 3). Five of these fragments (*Hin*dIII fragments 1 through 5) were identified in a clone bank by R.W. Lee. The 0.5 kb *Hin*dIII fragment 6 was not recovered during the screening of the *C. eugametos* library. A 1.2 kb *Pvu*II fragment of *C. eugametos* mtDNA (*Pvu*II fragment 4) that spanned the uncloned *Hin*dIII fragment and overlapped the adjacent *Hin*dIII fragments 1 and 4 was identified in a size-selected library of *C. eugametos* β-DNA. The recovery of this clone, therefore, completed the cloning of the entire mitochondrial genome of *C. eugametos*.

The availability of clones spanning the entire *C. eugametos* mitochondrial genome allowed the verification of the *Hin*dIII map and the validation of other restriction sites in this DNA by two approaches. The first involved Southern blot analysis of *Hin*dIII, *Acc*I, *Ava*I, *Bst*EII, *Eco*RI, *Bam*HI, *Sal* I, *Eco*RV and *Pvu*II single restriction digests and *Ava*I-*Bst*EII, *Eco*RI-*Sal*I and *Hin*dIII-*Acc*I double digests of the *C. eugametos* β-DNA fraction using cloned *C. eugametos* mtDNA as hybridization probe; the second approach involved the mapping of the restriction sites for these enzymes directly in the cloned DNA. These complementary approaches led to the identification and numbering of the *C. eugametos* mtDNA restriction fragments summarized in Table 1 and their ordering on the 24 kb circular map shown in Figure 3. Both mapping strategies gave consistent results, therefore, no rearrangement of sequences had occurred during the cloning process. The sizes of the larger fragments

Table 1
C. eugametos mitochondrial DNA restriction fragments
generated by *Hin*dIII, *Acc*I, *Ava*I, *Bst*EII, *Eco*RI, *Bam*HI, *Eco*RV and *Pvu*II.

Fragment Number	Size (kb)							
	*Hin*dIII	*Acc*I	*Ava*I	*Bst*EII	*Eco*RI	*Bam*HI	*Eco*RV	*Pvu*II
1	9.8	6.6	16.5	16.8	22.0	19.0	13.0	10.6
2	7.2	4.2	5.6	4.2	1.8	4.4	8.2	10.1
3	3.5	3.8	1.7	1.4			2.2	1.9
4	2.1	2.5	0.15	1.1				1.2
5	0.9	1.35						
5'		1.35						
5"		1.35						
6	0.5	0.85						
7		0.75						
7'		0.75						
Totals	24.0	23.5	23.8	23.5	23.8	23.4	23.4	23.8

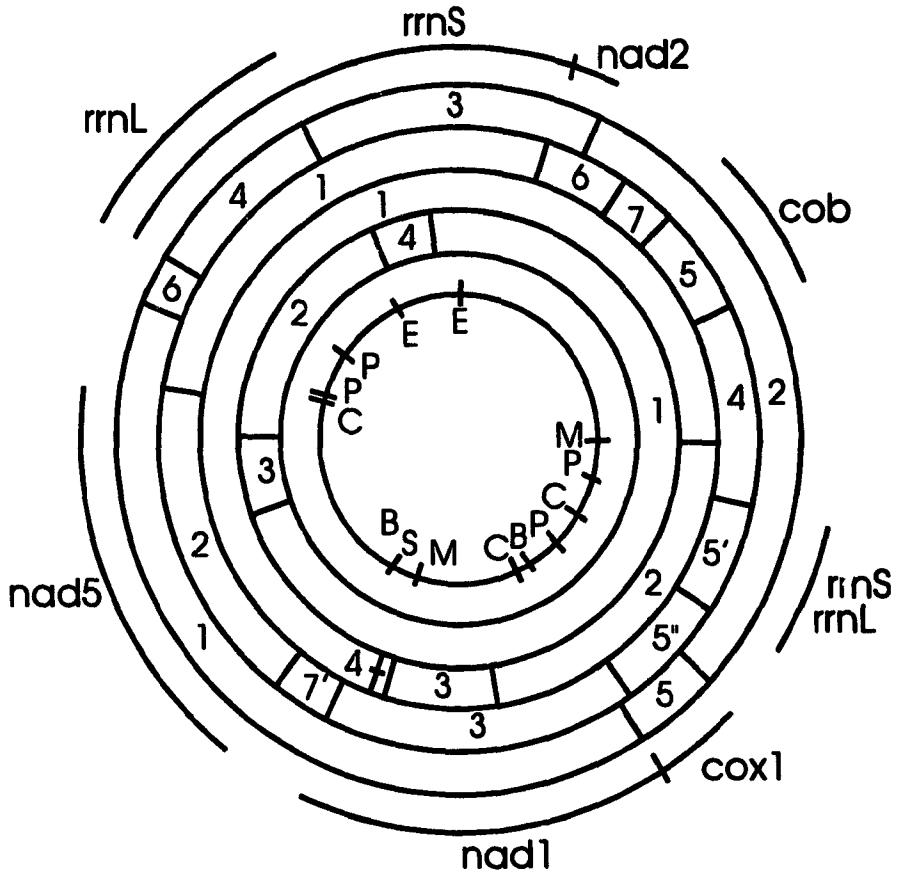


Figure 3. Physical map of the *C. eugametos* mitochondrial genome. The size of this genome is 24 kb. The four concentric circles starting from the outside represent the *Hin*dIII, *Acc*I, *Ava*I and *Bst*EII restriction site maps. Restriction site positions marked on the inner-most circle include, B, *Bam*HI; C, *Eco*RV; E, *Eco*RI; M, *Sma*I; P, *Pvu*II; and S, *Sal* I. Gene localizations represent the maximum extent of hybridization of the gene-specific probes employed.

reported in Table 1 and Figure 3 could not be determined directly by Southern blot analysis of single digests of the β-DNA fraction with the agarose gel concentrations employed and were, therefore, determined by summing fragment sizes after multiple restriction endonuclease digestions.

Localization of mitochondrial gene-hybridizing regions on the *C. eugametos* mtDNA map

Gene probes (Table 2) derived from *C. reinhardtii* and wheat mtDNA were employed to identify hybridizing regions of the *C. eugametos* mtDNA using filter-bound digests of the cloned mtDNA and/or β-DNA fraction. The results are summarized in Table 2. No hybridization was detected between the *C. reinhardtii nad2* and *nad6* probes, or the wheat *atp6*, *cob* and *cox2* probes, and the mtDNA in the β-DNA fraction of *C. eugametos*. Lee *et al.* (1991) were also unable to detect hybridization between these probes and the mtDNA in the β-DNA fraction of *C. moewusii*; however, using the same probes they were able to show hybridization of the *nad2* and *cob* probes with fragments of cloned *C. moewusii* mtDNA. Putative *nad2* and *cob* regions of the *C. eugametos* mitochondrial genome were identified by hybridization with the *C. moewusii nad2*- and *cob*-hybridizing regions.

Fragments of *C. eugametos* mtDNA that hybridized with the wheat mitochondrial *rrnS* and *rrnL* probes were identified using cloned mtDNA rather than the β-DNA fraction (chloroplast and mitochondrial DNA fraction) because these probes hybridized with chloroplast *rrnS* and *rrnL* genes more intensely than they hybridized with mitochondrial *rrnS* and *rrnL* rRNA genes. For the same reason, *C. moewusii* mtDNA fragments hybridizing with these probes were identified with cloned mtDNA (Lee *et al.*, 1991). The higher level of hybridization of the wheat mitochondrial rDNA probes with the chloroplast *rrnS* and *rrnL* genes of *C. eugametos* and *C. moewusii*, compared to their mitochondrial *rrnS* and *rrnL* genes, is not surprising in light of phylogenetic trees based on *rrnS* gene sequences.

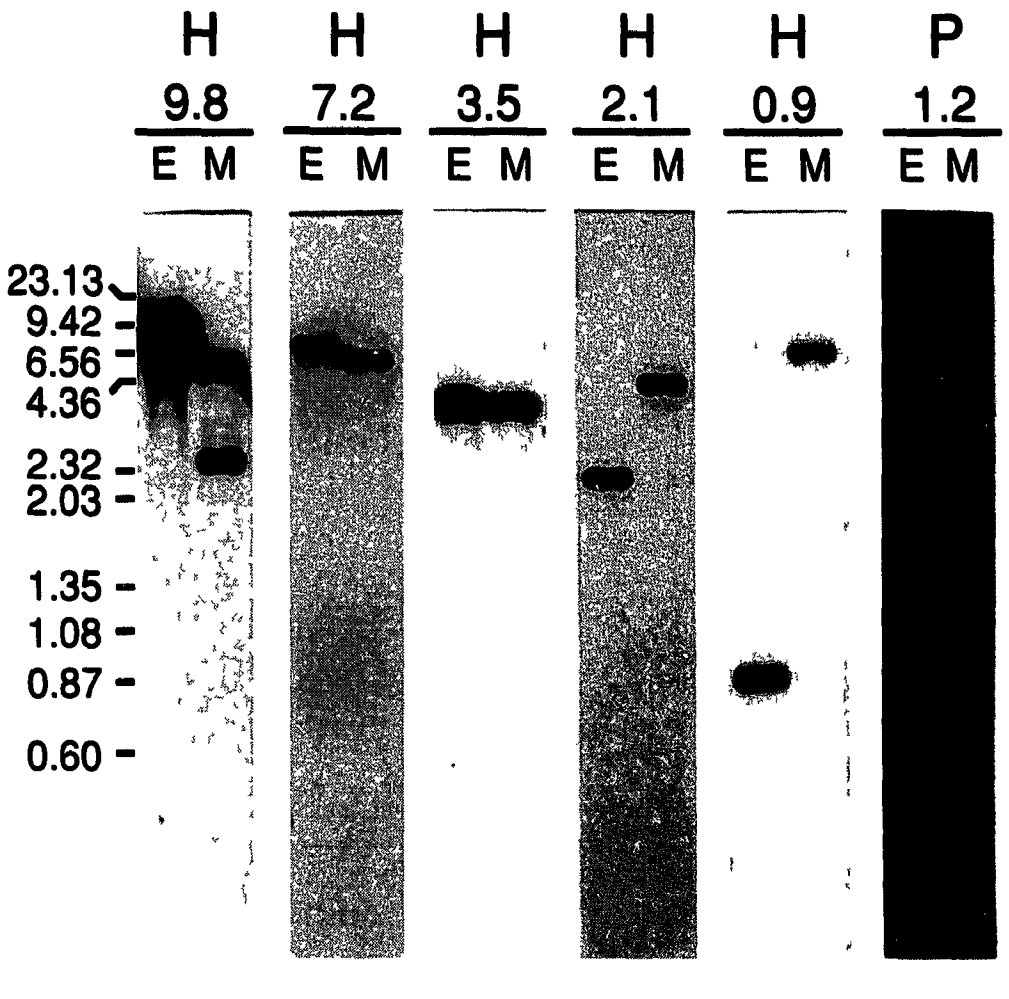


Figure 4. Southern blot analysis of the *Hin*dIII-digested β-DNA fractions of *C. eugametos* (E) and *C. moewusii* (M) with cloned fragments of *C. eugametos* mtDNA. The cloned *Hin*dIII (H) and *Pvu*II (P) fragments used as hybridization probes are identified by size in kb above each pair of autoradiograms. These correspond to *Hin*dIII fragments 1 through 5 and *Pvu*II fragment 4 (Table 1 and Figure 3), respectively. The molecular size markers indicated on the left were lambda DNA digested with *Hin*dIII and Φ X174 DNA digested with *Hae*III.

Table 2
Hybridization of wheat and *C. reinhardtii* mitochondrial gene probes to the *C. eugametos* β-DNA fraction or to cloned *C. eugametos* mtDNA.

Probe	Coding function	Source[a]	Filter bound DNA[b]	Hybridization[c]
nad1	Subunit 1 of NADH dehydrogenase	Cr	B	+
nad2	Subunit 2 of NADH dehydrogenase	Cr	B	-
nad5	Subunit 5 of NADH dehydrogenase	Cr	B	+
nad6	Subunit 6 of NADH dehydrogenase	Cr	B	-
cox1	Subunit I of cytochrome oxidase	Cr	B	+
cox2	Subunit II of cytochrome oxidase	Wheat	B	-
atp6	Subunit 6 of ATP synthetase	Wheat	B	-
cob	Apocytochrome b	Wheat	B	-
rrnS	SSU rRNA	Wheat	C	+
rrnL	LSU rRNA	Wheat	C	+
H5	SSU rRNA (S_3,S_4); LSU rRNA (L_1, L_2, L_4)	Cr	B,C	+
H8	LSU rRNA (L_7)	Cr	B,C	+

[a] Cr, *C. reinhardtii*
[b] B, β-DNA fraction; C, cloned *C. eugametos* mtDNA
[c] +, hybridization detected; –, no hybridization detected

These show a close relationship between the wheat mitochondrial *rrnS* gene and the corresponding genes from eubacteria and chloroplasts but only a distant connection of the wheat *rrnS* gene with the *C. reinhardtii* mitochondrial *rrnS* sequence (Gray *et al.*, 1989).

*Hin*dIII fragments of *C. reinhardtii* mtDNA, H5 and H8, were used as probes to further characterize rDNA-hybridizing regions of the *C. eugametos* mtDNA using the β-DNA fraction and cloned mtDNA. In *C. reinhardtii* mtDNA, *rrnS* and *rrnL* are subdivided into coding modules that are interspersed with each other and with protein-coding regions (Boer & Gray, 1988a). H5 is a 1.28 kb *Hin*dIII fragment containing part of the *rrnS* module S_3, and all of the *rrnS* and *rrnL* modules S_4, L_1, L_2 and L_4 as well as the elongator methionine tRNA; H8 is 659 bp *Hin*dIII fragment that encodes half of the *rrnL* module L_7 (Boer & Gray, 1988a). One region of H5 hybridization was localized to the same 1.35 kb *Acc*I fragment of *C. eugametos* mtDNA (*Acc*I fragment 5') identified with the wheat *rrnS* and *rrnL* probes. In addition, a region of weak H5 hybridization was localized to the 2.1 kb *Hin*dIII-*Acc*I fragment identified with the wheat *rrnS* probe. A region of strong H8 hybridization was localized to *Acc*I fragment 5'. Finally, a region of very weak H8 hybridization was localized to *Hin*dIII fragment 4. This 2.1 kb fragment also showed weak hybridization with the wheat *rrnS* probe but no hybridization with the wheat *rrnL* probe. Identical hybridization results were obtained with the H5 and H8 probes and corresponding fragments of the *C. moewusii* mtDNA as represented in Figure 5.

The mitochondrial genomes of *C. eugametos* and *C. moewusii* are co-linear

Figure 5 identifies nine fragments spanning the linearized mitochondrial genomes of *C. eugametos* and *C. moewusii*. Hybridization of *C. eugametos* and *C. moewusii* mtDNA clones to filter-bound *Hin*dIII, *Acc*I, *Ava*I and *Bst*EII digests of the β-DNA fraction of both species demonstrated that each of the fragments identified in the mitochondrial genome of one species has a counterpart at a similar position in the mitochondrial genome of the other species (Fig.

5). However, a 600 bp *Hin*dIII fragment of the *C. moewusii* mtDNA, within one of the compared regions, shows no hybridization with the *C. eugametos* mtDNA. The intra- and inter-specific hybridizations were equally strong under the conditions of high stringency employed in these experiments, thus confirming a considerable level of sequence similarity between the *C. eugametos* and *C. moewusii* mtDNAs and the equal enrichment of mtDNA in the β-DNA fraction of both species. Overall, these experiments support the co-linearity of the *C. eugametos* and *C. moewusii* mitochondrial genomes at the level of resolution examined. Of the 73 restriction sites identified in the *C. eugametos* and *C. moewusii* mtDNA, five are specific to *C. moewusii*, eight are specific to *C. eugametos* and 30 are common to both genomes.

Despite the obvious co-linearity of the *C. eugametos* and *C. moewusii* mitochondrial genomes, numerous restriction fragment length differences exist between them. Each of the nine compared fragments identified in the mtDNA of one of these species appears to differ in length from its counterpart in the other species by a value ranging from 0.1 to 2.3 kb. Hence, the 2 kb size difference between the *C. eugametos* and *C. moewusii* mtDNA is the net result of multiple addition-deletion differences rather than a single 2 kb difference. Size differences between the nine compared regions may, in some case, result from multiple addition-deletion differences within these regions.

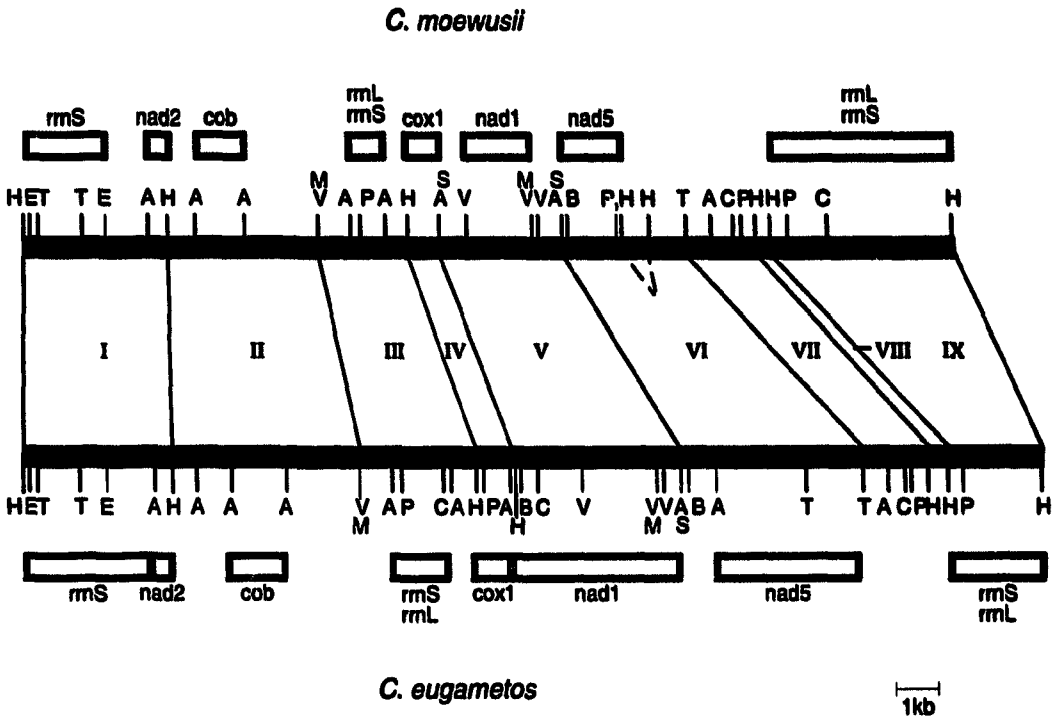


Figure 5. Alignment by cross-hybridization of the *C. eugametos* and *C. moewusii* mitochondrial genomes. The circular restriction maps were linearized at a common *Hin*dIII site and the cross-hybridizing regions connected. Restriction sites shown; A, *Acc*I; B, *Bam*HI; C, *Eco*RV; H, *Hin*dIII; M, *Sma*I; P, *Pvu*II; P_1, *Pst*I; S, *Sal* I; T, *Bst*EII; V, *Ava*I. Cross-hybridizing regions of the *C. eugametos* and *C. moewusii* mtDNAs were identified by Southern blot analysis of the β-DNA fractions and cloned mtDNA of *C. eugametos* and *C. moewusii* with probes derived from cloned *C. eugametos* and *C. moewusii* mtDNA. Length differences between these regions (*C. eugametos* length minus *C. moewusii* length) are as follows: I, +0.1 kb; II, +0.9 kb; III, +0.6 kb; IV, +0.1 kb; V, +1.4 kb; VI, +1.2 kb; VII, -0.1 kb; VIII, +0.1 kb; IX, -2.3 kb.

Discussion

The mitochondrial genome of *C. eugametos* has a 24 kb circular restriction map and thus is 2 kb larger than the circular mtDNA map of its interfertile partner *C. moewusii* (Lee *et al.*, 1991). While the restriction maps of these two algae are circular, the physical form of the mtDNA has not yet been determined. Southern blot hybridization analysis of the *C. eugametos* and *C. moewusii* mtDNA-enriched fraction with the cloned mtDNA fragments of *C. eugametos* and *C. moewusii* has revealed that these genomes are co-linear with respect to common sequence elements. The 2 kb size difference between them is the net result of multiple small addition/deletion differences and is not due to a single 2 kb size difference. Each of the nine compared restriction fragments in the mtDNA of one of these algae differs in size from the other by values ranging from 0.1 to 2.3 kb. Of the 73 restriction sites scored in the mtDNA of *C. eugametos* and *C. moewusii*, 13 are specific to *C. eugametos* or *C. moewusii* and 30 are common to both species.

Two regions of the *C. moewusii* mtDNA, hybridizing with both small and large subunit rRNA gene probes from wheat and/or *C. reinhardtii* mtDNA, are interrupted by regions hybridizing with a wheat *cob* probe and a *C. reinhardtii nad2* probe (Lee *et al.*, 1991); cross-hybridizing regions were identified in *C. eugametos* mtDNA with probes derived from the mtDNA of *C. moewusii*. The specific hybridization patterns of the rRNA gene probes cannot be explained by a simple duplication of a conventional rRNA operon in the *C. eugametos* and *C. moewusii* mtDNA. Rather, the results are more consistent with an rRNA gene structure resembling that of *C. reinhardtii* mtDNA, where small and large subunit rRNA-coding regions are subdivided into coding modules that are interspersed with each other and with protein-coding and tRNA genes (Boer & Gray, 1988a). In *C. eugametos/C. moewusii* mtDNA, however, it appears that the mitochondrial rRNA-coding regions are spread over a considerably larger distance (about 12 kb) compared to *C. reinhardtii* (6 kb) and are interrupted by different

protein-coding regions, i.e. *nad2* and *cob* versus *nad1* and *rtl*, respectively. Moreover, although the results support an identical arrangement of coding sequences within the rRNA-coding regions of *C. eugametos* and *C. moewusii* mtDNA, these coding regions appear to be rearranged relative to those of *C. reinhardtii* mtDNA. For example, two rRNA gene probes, H5 and H8, which originate from nearly opposite ends of the rRNA-coding regions of the *C. reinhardtii* mtDNA, hybridize strongly with single 1.35 and 0.8 kb *Acc*I fragments of the *C. eugametos* and *C. moewusii* mtDNAs, respectively (Fig. 5). In addition, *C. reinhardtii* probe H5, a 1.28 kb fragment, hybridized with two regions of the *C. eugametos* and *C. moewusii* mtDNAs that are separated by more than 5 kb in both DNAs. Finally, regions of the *C. eugametos* and *C. moewusii* mtDNAs represented by the 2.1 and 4.4 kb *Hin*dIII fragments, respectively, on the extreme right of the linear maps depicted in Figure 5, hybridized only very weakly with the *C. reinhardtii* and wheat probes. These observations may reflect either primary sequence divergence of functionally equivalent mitochondrial rRNA-coding regions or limited hybridization of the probe DNA to rRNA coding sequences involved in long-range interactions. Such interactions maintain secondary structure in functional rRNA (Gray & Schnare, 1990).

The *C. eugametos* and *C. moewusii* mitochondrial genomes appear to have an identical complement and an identical arrangement of protein-coding genes. The intensity of intra- and inter-specific hybridizations of the cloned *C. eugametos* and *C. moewusii* mtDNAs with *C. eugametos* and *C. moewusii* mtDNA fragments in β-DNA digests of these algae are indistinguishable, thereby supporting an extremely close sequence identity of these genomes. Although the *C. eugametos* and *C. moewusii* mitochondrial genomes have a complement of protein-coding genes similar to that of *C. reinhardtii* mtDNA, the order of these genes in *C. eugametos/C. moewusii* mtDNA, is completely rearranged relative to *C. reinhardtii* mtDNA. Moreover, our hybridization results suggest that while some of the protein-coding genes in *C. eugametos/C. moewusii* mtDNA, such as *cox1*, *nad1* and *nad5*, appear to have remained

similar to those of *C. reinhardtii* at the primary sequence level, others such as *nad2* and *cob* have diverged considerably. These results, together with those indicating extensive rearrangement of gene regions in the *C. eugametos/C. moewusii* mtDNA compared to *C. reinhardtii* mtDNA, are consistent with other evidence supporting a considerable evolutionary distance between *C. eugametos/C. moewusii* and *C. reinhardtii*.

The identification and mapping of physical differences between the mtDNAs of *C. eugametos* and *C. moewusii* thus add to the *Ava*I fragment differences described earlier (Lee *et al.*, 1990) a number of physical markers located throughout these molecules. It will be of interest to determine the molecular basis for these differences and to study their inheritance in interspecific crosses and hybrid backcrosses (Lee & Lemieux, 1990). In terms of their inheritance, it is important to know, for example, whether all of the newly identified markers are transmitted uniparentally from the mating type "plus" parent, as determined with the *Ava*I mtDNA markers, or if some alleles can be transmitted from the mating type "minus" parent and show a unidirectional spreading through all mtDNA molecules, in a manner similar to the mobile intron in the *cob* gene of the *C. smithii* mtDNA (Boynton *et al.*, 1987; Matagne *et al.*, 1988). A similar unidirectional spreading of certain ctDNA markers has been noted in interspecific crosses involving *C. eugametos* and *C. moewusii*; one of these markers has been shown to result from a mobile intron in the *rrnL* gene of the *C. eugametos* chloroplast (Lemieux & Lee, 1987; Gauthier *et al.*, 1991). Finally, the mtDNAs of *C. eugametos* and *C. moewusii* show an abundance of physical differences compared to the number of mtDNA differences between *C. reinhardtii* and *C. smithii*. This represents a potential advantage of the *C. eugametos-C. moewusii* system for studies of mitochondrial transmission genetics if conditions can be found that promote the biparental transmission of mtDNA in interspecific crosses with these algae.

Chapter 2

Comparative structure and genomic organization of the discontinuous mitochondrial ribosomal RNA genes of *Chlamydomonas eugametos* and *Chlamydomonas reinhardtii*.

Introduction

Small subunit (SSU) and large subunit (LSU) rRNAs in prokaryotic cells and in each of the protein-synthesizing compartments of eukaryotic cells are, generally, large continuous polyribonucleotide chains. Discontinuous or fragmented rRNAs, however, have been identified in the nucleo-cytoplasmic (Schnare *et al.*, 1982; White *et al.*, 1986; Spencer *et al.*, 1987; Schnare & Gray, 1990; Schnare *et al.*, 1990; Douglas *et al.*, 1991; Galvan *et al.*, 1991), mitochondrial (Seilhamer *et al.*, 1984; Schnare *et al.*, 1986; Heinonen *et al.*, 1987; Boer & Gray, 1988a; Vaidya *et al.*, 1989; Feagin *et al.*, 1992; Kairo *et al.* 1994) and chloroplast (Rochaix & Darlix, 1982; Lemieux *et al.*, 1989; Turmel *et al.*, 1991; 1993a) compartments of eukaryotic cells and in eubacteria (Doolittle, 1973; Burgin *et al.*, 1990; Skurnik & Toivanen, 1991). Discontinuous rRNAs can, by inter-molecular base-pairing, assume the same proposed secondary structures as their covalently continuous counterparts. In each example listed above, the discontinuities occur in variable regions of the rRNA molecules and are, therefore, confined to the periphery of the potential secondary structures (Gray & Schnare, 1995). Based on the conservation of sequences and the compensating changes that maintain helices, the catalytic core is preserved in both continuous and discontinuous rRNAs.

Although the majority of discontinuous rRNAs are derived from rRNA genes that are co-linear with the genes of unfragmented rRNAs, there are a few examples of discontinuous and scrambled rRNA gene pieces (Heinonen *et al.*, 1987; Boer & Gray, 1988a; Feagin *et al.*, 1992; Kairo *et al.*, 1994). Boer & Gray (1988a) found that the *C. reinhardtii* mitochondrial SSU and LSU rRNAs are encoded by four and eight gene pieces, respectively, the order of which is highly rearranged relative to the order in which the rRNAs appear in the proposed secondary structure. Furthermore, the SSU and LSU rRNA gene pieces are interspersed with each other and with protein-coding genes (Boer & Gray, 1988a; 1988b). Despite the unusual organization of the rRNA coding regions, the *C. reinhardtii* mitochondrial rRNA gene pieces are transcribed,

and transcript mapping and northern blot experiments identified 12 low-molecular-weight, abundant RNA species. Moreover, the conserved secondary structure core is preserved in the fragmented rRNAs, strongly supporting their role as the functional rRNAs in *C. reinhardtii* mitochondria.

Even though knowledge about mitochondrial SSU and LSU rRNA gene structure in other chlorophycean green algae is extremely limited, it is apparent that not all of these genes are discontinuous or discontinuous and highly scrambled like those in *C. reinhardtii* mtDNA. *Prototheca wickerhamii*, a colourless alga that is closely related to *Chlorella protothecoides* (Huss & Sogin, 1990), has conventional rRNA genes, that probably encode single, covalently continuous rRNA structures (Wolff & Kück, 1990; Wolff *et al.*, 1993). Southern blot analysis of the mtDNA from the exsymbiotic *Chlorella* strain N1a suggests that its mitochondrial SSU and LSU rRNA coding regions are not interspersed (Waddle *et al.*, 1990). A 2.0 kb mitochondrial LSU rRNA has been identified in *Scenedesmus obliquus*, but it is not known if this represents the entire mitochondrial LSU rRNA (Kück *et al.*, 1990).

Because of the conservation of rRNA sequence and potential secondary structure for all rRNAs, it is likely that all rRNA coding regions are related by descent (Gray *et al.*, 1984; Cedergren *et al.*, 1988). Due to the sporadic appearance of fragmented rRNAs in different lineages and genetic compartments, Clark (1987) and Gray & Schnare (1995) have proposed that the last common ancestor of all organisms and organelles had continuous rRNAs and that fragmentation of rRNA is a derived characteristic resulting from the acquisition of rRNA processing sites within variable rRNA domains. The fragmented and scrambled rRNA gene arrangements observed in the *C. reinhardtii* mtDNA are thought to have arisen by the rearrangement of gene pieces that were initially in the conventional 5' to 3' order. Because other chlorophycean green algae have conventional rRNA coding regions in their mtDNA, it is likely that the discontinuous and scrambled rRNA coding regions of *C. reinhardtii* mtDNA evolved from continuous rRNA coding regions present in the mitochondria of its green algal

ancestors; however, the phylogenetic position of the *C. reinhardtii* mitochondrial rRNA coding regions in global rRNA trees (Gray *et al.*, 1989) is sufficiently uncertain to leave open the possibility that they were acquired as either discontinuous or discontinuous and scrambled rRNA gene pieces in a separate endosymbiotic event relative to other green algae.

With the long-term goal of understanding the evolutionary history of scrambled, discontinuous rRNA coding regions in green algal mtDNA, I have undertaken a study of the mitochondrial rRNA genes and their transcripts in *Chlamydomonas eugametos*. This alga was selected because it is a distant relative of *C. reinhardtii* but shares with *C. reinhardtii* a common ancestor more recently than either of these algae share an ancestor with *P. wickerhamii*. Although *C. eugametos* and *C. reinhardtii* have been placed in the same genus, organismal and molecular data (Buchheim *et al.*, 1990; Turmel *et al.*, 1991; 1993a and references therein) support their placement in each of two major *Chlamydomonas* lineages. The number of nucleotide differences between both the chloroplast LSU rRNAs and the nuclear SSU rRNAs of *C. eugametos* and *C. reinhardtii* suggests an evolutionary distance between these algae equivalent to or greater than that observed between vascular and non-vascular land plants, as exemplified by tobacco and liverwort, respectively (Buchheim *et al.*, 1990; Buchheim & Chapman, 1991). The considerable evolutionary distance between *C. reinhardtii* and the interfertile close relatives, *C. eugametos* and *Chlamydomonas moewusii*, is also supported by differences in the mitochondrial genomes of these algae. The 15.8 kb linear mitochondrial genome of *C. reinhardtii* (Boer *et al.*, 1985; Michaelis *et al.*, 1990; Vahrenholz *et al.*, 1993) contrasts with the mitochondrial genomes of *C. eugametos* (Denovan-Wright & Lee, 1992) and *C. moewusii* (Lee *et al.*, 1991), which have circular maps of 24 and 22 kb, respectively. Hybridization of *C. reinhardtii* and wheat mitochondrial gene probes to *C. eugametos* and *C. moewusii* mtDNA supports the view that these DNAs have similar coding functions as the *C. reinhardtii* mtDNA; however, while gene order in the *C. eugametos* and *C. moewusii* mitochondrial genomes is co-linear, this order is highly rearranged relative to the

order of the genes in the *C. reinhardtii* mtDNA (Lee *et* al., 1991; Denovan-Wright & Lee, 1992). The numerous restriction fragment length differences between the mtDNAs of *C. eugametos* and *C. moewusii* are thought to be the result of optional introns and variations in intergenic regions (Denovan-Wright & Lee, 1992). A considerable sequence divergence between the *C. reinhardtii* and *C. eugametcs/C. moewusii* mitochondrial genes is suggested by the low level of hybridization of *C. reinhardtii* mitochondrial gene probes to *C. eugametos* or *C. moewusii* mtDNAs. Although hybridization of *C. reinhardtii* and wheat mitochondrial rRNA and protein-coding domains to the *C. eugametos/C. moewusii* mtDNAs supports a modular rRNA gene arrangement, both the organization of the rRNA coding regions and the particular protein-coding genes interspersed with these rRNA modules is different from that described for *C. reinhardtii* (Denovan-Wright & Lee, 1992; 1993).

In this chapter, I describe the modular, dispersed organization of the mitochondrial rRNA genes in *C. eugametos* and the structure of their low-molecular-weight transcripts. Northern blot analysis has established that the mitochondrial rRNA transcripts are of similar size in *C. eugametos* and *C. moewusii.* Because *C. eugametos* and *C. reinhardtii* represent two main lineages within the genus *Chlamydomonas* and because they each possess highly fragmented and scrambled mitochondrial rRNA gene pieces, I propose that this unusual rRNA gene arrangement was derived in the genus *Chlamydomonas* after its divergence from a chlorophycean green algal ancestor that possessed continuous mitochondrial rRNA genes. Differences in the structure and organization of the mitochondrial rRNA coding regions of *C. eugametos* and *C. reinhardtii* suggest that the processes giving rise to this unusual rRNA gene structure have been ongoing since the divergence of these two algae.

Materials and Methods

Cloning and DNA sequencing

*Five Hin*dIII DNA fragments and a *Pvu*II DNA fragment previously cloned in pBluescript II SK (+) (Stratagene, La Jolla, Ca.) and spanning the entire 24 kb mitochondrial genome of *C. eugametos* (Denovan-Wright and Lee, 1992) were employed in this study. In addition, a 7.2 kb *Eco*RI fragment of the *C. reinhardtii* mtDNA cloned in pUC9 and referred to as E1 by Boer and Gray (1988a) was used as a hybridization probe in northern blot analysis. Overlapping deletion subclones were generated using the exonuclease III / mung bean nuclease method (Henikoff, 1984). The methods employed in the production of recombinant phagemids and in the isolation of single-stranded DNA were as described by Karger (1990). DNA sequences of both strands were determined by the dideoxy chain termination method (Sanger *et al.*, 1977) using [α-^{35}S] dATP (1000 Ci/mmol), purchased from Amersham, and T7 polymerase, using the protocol and reagents provided by Pharmacia LKB Biotechnologies with the T7 sequencing kit. Sequencing reaction products were resolved on 6% polyacrylamide, 7 M urea gels using a 0.5-2.5 X TBE buffer gradient (Biggin *et al.*, 1983). Oligonucleotide primers employed to initiate the polymerization reactions were either the -20 universal primer, SK primer or KS primer (Stratagene, La Jolla, Ca.), and in some cases oligonucleotides specific to *C. eugametos* mtDNA.

Isolation of total cellular RNA

Using the method of Turmel *et al.* (1988), total cellular RNA was isolated from wild-type synchronized, phototrophic cultures (Lemieux *et al.*, 1980) of *C. eugametos* (UTEX 9), *C.*

moewusii (UTEX 97) and *C. reinhardtii* (137c, *mt*$^+$) six hours after the onset of the light period when the culture densities were between 2.5 and 4 x 10^6 cells/ml.

Northern blot analysis

Aliquots of total RNA (10 μg) were either glyoxalated and subjected to agarose gel electrophoresis (Maniatis *et al.*, 1982) or heat denatured in the presence of 7M urea and subjected to acrylamide gel electrophoresis (Schnare & Gray, 1990). The RNAs that were electrophoretically separated in agarose gels were transferred to Hybond-N membranes (Amersham) by vacuum blotting using conditions recommended by the manufacturer (Pharmacia). RNAs that had been resolved by polyacrylamide gel electrophoresis were transferred to Hybond-N membranes using a transblot apparatus (BioRad) in 0.5X TBE buffer (1X TBE = 100 mM Tris, 90 mM boric acid, 1 mM Na_2EDTA, pH 8.3) for 60 min at 108 volts. After transfer, the RNA was covalently cross-linked to the membranes by UV illumination at 1200 uW/cm^2 for 3 min and then baked at 80°C for 2 h under vacuum.

Probes used for northern blot analysis were isolated using glass matrix (Geneclean, Bio101) after the appropriate restriction endonuclease digestion of cloned DNA and electrophoretic separation in agarose gels (Maniatis *et al.*, 1982). The filter-bound RNA was allowed to hybridize with DNA restriction fragments that were radio-labelled by random priming (Feinberg & Volgelstein, 1983, 1984) using [α-^{32}P] dCTP (3000 Ci/mmol; Amersham) and Klenow DNA polymerase (Amersham). The filters were pre-hybridized for 4 h at the hybridization temperature (42°C) in 5X SSPE (20X SSPE = 3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.4), 50% v/v deionized formamide, 5X Denhardt's solution (1X Denhardt's solution = 0.1 % w/v polyvinylpyrollidone, 0.1% w/v bovine serum albumin, 0.1% w/v Ficoll) and 100 μg/ml denatured salmon sperm DNA. Heat-denatured radio-labelled probe was added to a final concentration of 2.5 ng/ml and the hybridization was allowed to proceed at 42°C for 48

h. The filters were washed twice in 5X SSPE at 42°C, twice in 1X SSPE, 0.1% w/v SDS at 42°C and twice in 0.1X SSPE, 0.1% SDS at room temperature. Higher stringency washes employed the same buffer solutions but the temperature was raised to 65°C.

RNA sequencing

Positions of the 5' termini of all mitochondrial rRNA species were determined by primer extension analysis using oligonucleotides complementary to the regions downstream from these termini. RNA sequencing was employed to determine the exon boundaries and internal sequence of each mitochondrial rRNA species. The sequencing reactions were performed as described by Geliebter (1987), with the exception that actinomycin D was omitted from the reactions, using AMV reverse transcriptase (Pharmacia), an appropriate oligonucleotide to initiate cDNA synthesis and total cellular *C. eugametos* RNA (10 μg) as the template . Primer extension and RNA sequencing reaction products were subjected to electrophoresis in 6% polyacrylamide, 7 M urea, 0.5-2.5 X TBE gradient sequencing gels (Biggin *et al.*, 1983). The latter were produced by the method of Geliebter (1987) except that the ddNTPs in the RNA sequencing reactions were omitted. Molecular weight markers included in these gels were dideoxy sequencing reactions of template M13 mp18 initiated with the universal -20 primer using 3000 Ci/mmol [α-^{32}P] dATP (Amersham) and T7 polymerase (Pharmacia).

The 3' terminal nucleotide of each rRNA species was estimated from the size of the transcripts detected in northern blot analysis and the position of 5' terminal nucleotides as they map to the DNA sequence.

Results

Structure and organization of the *C. eugametos* mitochondrial rRNA coding domains

Hybridization analysis with wheat and *C. reinhardtii* mitochondrial rRNA gene probes (Chapter 2) and sequence analysis of over 20 kb of the *C. eugametos* mitochondrial genome have led to the identification of nine rRNA coding segments that share sequence similarity to rRNA domains that are highly conserved in other organisms (Fig. 6 Ia). These include three SSU and six LSU rRNA gene pieces that are designated S_1-S_3 and L_1-L_6, respectively; the numbering of the gene pieces reflects the order in which homologous SSU and LSU coding sequences appear in conventional rRNA genes. The SSU and LSU rRNA gene pieces are distributed into four regions (1-4, Fig. 6 Ia) spanning 15 kb of the *C. eugametos* mitochondrial genome. They are extensively rearranged relative to the usual transcriptional order of the rRNA domains, and the SSU and LSU rRNA gene pieces are interspersed with each other. The DNA sequence of the four rRNA coding regions of *C. eugametos* mtDNA is presented in Figure 7.

Transcript analysis and secondary structure modelling of *C. eugametos* mitochondrial rRNA

Northern blot analysis was used to determine the size of the transcripts produced from the nine rRNA coding domains identified by sequence comparisons. Equivalent amounts of total cellular RNA from *C. eugametos, C. moewusii* and *C. reinhardtii* were fractionated by gel electrophoresis, transferred to nylon membranes and hybridized with probes derived from the *C. eugametos* entire mtDNA under high stringency hybridization conditions. Probes I - VI (Fig. 6 Ia) identified small strongly hybridizing *C. eugametos* RNAs between 100 and 700

Figure 6. I. Comparative organization of the rRNA gene pieces (solid boxes) in the *C. eugametos* (a) and *C. reinhardtii* (b) mtDNA and II. Northern blot analysis of the total cellular RNA from *C. reinhardtii* (R), *C. eugametos* (E) and *C. moewusii* (M). The organization of the *C. eugametos* mitochondrial SSU and LSU rRNA gene pieces (Ia), designated S_1-S_3 and L_1-L_6, respectively, is shown on the 24 kb circular mtDNA map numbered in the 5' to 3' order in which the corresponding RNAs associate in their proposed secondary structure. The hatched regions within the rRNA gene pieces S_2, L_5 and L_6 denote the positions of group I introns. The restriction sites determined during the physical mapping of the *C. eugametos* mtDNA include *Acc*I, A; *Ava*I, V; *Bst*EII, T; *Eco*RI, E; *Hin*dIII, H; *Pvu*II, P; and *Sma*I, M. One additional *Ava*I restriction site, identified by sequence analysis, is included on this map. The rRNA gene pieces are clustered into four regions designated by Arabic numerals inside the circular map as follows: region 1, L_3 and L_2; region 2, L_6 and S_2; region 3, L_4, S_1 and L_1; region 4, L_5 and S_3. All gene pieces are transcribed from the same strand and the orientation of transcription is clockwise on the circular map. The restriction fragments, indicated by Roman numerals outside the circular map, were used for Northern blot hybridizations (II). The organization of the *C. reinhardtii* mitochondrial SSU and LSU rRNA gene pieces, designated S_1-S_4 and L_1-L_8, respectively, according to Boer & Gray (1988a) is shown on the 15.8 kb linear mtDNA map (Ib). Common designations of mitochondrial rRNA gene pieces in *C. eugametos* and *C. reinhardtii* do not reflect equivalent units of coding information (see Fig. 10). Northern blot analysis of total cellular RNA that was either glyoxalated and subjected to agarose gel electrophoresis (IIa) or heat denatured in the presence of 7M urea and subjected to polyacrylamide gel electrophoresis (IIb). The *C. eugametos* mitochondrial DNA probes, indicated above each panel include for (IIa): I, 1.5 kb *Bst*EII-*Hin*dIII fragment; II, 1.2 kb *Pvu*II fragment; III, 2.1 kb *Hin*dIII fragment; IV, 1.7 kb *Eco*RI fragment; VI, 3.2 kb *Sma*I-*Hin*dIII fragment and for (IIb): V, 4.0 kb *Hin*dIII-*Sma*I fragment. The *C. eugametos* and *C. moewusii* SSU (S_1-S_3) and LSU (L_1, L_2 and L_4-L_6) rRNA transcripts and the positions of the RNA size markers (Bethesda Research Laboratories) in kb (IIa) and nucleotides (IIb) are indicated.

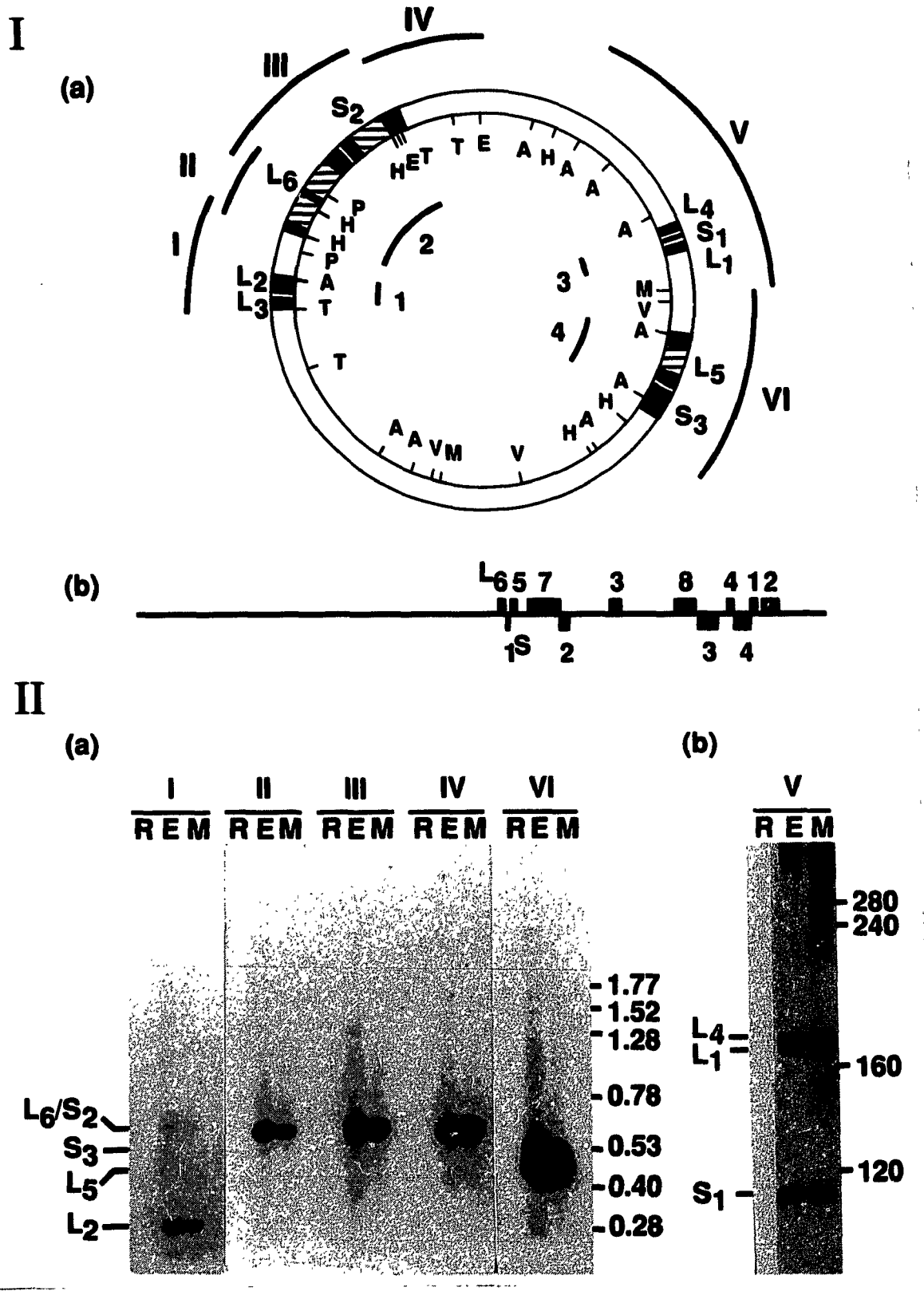

I
(a)
IV
III
II
I
V
VI
S_2
L_6
L_2
L_3
L_4
S_1
L_1
L_5
S_3
1
2
3
4
(b)
L_6 5 7 3 8 4 1 2
1S 2 3 4
II
(a)
I II III IV VI
REM REM REM REM REM
L_6/S_2
S_3
L_5
L_2
1.77
1.52
1.28
0.78
0.53
0.40
0.28
(b)
V
REM
280
240
L_4
L_1
160
120
S_1

Figure 6

Figure 7. DNA sequence of the *C. eugametos* mitochondrial DNA regions 1 through 4 encoding scrambled SSU and LSU rRNA gene pieces. SSU and LSU gene pieces are enclosed by solid lines, while group I introns within gene pieces S_2 (SSU.m1), L_5 (LSU.m1) and L_6 (LSU.m2 and LSU.m3), are enclosed by broken lines. The 5' and approximate 3' terminal nucleotide of each rRNA gene piece is indicated. The accession numbers of these DNA sequences are listed in the GenBank data library as follows: region 1, L28928; region 2, L28929; region 3, L28930; region 4, L28931.

Region 1

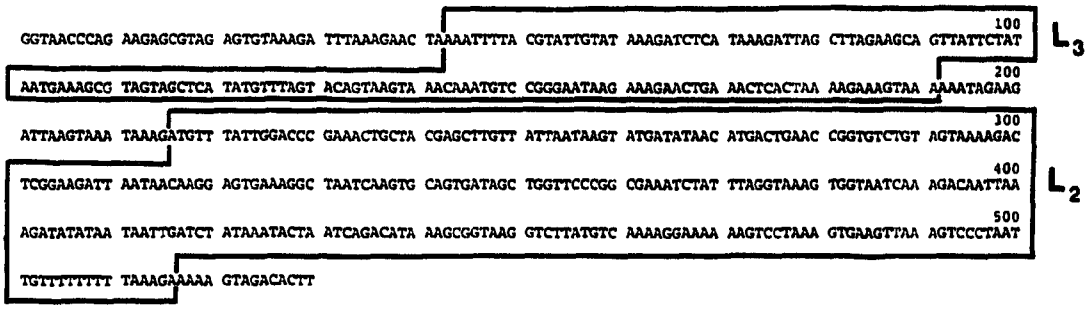


Figure 7

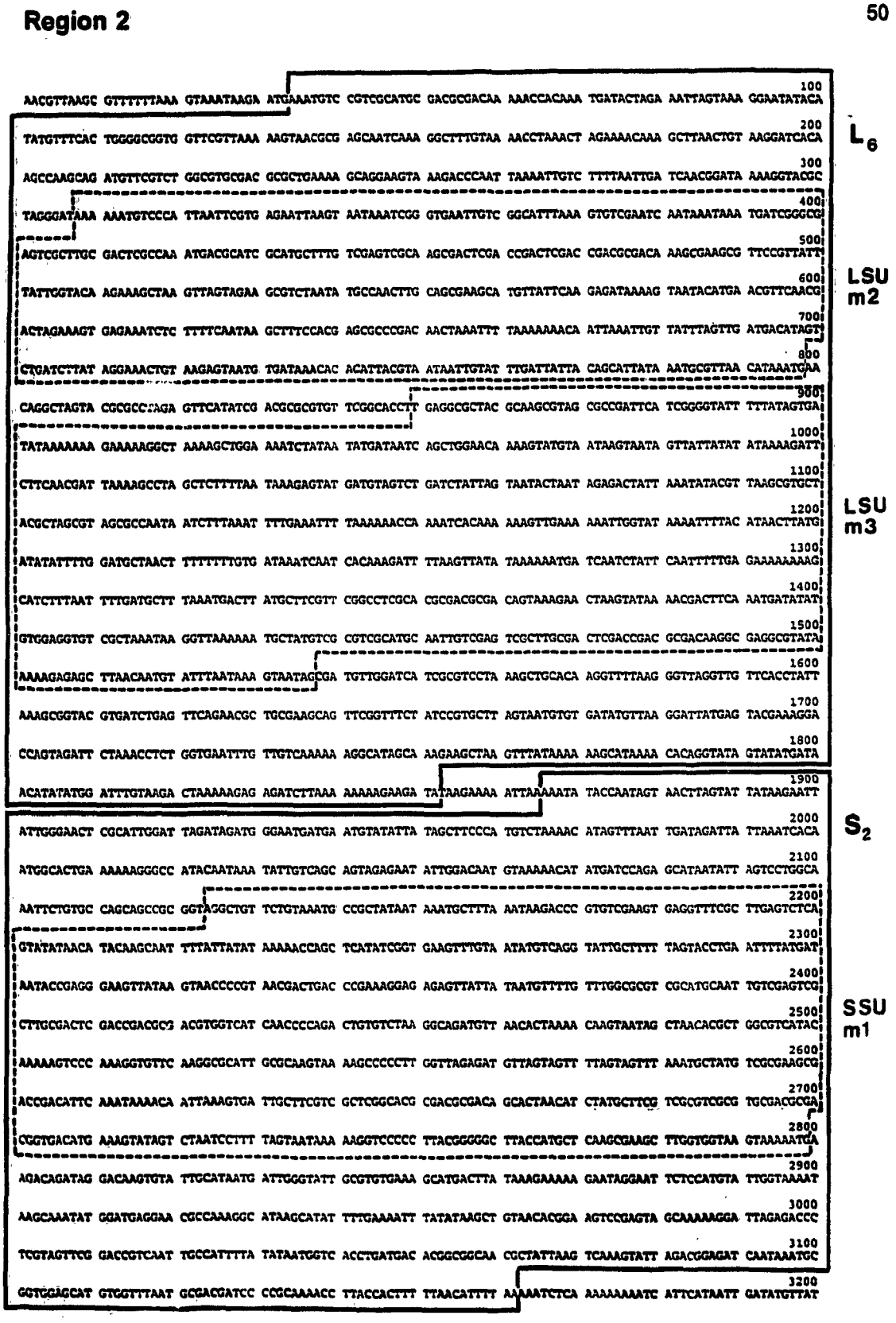
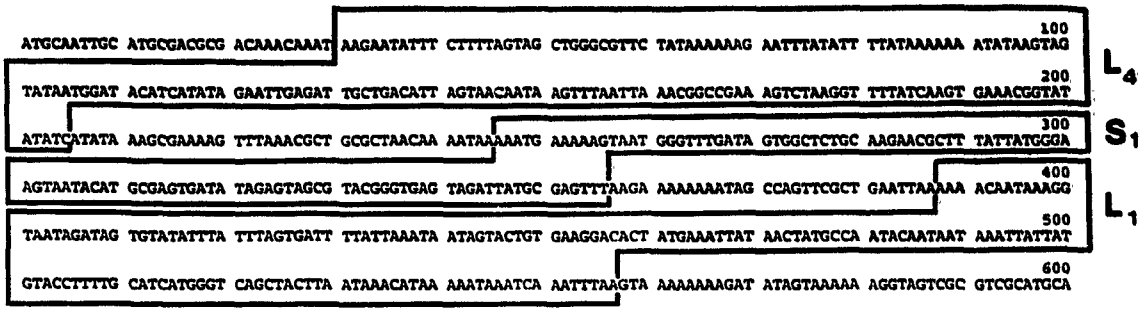


Figure 7 (continued)

Region 3

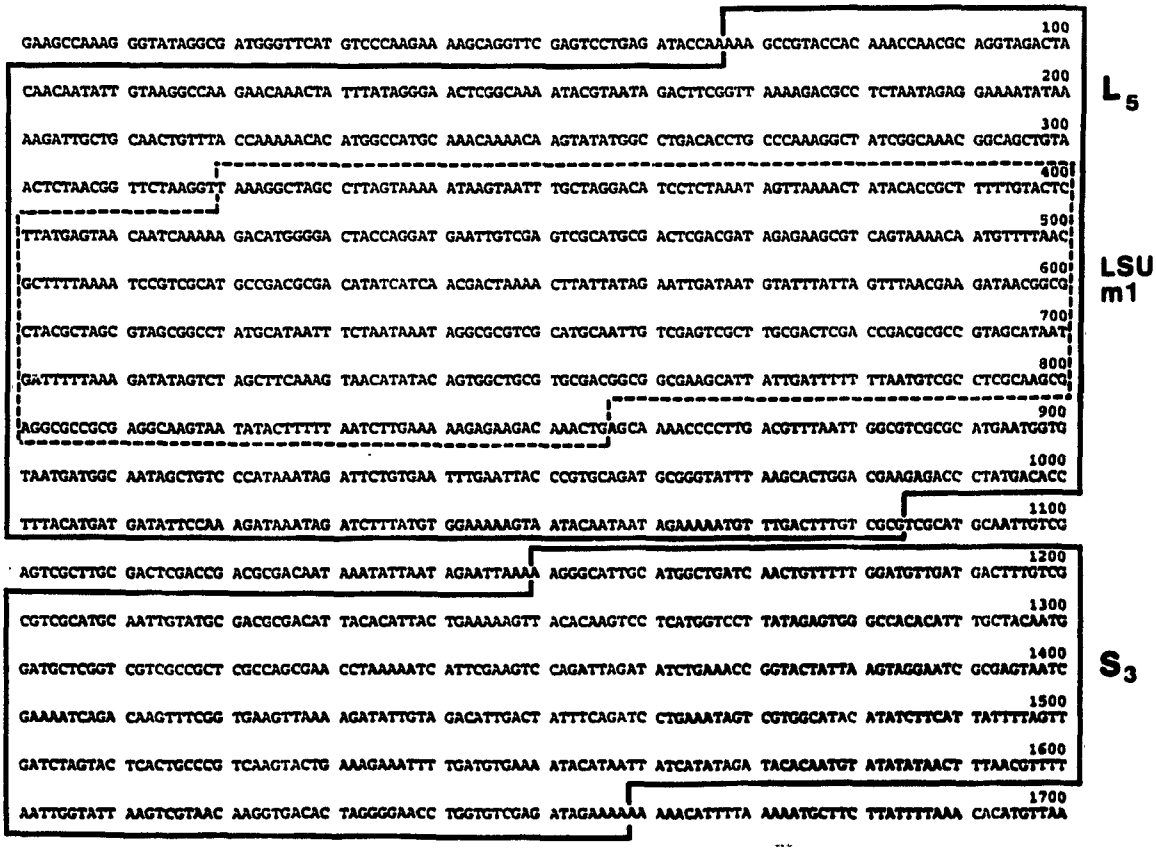


L_4

S_1

L_1

Region 4



LSU m1

S_3

Figure 7 (continued)

nucleotides in length (Fig. 6 II), that correspond to the length of the *C. eugametos* mitochondrial rRNA gene pieces. The mature rRNAs encoded by L_6 and S_2 co-migrated in these gels and their identities were established by probes II and IV, which were specific to L_6 and S_2, respectively, and by probe III, which identified both rRNAs. The identity and size of the L_3 transcript and confirmation of the other rRNA transcripts, as summarized in the legend of Figure 6, were established using rRNA-specific oligonucleotide probes against northern blots of total RNA fractionated by polyacrylamide gel electrophoresis (data not shown). The *C. eugametos* probes strongly hybridized with similar-size RNA fragments in the *C. moewusii* RNA preparation, but failed to hybridize with any RNA fragments in the *C. reinhardtii* RNA preparation, thus supporting the similarity of sequence and position of fragmentation sites between the *C. eugametos* and *C. moewusii* mitochondrial rRNAs and their considerable sequence divergence from those of *C. reinhardtii.* The 7.2 kb *Eco*RI insert of the clone pE1 derived from *C. reinhardtii* mtDNA (Boer and Gray, 1988a), which contains all of the rRNA gene pieces, was used as a control for the presence of *C. reinhardtii* mitochondrial rRNA; the E1 probe annealed with low-molecular-weight transcripts in *C.reinhardtii* total RNA and the hybridization patterns were consistent with those reported previously (Boer and Gray, 1988a). The E1 probe did not hybridize with the *C. eugametos* or *C. moewusii* RNA (data not shown).

The 5' terminal nucleotide of each of the SSU and LSU rRNAs was determined by reverse transcriptase-catalyzed dideoxy sequencing and primer extension reactions, and the 3' terminus of each rRNA species was estimated on the basis of its size as detected in northern blot analysis and the experimentally determined position of its 5' terminal nucleotide (Table 3). The terminal 5' nucleotide and approximate 3' nucleotide of each rRNA species are indicated in the DNA sequence in Figure 7 and on the secondary structures in Figure 8. All *C. eugametos* mitochondrial rRNA coding regions are transcribed from the same DNA strand. No large transcripts have been detected in northern blot analysis nor have primary transcripts been identified by guanylyltransferase capping experiments; therefore, the number or size

Table 3
Estimated size of rRNA fragments encoded by the *C. eugametos* mitochondrial DNA.

RNA species	Length (nt)
S_1	110
S_2	620
S_3	510
Total SSU	1240
L_1	170
L_2	300
L_3	170
L_4	175
L_5	480
L_6	640
Total LSU	1935

of the mitochondrial transcriptional units has not been determined. However, the proximity of the terminal nucleotides of L_6 and S_2 and of L_4, S_1 and L_1 within the DNA sequence of regions 2 and 3, respectively, suggests that these transcripts are derived from a larger precursor and are not produced by separate transcriptional events. Transcriptional mapping of *C. reinhardtii* mtDNA involving S1 nuclease protection and primer extension experiments (Gray & Boer, 1988) revealed extensive co-transcription of gene clusters including SSU and LSU rRNA gene pieces and interspersed tRNA and protein-coding genes. It remains to be determined whether these *C. reinhardtii* co-transcripts, not visible by northern blot analysis, are in turn derived from a larger precursor that includes all of the mitochondrial rRNA modules.

The proposed secondary structures of the *C. eugametos* mitochondrial SSU and LSU rRNAs have been modelled after the homologous *Escherichia coli* structures and are shown in Figure 8. These structures duplicate potential secondary structures of conventional rRNAs in that conserved domains interact by base-pairing to form the universal core of the SSU and LSU rRNA molecules. The termini of all *C. eugametos* mitochondrial rRNA transcripts are confined to the periphery of the proposed SSU and LSU rRNA structures in variable domains, wherein lie discontinuities in other fragmented rRNAs (see Gray & Schnare, 1995). In the *C. eugametos* rRNA gene piece S_3 (Fig. 8a), there appears to be an insertion of nucleotides following nucleotide 1375, relative to the numbering of the *E. coli* SSU rRNA. The position and proposed secondary structure of this 71-nt insertion, which has not previously been identified as a variable rRNA domain, is indicated in the SSU rRNA secondary structure model in Figure 8a. Reverse transcriptase sequencing of this region of the SSU rRNA demonstrated that the extra rRNA sequence is present in the mature rRNA transcript.

Overall, the results of northern blot analysis and secondary structure modelling of the *C. eugametos* mitochondrial rRNA transcripts indicate that these gene pieces, like those of *C. reinhardtii* (Boer & Gray, 1988a), are expressed and that collectively, their small transcripts

Figure 8. Potential secondary structures of the *C. eugametos* mitochondrial rRNAs based on the *E. coli* rRNA models (Gutell *et al.*, 1992b). (a) SSU rRNA, (b) 5' half and (c) 3' half of the LSU rRNA. Canonical base-pairs (C:G, A:U) are indicated by short lines, G:U pairs are indicated by small black dots, A:G pairs are indicated by open circles and non-canonical pairs are indicated by large black dots. Teritary interactions are connected with thicker and longer solid lines. The 5' and approximate 3' terminal nucleotide of each rRNA fragment is indicated. Variable SSU and LSU rRNA domains, defined by Gray *et al.* (1984) and Gray & Schnare (1995) associated with points of fragmentation in *C. eugametos* and/or *C. reinhardtii*, are enclosed by solid lines; variable rRNA domains that are discontinuous in both algae are marked with an asterisk. The insertion sequence of 71 nucleotides identified in S_3 within a region not identified previously as a variable SSU domain is enclosed by a broken line. Small boxes linked by lines indicate potential regions of base-pairing according to the *E. coli* rRNA models. The position of the CeSSU.m1 (SI1) group I intron splice site in the '530' loop of the S_2 gene piece and the positions of the intron splice sites within the *C. eugametos* rRNA gene pieces L_5 and L_6 are indicated as follows: CeLSU.m1, LI1; CeLSU.m2, LI2; CeLSU.m3, LI3. Although discontinuities in both algal coding regions are found exclusively in variable rRNA domains (as is characteristic of other discontinuous rRNAs) and many of these sites are in corresponding variable regions in the two algae, several variable rRNA regions are interrupted in one alga but not the other (see Fig. 10).

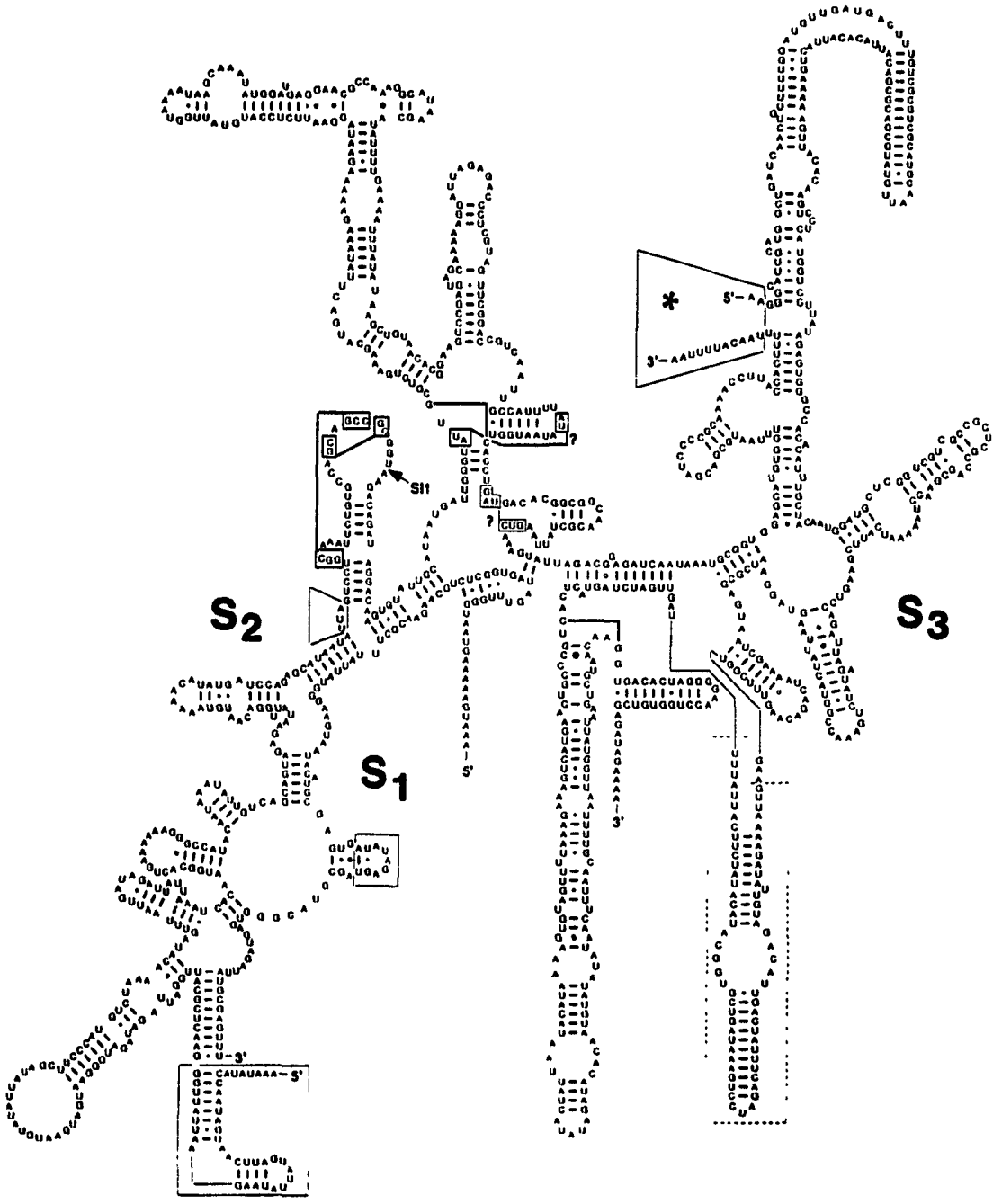


Figure 8 (a)

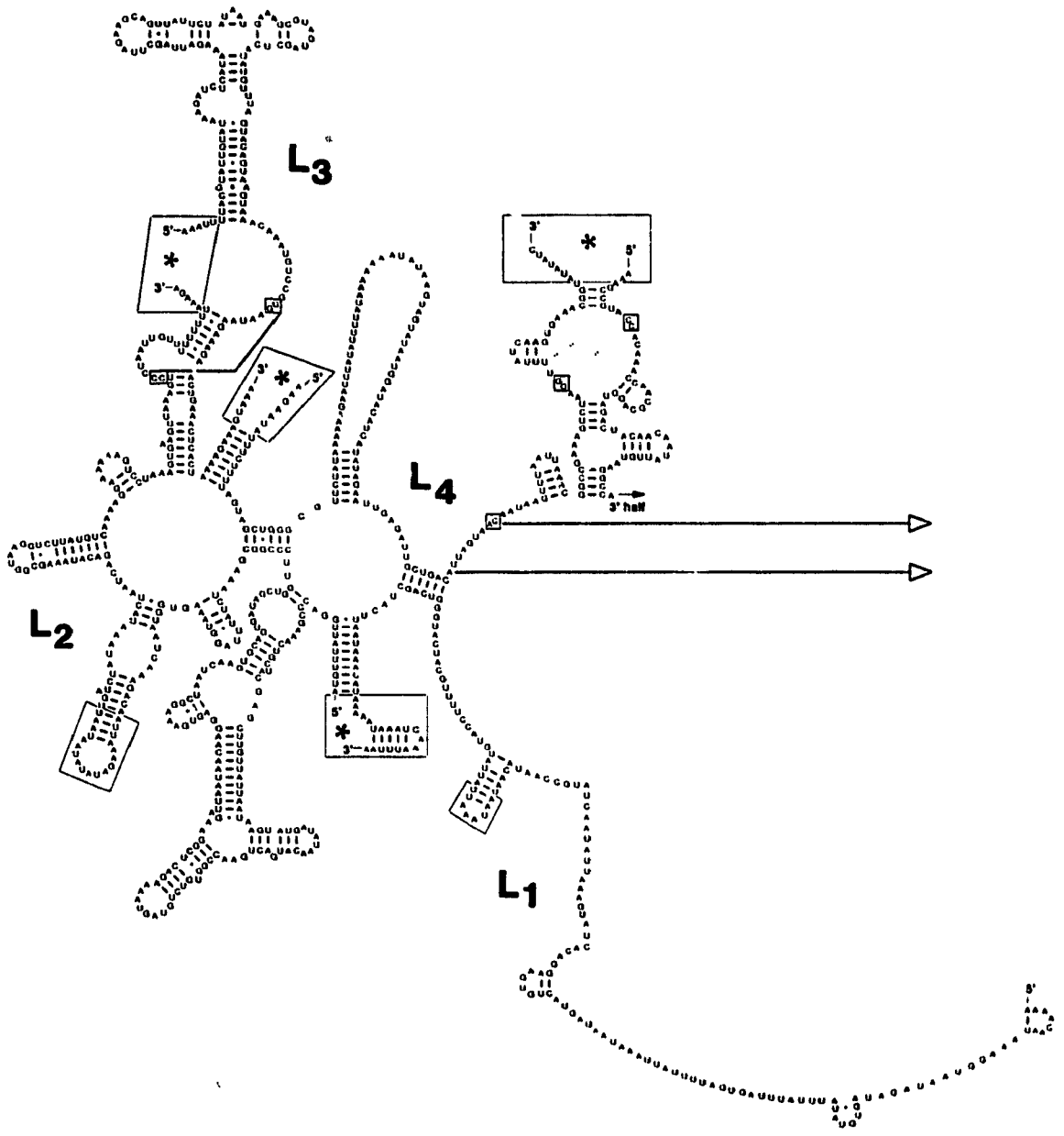


Figure 8 (b)

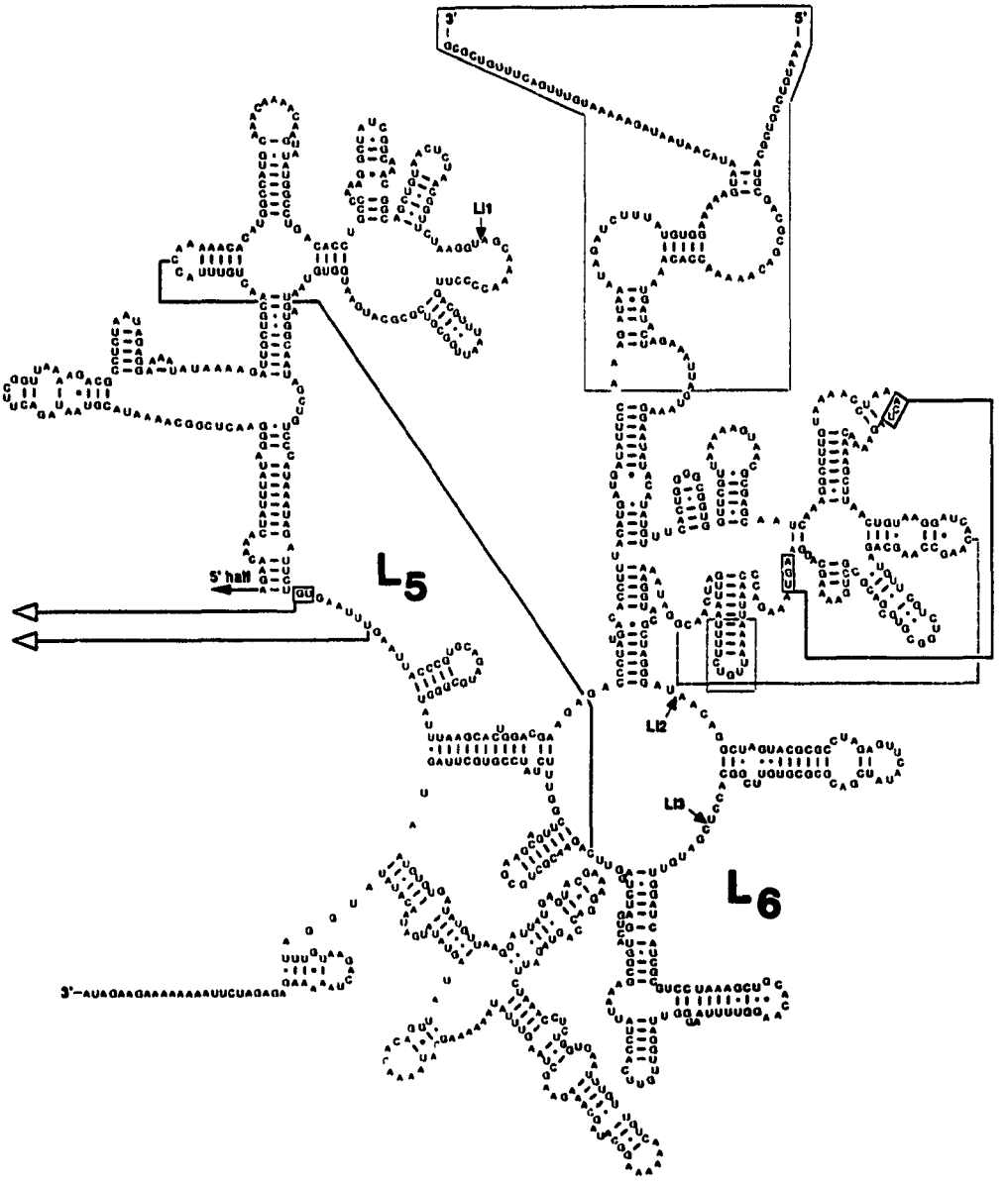


Figure 8 (c)

could reconstitute the functional rRNA core of *Chlamydomonas* mitochondrial ribosomes. These observations argue against the hypothesis that scrambled, modular rRNA genes in *Chlamydomonas* mtDNA are pseudogenes that have become discontinuous and dispersed in the genome after the loss of mitochondrial rRNA function. Northern blot analysis with clones spanning the entire mtDNA of *C. eugametos* and determination of the entire sequence of the mtDNA of *C. reinhardtii* (Ma *et al.*, 1992b and references therein) are inconsistent with the possibility that conventional rRNA genes are encoded elsewhere in the mtDNA of *C. eugametos* or *C. reinhardtii*.

Group I introns in the *C. eugametos* mitochondrial rRNA genes

I have identified four intervening sequences in the *C. eugametos* mitochondrial rRNA gene pieces (Fig. 6 Ia & Fig. 7). One was identified in the coding region of S_2 within the '530 loop', another in the region of gene piece L_5 encoding domain VI (Noller, 1984) and two in the region of gene piece L_6 encoding the peptidyl transferase centre; these intervening sequences are designated CeSSU.m1, CeLSU.m1, CeLSU.m2 and CeLSU.m3, respectively (Fig. 9). The designations reflect the organism, gene and genetic compartment where the introns were found. Relative to the numbering of the *E. coli* rRNAs, CeSSU.m1 (676 nt) follows nucleotide 531 of the SSU (16S) rRNA while CeLSU.m1(537 nt), CeLSU.m2 (491 nt) and CeLSU.m3 (688 nt) follow nucleotides 1931, 2449 and 2500 of the LSU (23S) rRNA, respectively. RNA sequencing demonstrated that all four of the *C. eugametos* intervening sequences are absent from the mature rRNA transcripts and the exons are covalently religated (data not shown). The intervening sequences possess the conserved structural sequences and predicted splice sites of group I introns (Michel & Dujon, 1983; Cech, 1988) and conform to the secondary structure models proposed by Burke *et al.* (1987) . I have not detected open reading frames

Figure 9. Potential secondary structures of the group I intron sequences encoded by the mitochondrial SSU and LSU rRNA gene pieces of *C. eugametos*. The group I intron structures are based on the model proposed by Burke *et al.* (1987). Nucleotides within the introns are indicated by upper-case letters, while nucleotides within the exon sequences are indicated by lower-case letters. Group I introns have been assigned to different subclasses according to the scheme of Michel and Westhof (1990), which is based on characteristic nucleotide or helical structures: (a) CeSSU.m1, subclass Group IA1; (b) CeLSU.m1, subclass Group IA1; (c) CeLSU.m2, subclass Group IA1; and (d) CeLSU.m3, subclass Group IB.

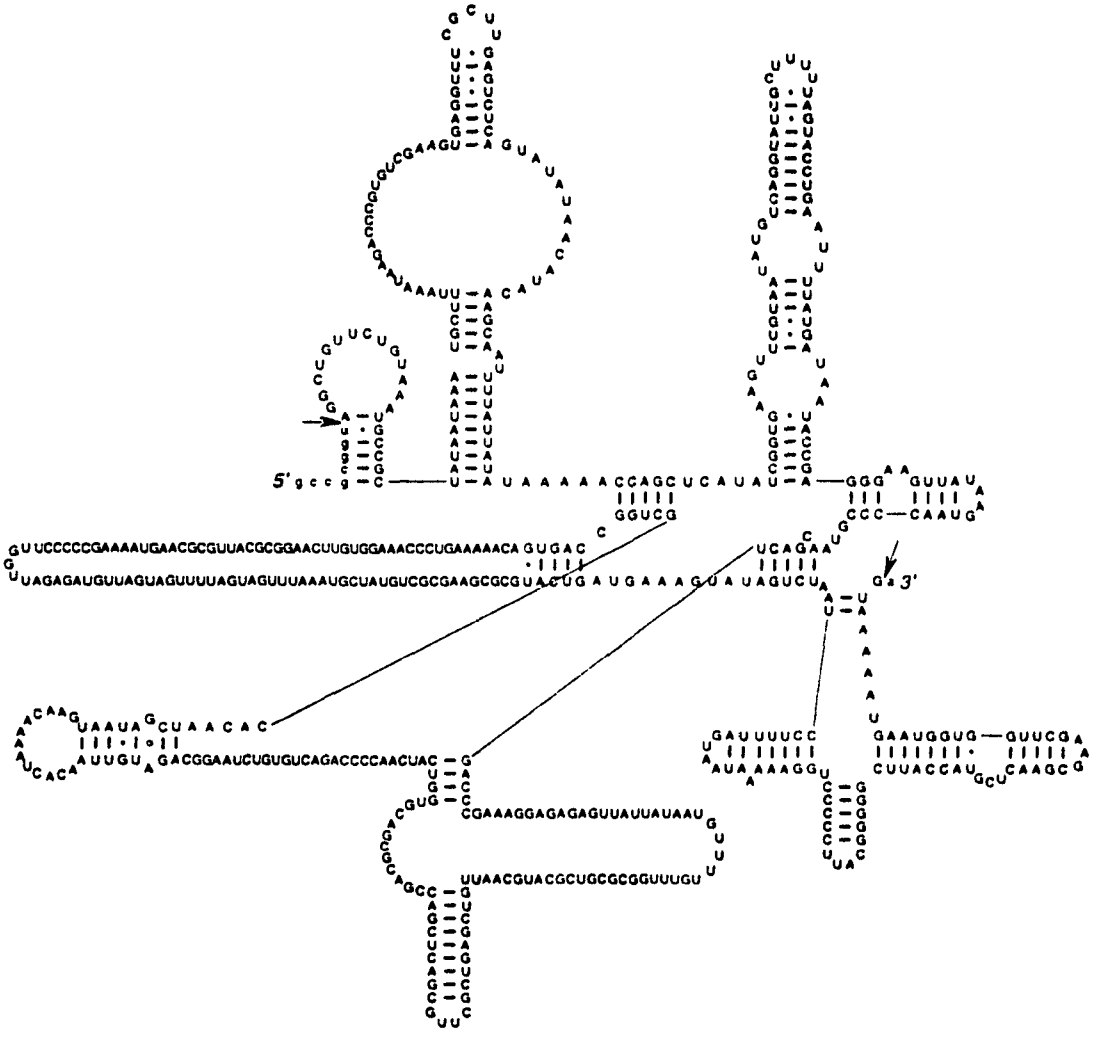


Figure 9 (a)

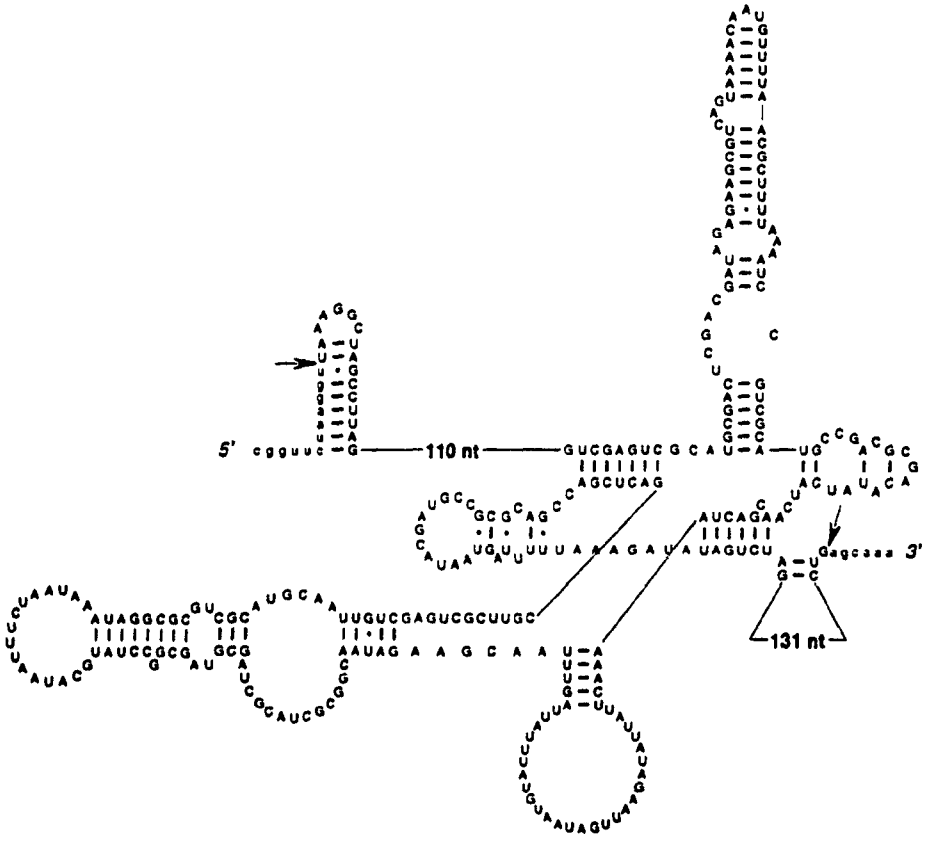


Figure 9 (b)

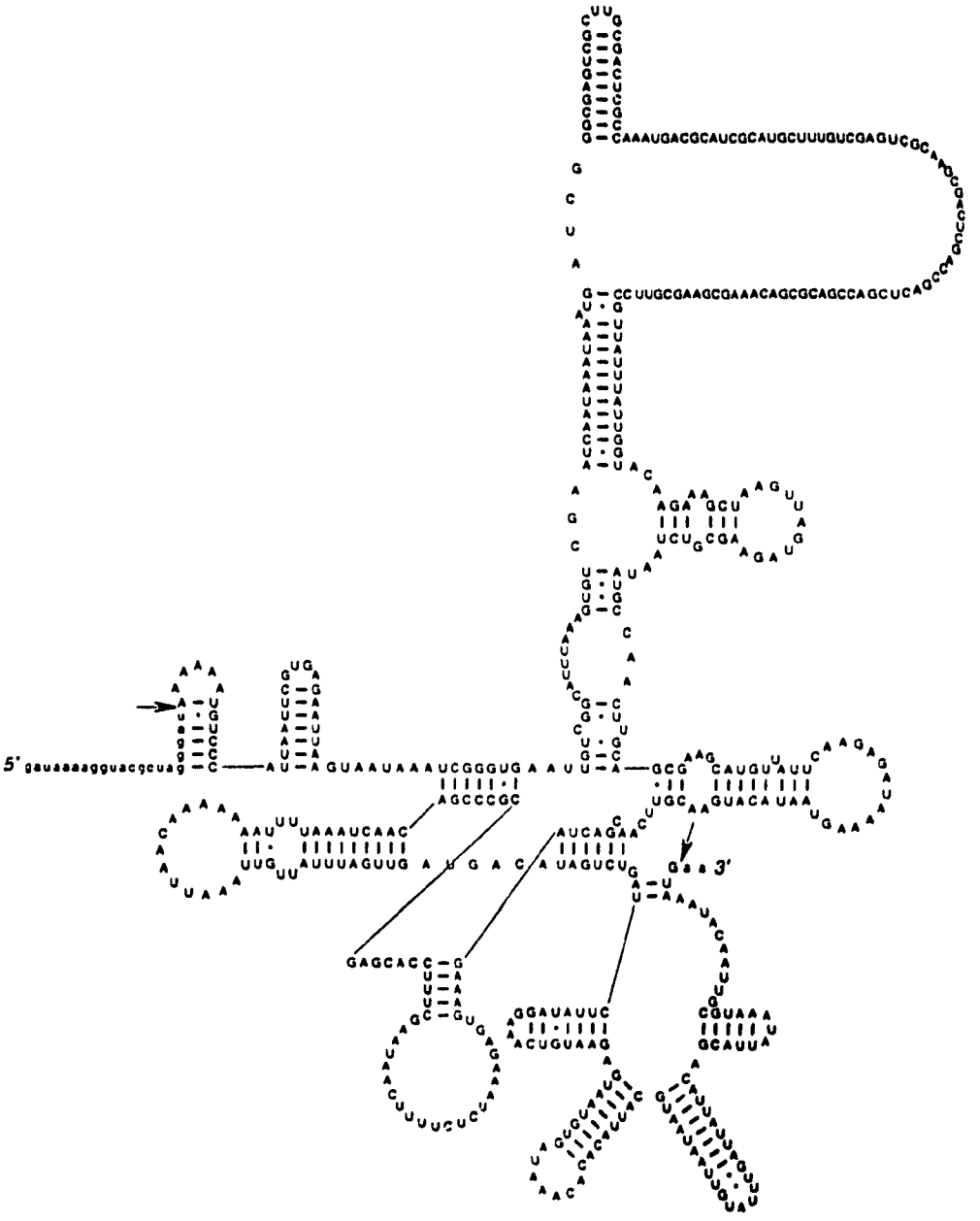


Figure 9 (c)

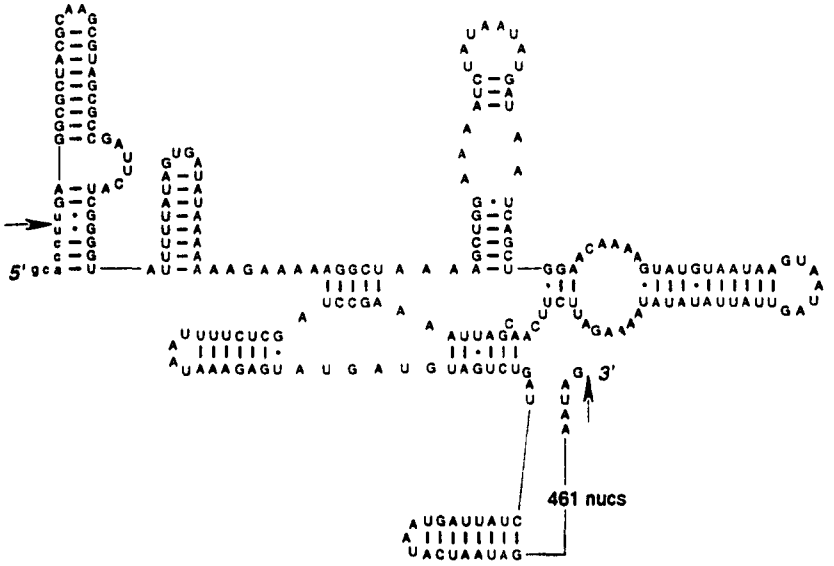


Figure 9 (d)

in any of the *C. eugametos* rRNA introns that share sequence similarity with other intron-encoded ORFs.

Unlike the points of discontinuity in the variable rRNA domains corresponding to the ends of the gene pieces, all four group I introns in the *C. eugametos* rRNA gene pieces are located in conserved regions at positions sporadically populated by group I introns in other rRNA genes (see Gray & Schnare, 1990; 1995 and references therein, Turmel *et al.*, 1991; 1993a and Kück *et al.*, 1990). Although the introns in the *C. eugametos* mitochondrial rRNA coding regions share similarity in their core structures and are found at identical positions in other mitochondrial and chloroplast rRNA genes, it is impossible from present data to deduce whether these introns have been retained from an ancestral LSU rRNA gene or whether inter-organismal or inter-organelle transfer has led to the incorporation of homologous introns at identical gene positions. Recently, Turmel *et al.* (1993b) employed the polymerase chain reaction to identify one complete intron and part of another in the *C. moewusii* mitochondrial LSU rRNA coding regions that correspond to the CeLSU.m2 and CeLSU.m3 introns described here. Considering the interfertile and likely conspecific nature of *C. eugametos* and *C. moewusii* (Gowans, 1963; Cain, 1979; Lee & Lemieux, 1990), it is not surprising that there are only minor sequence differences between the corresponding introns of these algae. Although group I introns have been found in other SSU rRNA genes (Sogin & Edman, 1989; Dávila-Aponte *et al.*, 1991; Wilcox *et al.*, 1992; Oda *et al.*, 1992), the intron splice site in the universal '530' loop of the *C. eugametos* mitochondrial SSU rRNA is only the second such example in this region of an SSU rRNA. Durocher *et al.* (1989) identified an intron in the same subgroup at the cognate position of the chloroplast SSU rRNA gene of *C. moewusii*, thereby supporting a possible inter-organelle transfer of this intron. There are no introns in the *C. eugametos* chloroplast SSU rRNA gene.

Discussion

Comparative structure and genomic organization of the rRNA coding regions

The *C. eugametos* mitochondrial rRNA gene pieces, like those reported for *C. reinhardtii* (Boer & Gray, 1988a), are transcribed and, collectively, the individual rRNAs conform to conventional rRNA secondary structure models. These observations support the hypothesis that small interacting RNAs reconstitute the functional ribosome core in *Chlamydomonas* mitochondria. The data also argue against the hypothesis that scrambled, modular rRNA genes are pseudogenes that have become fragmented and dispersed in the genome after the loss of mitochondrial rRNA function. It is also unlikely that, as a result of RNA isolation, degradation of larger rRNA molecules is an explanation for the small size of the *Chlamydomonas* mitochondrial rRNAs, because each of the individual rRNAs in both *C. eugametos* (this work) and *C. reinhardtii* (Boer & Gray, 1988a) is homogenous in size and because the cytosolic and chloroplast rRNAs, observed following gel electrophoresis and staining, are not degraded. Moreover, intact mitochondrial protein-coding transcripts were detected by northern blot analysis of the total cellular RNA samples used to identify the mitochondrial rRNA pieces in both *C. eugametos* (unpublished data) and *C. reinhardtii* (Boer *et al.*, 1985; Boer & Gray, 1988a). Conversely, the inability to detect abundant high-molecular-weight rRNA transcripts with the *C. eugametos* or *C. reinhardtii* mtDNA probes precludes the possibility that trans-splicing, a phenomenon observed in some mitochondrial, chloroplast and nucleus-encoded transcripts (reviewed by Bonen, 1993), creates covalently continuous rRNA molecules from discontinuously encoded transcripts. Finally, northern blot analysis with clones spanning the entire mtDNA of *C. eugametos* and determination of the entire sequence of the mtDNA of *C. reinhardtii* (Pratje *et al.*, 1984; Boer *et al.*, 1985; Boer & Gray, 1986; 1988a; 1988b; 1988c; 1989; Pratje *et al.*, 1989; Michaelis *et al.*, 1990; Ma *et al.*, 1992a; 1992b;

Vahrenholz *et al.*, 1993) are inconsistent with the possibility that conventional rRNA genes are encoded elsewhere in the *C. eugametos* or *C. reinhardtii* mtDNAs.

Although *C. eugametos* and *C. reinhardtii* share the unusual feature of highly fragmented and scrambled mitochondrial rRNA gene pieces, there are considerable differences in the distribution and organization of the rRNA coding information in the two algal mtDNAs. The *C. eugametos* rRNA gene pieces contain four group I introns, the gene pieces are dispersed over 15 kb of the mitochondrial genome (in contrast to the gene pieces of *C. reinhardtii*, which contain no introns and are dispersed over 6 kb) and the 5' to 3' order of the *C. eugametos* gene pieces appears highly rearranged relative to that of *C. reinhardtii* (Fig. 6 I). Previous work established that the particular protein-coding domains interspersed with the rRNA gene pieces in *C. eugametos* mtDNA differ from those interspersed with the rRNA gene pieces in *C. reinhardtii* (Boer & Gray, 1988a; Chapter 1). Finally, the distribution of coding information among the mitochondrial rRNA gene pieces is different in the two algae. This is illustrated by Figure 10, which identifies the positions of discontinuities in the *C. eugametos* and *C. reinhardtii* mitochondrial rRNAs. Although discontinuities in coding regions of both algae are found exclusively in variable rRNA domains (as is characteristic of other fragmented rRNAs) and many of these sites are in corresponding variable regions in the two algae, several variable rRNA regions are interrupted in one alga but not the other. I note that corresponding variable regions in the two algae are sufficiently different in length and sequence to obscure information about the possible common or independent evolutionary origin of shared breakpoints in these regions.

No simple rearrangement of gene pieces can inter-convert the two gene arrays in these algal mtDNAs. For example, the 5' ends of the SSU and LSU rRNAs are encoded by S_1 and L_1, respectively, in both *C. eugametos* and *C. reinhardtii* (Fig. 10; Table 4). S_1 and L_1 are adjacently encoded in *C. eugametos* mtDNA, but are encoded at opposite ends of the cluster of rRNA gene pieces in *C. reinhardtii* (Fig. 6 I). If the circular mtDNA of *C. eugametos* were

Figure 10. Relationship between the discontinuities and group I intron splice sites in the SSU and LSU mitochondrial rRNAs of *C. eugametos* (Ce) and *C. reinhardtii* (Cr) relative to the conserved (solid) and variable (open) rRNA regions described by Gray & Schnare (1995) and drawn to the scale of the *E. coli* rRNAs. The fourth open box from the left end of the LSU rRNA represents three closely spaced variable regions identified by Gray & Schnare (1995). Stippled regions represent the terminal nucleotides missing from the *Chlamydomonas* mitochondrial LSU rRNAs relative to the *E. coli* 23S rRNA. The points of discontinuity in the rRNAs of *C. eugametos* and *C. reinhardtii* are shown by arrows above and below each rRNA, respectively. Open triangles represent the positions of intron splice sites in the *C. eugametos* rRNA. The SSU and LSU rRNA pieces and the intron splice sites are presented in the 5' to 3' order that they appear in the secondary structure models.

Table 4

Distribution of homologous coding information in the *C. eugametos* and *C. reinhardtii* mitochondrial rRNA gene pieces.

C. eugametos		*C. reinhardtii*	
Gene piece[1]	Coding information[2]	Gene piece	Coding information
S_1	AB	S_1	A
S_2	CD	S_2	BC
S_3	E	S_3	D
		S_4	E
L_1	FG	L_1	F
L_2	HI	L_2	G
L_3	J	L_3	H
L_4	K	L_4	I
L_5	L	L_5	J
L_6	MN	L_6	K
		L_7	LM
		L_8	N

[1] The gene pieces of the SSU and LSU have been numbered sequentially in the order that homologous sequences appear in the 5' to 3' order in conventional rRNA genes.

[2] The rRNA coding information has been designated by capital letters to indicate the information between any two beakpoints in comparisons of the mitochondrial rRNA of *C. eugametos* and *C. reinhardtii.* The lettered blocks of coding information using the co-ordinates of the *E. coli* 16S (A through E) and 23S rRNA (F through N) are as follows A, 1-60; B, 110-143; C, 220-405; D, 500-990; E, 1045-1540; F, 430-536; G, 557-601; H, 656-860; I, 920-1020; J, 1030-1170; K, 1179-1350; L, 1595-2200; M, 2223-2404; N, 2413-2835.

linearized between these two gene pieces, L_1 would be upstream of S_1, relative to the direction of transcription and, therefore, in the opposite order relative to S_1 and L_1 in *C. reinhardtii*. The only adjacent rRNA gene pieces encoding homologous information in the same relative orientation in the two algae are L_4 and the 5' portion of S_1 from *C. eugametos* and L_6 and S_1 from *C. reinhardtii* (Fig. 6 I). It is not yet possible to determine whether this similar arrangement of information was inherited from a common ancestor or whether homologous coding regions became juxtaposed by chance in both algae. Evidence for the former possibility would support the hypothesis that the last common ancestor of these algae had fragmented and scrambled mitochondrial rRNA genes. Finally, we note that L_1 of *C. eugametos* encodes the structural equivalent of the *C. reinhardtii* gene pieces L_1 and L_2. Although L_1 is encoded upstream of L_2 in the *C. reinhardtii* mtDNA, a single nucleolytic scission separates the two mature RNAs (Boer & Gray, 1991).

An insertion within domains that have not previously been identified to be variable was found in the mitochondrial SSU rRNAs of *C. eugametos* and *C. reinhardtii* and LSU rRNA of *C. reinhardtii*; this feature is not shared by other rRNAs. However, the position of the SSU insertion is different in the two algal mitochondrial rRNAs; these follow nucleotides 1375 and 1314 in *C. eugametos* and *C. reinhardtii*, respectively, relative to the numbering of the *E. coli* SSU rRNA. The hypothetical imperfect helices that can be formed by these apparently unrelated insertion sequences do not alter the secondary structure modelling of the two universal rRNA cores.

The 5' and 3' terminal sequences found in most LSU rRNAs are not encoded in the mitochondrial genome of *C. reinhardtii* (Boer & Gray, 1988a; Michaelis *et al.*, 1990), vertebrates and many fungi and protists (Gutell *et al.*, 1992a). Similarly, these 5' and 3' LSU rRNA terminal domains are not encoded in the mtDNA of *C. eugametos*. We have not

detected, by hybridization or DNA sequencing, any rRNAs that could potentially contribute these 5' and 3' terminal domains to the LSU rRNA of *C. eugametos*.

Boer & Gray (1988a) described two small RNAs, designated L_2b and L_3a, that are co-transcribed with the L_2 and L_3 LSU rRNAs from the *C. reinhardtii* mtDNA; these two RNA species are thought to be in a variable rRNA domain corresponding to divergent region D3 as defined by Michot *et al.* (1984). L_2b and L_3a do not have homologs in other LSU rRNAs and coding regions with sequence similarity to L_2b and L_3a have not been identified in the *C. eugametos* mtDNA nor have small transcripts been detected in this alga that could contribute comparable sequences. The functional significance of the L_2b and L_3a rRNA modules in *C. reinhardtii* remains obscure.

Evolution of *Chlamydomonas* mitochondrial rRNA genes

Differences in the distribution and organization of the mitochondrial rRNA coding information between *C. eugametos* and *C. reinhardtii* have allowed me to speculate on the evolution of their discontinuous, scrambled structure. I assume that this unusual gene structure arose from conventional, continuous rRNA genes (see Gray & Schnare, 1995) by two processes: the introduction of processing signals and the scrambling of coding regions defined by these signals. It seems likely that the introduction of processing signals preceded the scrambling of the rRNA coding regions because the 5' to 3' order of the conserved sequences in mature rRNA transcripts must be maintained if these rRNAs are to participate in the inter- and intra-molecular base-pairing necessary to form the secondary and tertiary structures that are compatible with rRNA function. Moreover, the observation that most discontinuous rRNAs have gene structures that are co-linear with conventional rRNAs (see Gray & Schnare, 1995) and that not many discontinuous rRNA coding regions are also scrambled suggests that the introduction of processing signals and the scrambling of the coding regions are separate

events. Given these assumptions, the simplest explanation for the comparative features of the *C. eugametos* and *C. reinhardtii* mitochondrial rRNA coding regions is that i) the last common ancestor of these algae had discontinuous (and possibly scrambled) rRNA genes in its mitochondrial genome, ii) some or all of the breakpoints in corresponding variable regions in the two algal mitochondrial rRNAs were inherited from this ancestor, iii) the unique rRNA breakpoints were derived independently since the divergence of the lineages leading to *C. eugametos* and *C. reinhardtii*, and iv) the processes leading to the division of the rRNA into separate transcripts and the rearrangement of the information encoding these rRNAs have been ongoing since the divergence of the lineages leading to *C. eugametos* and *C. reinhardtii*.

I do not favour the hypothesis that the last common ancestor of *C. eugametos* and *C. reinhardtii* had continuous rRNA genes in its mitochondria and that the interruption of these genes occurred independently in the two algae. The mitochondrial rRNAs of *C. eugametos* and *C. reinhardtii* each have 30 of the rRNA variable regions identified by Gray & Schnare (1995). Seven and ten of these variable regions are discontinuous in *C. eugametos* and *C. reinhardtii*, respectively; five corresponding variable regions are discontinuous in both algae. Assuming that the number of unbroken variable regions in the common ancestor of the two species was 30 and the variable regions that became discontinuous arose at random in the lines leading to *C. eugametos* and to *C. reinhardtii*, the probability that five or more corresponding variable regions would be interrupted in both algae by chance alone is unlikely (P = 0.024). If the discontinuities could arise only in 18 of the 30 variable rRNA regions that have been reported to be discontinuous in these and other rRNAs (Gray & Schnare, 1995), this probability would increase to P = 0.278 (see Appendix). The assumption that each variable region within the mitochondrial rRNA of these algae had an equal chance of becoming discontinuous seems reasonable in light of the fact that very few rRNAs are discontinuous in comparison to the total number of rRNAs described, and that among these few discontinuous rRNAs, the fragmentation sites appear to be distributed randomly amongst the 18 variable

rRNA domains. The hypothesis that at least some of the variable regions that are interrupted in both *C. eugametos* and *C. reinhardtii* were inherited from a common ancestor is further supported by the fact that one of the common SSU and two of the four common LSU interrupted variable regions have not previously been reported to be sites of discontinuity in other fragmented rRNAs. For this analysis, I assumed that each site of rRNA discontinuity arose independently regardless of the state of other variable regions within a given rRNA molecule. Overall, these results suggest that the process of mitochondrial rRNA fragmentation began in the *Chlamydomonas* lineage after its divergence from other chlorophycean algae, such as *P. wickerhamii*, which have conventional rRNA structures, but before the divergence of the lineages leading to *C. eugametos* and *C. reinhardtii*.

To appreciate the evolutionary history of the *Chlamydomonas* mitochondrial rRNA genes, it is necessary to understand the processing pathways that produce the mature rRNAs. Detailed transcriptional mapping of *C. reinhardtii* mtDNA involving S1 nuclease protection and primer extension experiments (Gray & Boer, 1988) revealed extensive co-transcription of gene clusters including SSU and LSU rRNA gene pieces and interspersed tRNA and protein-coding genes, although single transcripts containing all of the rRNA fragments have not been identified. Similarly, no large transcripts were observed following northern blot analysis with the *C. eugametos* mitochondrial gene probes. However, I suggest that there is also extensive co-transcription of the rRNA gene pieces in the *C. eugametos* mitochondria because, as in *C. reinhardtii*, many of the 5' and 3' termini of the mature rRNAs are in close proximity as they map to the genome.

In an effort to identify possible mitochondrial rRNA processing signals, I compared the sequences surrounding the *C. eugametos* and *C. reinhardtii* mtDNA regions where the mature rRNA termini map. No conservation in the sequences or potential secondary structures within proposed precursor transcripts corresponding to these regions were detected in either alga or between the algae other than the presence of an adenosine at the 5' end of each

mature rRNA. Comparisons of the sequences that surround the *Chlamydomonas* rRNA processing sites using the program CLUSTAL (Higgins & Sharp, 1988) failed to align any sites of cleavage. In addition, comparison of the number of nucleotides between comparable processing sites in the two algae and nucleotides involved in base-pairing in secondary structure models did not reveal a simple relationship between sites of rRNA processing and the adjacent conserved helical structures in the rRNA. Together, these analyses suggest that there is no conservation of the primary sequences and potential secondary structures around rRNA processing sites in these algae. However, short dispersed repeats, containing a consensus palindromic motif and identified mainly in spacer regions of *C. reinhardtii* mtDNA, may play a role in post-transcriptional processing (Boer & Gray, 1991). Although I have not identified dispersed repeats in the mtDNA of *C. eugametos* that share sequence similarity with those found in the *C. reinhardtii* mtDNA, a family of dispersed repeats with similar features has been identified (Denovan-Wright and Lee, unpublished results).

Similarly, Turmel *et al.* (1993a) observed no within- or between-species conservation in the sequences or potential secondary structures that flank the three processing sites in fragmented chloroplast LSU rRNAs in several species of *Chlamydomonas.* They proposed that the liberation of the different ITSs separating the chloroplast LSU rRNAs of a given *Chlamydomonas* species was either directed by different recognition signals or by a common signal related to the three-dimensional shape of the ribosome. According to the latter possibility, they suggest that the ITSs may be removed, after ribosome assembly, by non-specific ribonucleases (RNases) that act on RNA sequences protruding from the ribosomal surface. The evolutionary implication of this model is that variable rRNA domains that become expanded and protrude from the ribosome core would be converted to ITSs and that ITSs that become sufficiently reduced in size would not be processed but return to being continuous variable rRNA domains. However, the observation that no simple relationship exists between the site of cleavage and the conserved helical domains of fragmented rRNAs

and that no (or little) heterogeneity is observed at the termini of these fragments (Fujiwara & Ishikawa, 1986; Schnare *et al.*, 1986; Heinonen *et al.*, 1987; Schnare & Gray, 1990; Turmel *et al.*, 1993a) suggests that some factor in addition to ribosome architecture is directing the endonucleolytic cleavages. I suggest, therefore, that RNA sequences that extend beyond the protected core of the ribosome are susceptible to endonucleolytic attack by site-specific RNases resident within the cell or sub-cellular compartment. Because of the diversity of RNases, any extruded RNA sequence peripheral to the ribosome core could contain within it a sequence or structure recognized as a unique substrate for a particular enzyme (thereby giving rise to highly accurate cleavage). Natural selection against the evolution of RNase target sites in unprotected RNA coding regions, such as protein-coding sequences, could limit the nucleolytic attack of these sequences. Assuming that the precursor of the *Chlamydomonas* mitochondrial SSU and LSU rRNA fragments can interact with ribosomal proteins and form a "pre-ribosomal" complex, despite the scrambled order of the rRNA gene pieces, one can envision the removal of sequences that protrude from the ribosomal surface. Moreover, the order of the rRNA modules within the RNA precursor(s) would be limited to those that allow assembly of pre-ribosomes which in turn could be modified by processing to form functional ribosomes. Alternatively, Boer & Gray (1988a) suggested that the post-transcriptional processing of the rRNA fragments may occur before the assembly of *Chlamydomonas* mitochondrial ribosomes. An attractive feature of an rRNA processing model that involves the removal of sequences that protrude from the pre-ribosome surface is that it could allow for the scrambling of rRNA gene pieces, such as observed in the *Chlamydomonas* mitochondria, without the need to evolve new primary sequence signals prior to rearrangement of the gene pieces.

An open reading frame with sequence similarity to reverse transcriptase-like proteins was found in the mitochondrial genome of *C. reinhardtii*, leading to the intriguing possibility that reverse transcription could be responsible for the scrambling of the mitochondrial rRNA genes

(Boer & Gray, 1988a). Reverse transcription of part of an rRNA molecule followed by the reintegration of this coding information into a novel genomic location and subsequent selection for a small genome could alter the order of the coding domains. However, no such reverse transcriptase-like open reading frame has been found in the mtDNA of *C. eugametos* despite weak Southern hybridization of a probe derived from the regions encoding the putative mitochondrial reverse transcriptase of *C. reinhardtii* (Denovan-Wright & Lee, 1992 and unpublished data). Moreover, the presence of four group I introns in the rRNA gene pieces in *C. eugametos* would suggest that if reverse transcription were involved in the rRNA scrambling, then all the introns were acquired after the rRNA gene rearrangements in *C. eugametos* had occurred.

I favour the view that discontinuous rRNA coding regions in *Chlamydomonas* mitochondria became scrambled by recombination between non-homologous regions of mtDNA molecules. Sequences with limited similarity such as the dispersed repeated elements observed in *C. eugametos* (unpublished results) and *C. reinhardtii* (Boer & Gray, 1991) mtDNA could act as substrates for such recombination. Following sorting out of mitochondrial genomes and the formation of cells homoplasmic for the rearranged mtDNA, selection for a compact genome could result in mtDNA genomic streamlining in those cells with mitochondrial genomes containing extra rRNA coding modules; homoplasmic segregants lacking a full complement of mitochondrial rRNA coding regions would die. Scenarios involving the scrambling of the rRNA coding modules by intra-molecular recombination seem less likely; such events would either divide the mtDNA into separate pieces or invert the orientation of the coding modules, a process that is inconsistent with the observation that all rRNA modules in *C. eugametos* and *C. reinhardtii* mtDNA are transcribed from the same strand of DNA.

The differences observed in the distribution and organization of the mitochondrial rRNA coding information between *C. eugametos* and *C. reinhardtii* make it difficult to fully appreciate the evolutionary pathways that have led to their unusual gene structures. For a deeper

understanding of these pathways it may be helpful to examine the mitochondrial rRNA genes of *Chlamydomonas* taxa allied more closely to *C. eugametos* or *C. reinhardtii*.

Chapter 3

Mitochondrial ribosomes in *Chlamydomonas*

Introduction

Mitochondrial and chloroplast genomes are expressed by transcriptional and translational systems resident within their respective subcellular compartments (Kitakawa & Isono, 1991). The rRNAs and, in some cases, a few ribosomal proteins are encoded by the organelle genome. Other components of the organellar protein-synthesizing machinery are encoded by the nucleus and imported into chloroplasts and mitochondria (Schmidt *et al.*, 1985; Tzagoloff & Myers, 1986). Studies on the physicochemical and functional properties of chloroplast and mitochondrial ribosomes indicate that basic differences exist between these ribosomes and those of the eukaryotic cytoplasm (Leaver, 1976; Stevens *et al.*, 1976). Chloroplast ribosomes as a group are relatively uniform and show more similarities to prokaryotic ribosomes than do mitochondrial ribosomes, which are structurally and functionally diverse and which overall show no strong resemblance to either prokaryotic or eukaryotic ribosomes (Stevens *et al.*, 1976). In terms of their apparent sedimentation coefficent, the most common criterion for distinguishing different ribosome types, chloroplast ribosomes sediment like the 70S ribosomes of prokaryotes while mitochondrial ribosomes are reported to be 55-60S in metazoan animals (O'Brien *et al.*, 1971;Rabbits & Work, 1971; O'Brien, 1972), 70-74S in fungi and *Euglena* (Avadhani & Buetow, 1972; Vignais *et al.*, 1972; Pinel *et al.*, 1986; Leaver & Harmey, 1973) and 77-80S in ciliate protozoans and plants (Chi & Suyama, 1970; Vasconcelos & Bogorad, 1971; Tait & Knowles, 1977). Mitochondrial ribosomes from chlorophycean green algae have not been characterized.

Extensive studies of total cellular ribosomes from *C. reinhardtii* by sedimentation in sucrose density gradients have revealed only two ribosome classes (Hoober & Blobel, 1969; Bourque *et al.*, 1971; Boynton *et al.*, 1972; Chua *et al.*, 1973; Conde *et al.*, 1975; Bartlett *et al.*, 1979). The cytoplasmic ribosomes represent about 60% of the total ribosome population and have an apparent sedimentation coefficient of 80-83S. At Mg^{2+} concentrations below 2 mM

these ribosomes separate into subunits of 57-60S and 37-40S (Hoober & Blobel, 1969). Chloroplast ribosomes account for about 40% of the total ribosome population and have an apparent sedimentation coefficient of 70S; however, under some conditions they appear to sediment more quickly. At Mg^{2+} concentrations below 25 mM, chloroplast ribosomes fractionate as a heterogenous population of molecules with lower sedimentation values than 70S monosomes, presumably as the result of conformational changes, and they begin to dissociate into subunits of 50-54S and 33-41S (Hoober & Blobel, 1969; Bourque *et al.*, 1971). At Mg^{2+} concentrations below 2 mM, chloroplast ribosomes become completely dissociated into subunits (Hoober & Blobel, 1969).

Although there is considerable indirect evidence for the existence of ribosomes and for a functioning protein-synthesizing system in *Chlamydomonas* mitochondria (Conde *et al.*, 1975; Boynton *et al.*, 1973; Boer & Gray, 1988a; Chapter 2), direct support for these presumptions is lacking. Particles resembling ribosomes were observed in electron micrographs in association with mitochondrial membranes in *C. reinhardtii*; these particles were smaller than the chloroplast and cytosolic ribosomes and less abundant (Harris, 1989; Boynton, unpublished). In *C. reinhardtii* and *C. eugametos*, the mitochondrial SSU and LSU rRNAs are discontinuously encoded in separate gene pieces that are scrambled in order and are interspersed with each other and with protein-coding genes and tRNA genes (Boer & Gray, 1988a; Denovan-Wright & Lee, 1993). The small transcripts encoded by the *Chlamydomonas* mitochondrial rRNA gene pieces have the potential to form standard rRNA secondary structures through inter-molecular base pairing, and are thought to function in the *Chlamydomonas* mitochondrial ribosomes as a non-covalent network of small RNAs. Moreover, the mitochondrial rRNAs of *C. reinhardtii* are more abundant than the protein-coding transcripts, as would be predicted for functional rRNA molecules (Boer & Gray, 1988a). The mitochondrial genomes of *C. reinhardtii* and *C. eugametos* encode several electron transport chain proteins including apocytochrome b, cytochrome c oxidase subunit I and several subunits of NADH dehydrogenase that function in

respiration and are commonly found in other mitochondrial genomes (Michaelis *et al.*, 1990 and references therein; Denovan-Wright & Lee, 1993). The fact that these genes are encoded by the mtDNA suggests that inner mitochondrial membrane proteins are produced by a translation system resident within the mitochondria, unlike the vast majority of mitochondrial proteins, which are imported into mitochondria after synthesis on cytosolic ribosomes (reviewed in Glick & Schatz, 1991). The elimination of mtDNA during cell growth in the presence of ethidium bromide or acriflavin leads to cell death after only a few generations (Alexander *et al.*, 1974; Gillham *et al.*, 1987a), thereby suggesting that one or more mtDNA-encoded gene products is essential for cell viability in *Chlamydomonas*. However, the possibility has not been formally eliminated that the scrambled modular rRNA genes are pseudogenes that have become fragmented and dispersed in the mitochondrial genomes of *Chlamydomonas* after the loss of mitochondrial rRNA function. It remains to be established that the small RNAs encoded by these scrambled, modular gene pieces are in fact associated with RNP particles and that these particles function in protein synthesis.

As a first step in this regard, I report the association of *C. eugametos* mitochondrial SSU and LSU rRNA sub-fragments with putative mitochondrial ribosomes and large and small ribosomal subunits in preparations of total cellular ribosomes fractionated by sucrose density gradient centrifugation. The putative mitochondrial ribosome monomers and subunits, which sediment more slowly than (and independently of) their cytoplasmic and chloroplast counterparts, were identified by northern hybridization with mtDNA clones or oligonucleotides specific to the mitochondrial SSU and LSU rRNA sub-fragments of *C. eugametos.*

Materials and Methods

Cell culture and harvesting of total cellular ribosomes

Synchronous, phototrophic cultures of wild-type *C. eugametos* (UTEX 9) and *C. reinhardtii* (137c, mt^+) were grown to a cell density of 3-4 x 10^6 cells/ml as previously described (Lemieux *et al.*, 1980). Six hours after the onset of the light period (L-6), between 1 x 10^{10} and 1.6 x 10^{10} cells were subjected to centrifugation at 5000 x *g* for 5 min at 4°C and resuspended to a final concentration of 2 x 10^9 cells/ml in TKM buffer (25 mM Tris-HCl, pH 7.8, 25 mM KCl, 25 mM MgOAc; 5 mM glutathione). The cells were lysed by two passages through a French Pressure cell (Aminco) at 5000 psi. The cell lysate was subjected to centrifugation at 40,000 x *g* for 30 min at 4°C; the resulting supernatant was designated as the 40s fraction. Sixty A_{260} units of the 40s fraction or of the pellet resulting from centrifugation of the 40s fraction through a 1.85 M sucrose cushion (134,000 x *g* for 4 h at 4°C) were layered on top of sucrose step gradients composed of 9, 14, 19, 24, 29 and 34% sucrose in either TKM buffer or TK buffer (25 mM Tris-HCl, pH 7.8, 25 mM KCl; 5 mM glutathione) containing either 0 or 5 mM MgOAc. After centrifugation at 22,500 rpm at 2°C for the times indicated in a Beckman SW28 rotor, 0.5 ml fractions of the sucrose gradients were collected using a syringe pump to upwardly displace the gradient, and aliquots of each fraction were analyzed spectrophotometrically. Another 5 μl aliquot of each fraction was denatured by incubation at 50 °C for 60 min in the presence of glyoxal (Maniatis *et al.*, 1982), diluted with 20X SSPE (3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.4) to a final concentration of 5X SSPE, and applied to a Hybond N (Amersham) membrane in a slot blot manifold. Each slot blot was rinsed twice with 20X SSPE, allowed to air dry and baked at 80°C under vacuum for 2 h. All solutions and glassware were treated to inhibit the activity of RNases (Maniatis *et al.*, 1982).

Hybridization

C. eugametos mitochondrial ribosomes were identified using hybridization probes derived from clones of the *C. eugametos* mtDNA and synthetic oligonucleotide probes. The 2.1 kb *Hind*III (probe III) and 3.2 kb *Sma*I-*Hind*III (probe VI) DNA fragments of *C. eugametos* (Fig. 6) were isolated from agarose gels using glass matrix (Geneclean, Bio101) after the appropriate restriction endonuclease digestion of recombinant clones. The sequences of the *C. eugametos* mitochondrial rRNA-specific oligonucleotide probes and their corresponding co-ordinates relative to the numbering of the *E. coli* rRNAs are as follows:

S_1	5' CTCGCATAATCTACTCACCCG 3'	*E. coli* 16S rRNA 110-128
S_2	5' TCACACGCAATACCCAATCAT 3'	*E. coli* 16S rRNA 560-582
L_4	5' AACCTTAGACTTTCGGCCGTTT 3'	*E. coli* 23S rRNA 1292-1313
L_5	5' CATTACACCATTCATGCGCG 3'	*E. coli* 23S rRNA 2020-2039
L_6	5' CCCTTAAAACCTTGTGCAGC 3'	*E. coli* 23S rRNA 2526-2545

Filter-bound RNA isolated from the sucrose gradients was allowed to hybridize with DNA restriction fragments that were radio-labelled by random priming (Feinberg & Volgelstein, 1983, 1984) using [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) and Klenow polymerase (Amersham) or with oligonucleotide probes that were 5' end-labelled using T4 polynucleotide kinase (Pharmacia) and [γ-^{32}P]ATP (3000 Ci/mmol; Amersham) as described in Sambrook *et al.* (1989). The filters were pre-hybridized for 4 h at the hybridization temperature (42°C) in 5X SSPE (20X SSPE= 3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.4), 50% v/v deionized formamide, 5X Denhardt's solution (1X Denhardt's solution = 0.1 % w/v polyvinylpyrollidone, 0.1% w/v bovine serum albumin, 0.1% w/v Ficoll) and 100 μg/ml denatured salmon sperm DNA. Hybridization was allowed to proceed at 42°C for 48 h. The filters probed with random-primed DNA fragments were washed twice in 5X SSPE at 42°C and twice in 1 X SSPE, 0.1% SDS at 42°C and twice in 0.1X SSPE, 0.1% SDS at room

temperature. The final low-salt washes were omitted in northern hybridizations using oligonucleotide probes. The filters were exposed to Kodak X-Omat AR film at -70°C with intensifying screens.

Isolation of RNA from ribosome fractions.

RNA was extracted from sucrose gradient fractions by phenol extraction. *C. eugametos* RNA (500 ng) isolated from fractions of the 25 mM Mg^{2+} sucrose gradient or total cellular RNA was 3' end-labelled using [5'-^{32}P]pCp (3000 Ci/mmol; Amersham) and T4 RNA ligase (Pharmacia) as described in Peattie (1979). The radio-labelled RNA was precipitated with ethanol, lyophilized, resuspended in urea stop dye and subjected to electrophoresis in 6% polyacrylamide, 7M urea gels (Schnare & Gray, 1990). Following electrophoresis, the gel was exposed to Kodak XOMAT film for 10 to 30 min.

Isolation of mitochondrial-enriched RNA from wild-type *C. eugametos*

A mitochondrial sub-cellular fraction was isolated from synchronous, phototrophically grown wild-type *C. eugametos* cells. The cells were collected at the beginning of the light period (L-0) when the culture density was 2.7 x 10^6 cells/ml. The cell pellet was washed twice by resuspending the cells in buffer A (300 mM mannitol, 3 mM EDTA, 0.1% bovine serum albumin, 1 mM β-mercaptoethanol, 50 mM Tris, pH 8.0) and subjecting the suspension to centrifugation at 6000 x *g*. The cells were resuspended to a final density of 1.4 x 10^8 cells/ml in buffer A and lysed at 3000 psi in the French pressure cell into an equal volume of cold buffer A. The minimum cell pressure required to lyse 95% of the cells leaving 5% of the cells undamaged or slightly cracked as judged by light microscopy was chosen to minimize the damage to mitochondria. The cell suspension was subjected to two rounds of centrifugation at

3000 x *g* for 15 min and the resulting supernatant was subjected to centrifugation at 10,000 x *g* for 20 min at 4°C. The crude mitochondrial pellet was resuspended in 2.5 ml of buffer A and 10 ml of buffer B (150 mM NaCl, 100 mM EDTA, 10 mM Tris, pH 8.0) and centrifuged twice at 10,000 x *g* for 20 min at 4°C. The mitochondrial pellet was incubated on ice for 15 min after the addition of 0.2% SDS, 100 mM Tris, pH 9.0. The RNA was then purified by phenol extraction as described by Turmel *et al.*(1988) and subjected to denaturing polyacrylamide gel electrophoresis (as described above) and ethidium bromide staining.

Results

Identification of the *C. eugametos* mitochondrial rRNA-hybridizing fractions within the population of total cellular ribosomes

Total cellular ribosomes were isolated from a synchronous cell culture of *C. eugametos* and partially purified by subjecting the 40s supernatant fraction to centrifugation through a 1.85 M sucrose cushion. During ribosome isolation and fractionation, the concentration of Mg^{2+} was maintained at 25 mM. This concentration was chosen because chloroplast and cytosolic monosomes are stable under these conditions (Hoober & Blobel, 1969; Conde *et al.*, 1975). Spectrophotometric analysis of aliquots of the sucrose gradient revealed two UV-absorbing peaks. Calculation of the apparent sedimentation coefficients (McEwen, 1967) of these UV-absorbing zones within the gradient indicated that these peaks are most likely 69-70S chloroplast and 83S cytosolic ribosomes. No other UV absorbance peaks significantly above background absorbance were observed within the sucrose gradients (Fig. 11). This result was anticipated in view of the fact that mitochondrial ribosomes have not been observed previously using this type of analysis.

Northern hybridization analysis was employed to identify mitochondrial rRNA-hybridizing fractions within the sucrose gradient of total cellular ribosomes; these experiments used *C. eugametos* mtDNA fragments (Fig. 6) and oligonucleotides specific to individual *C. eugametos* mitochondrial rRNAs as hybridization probes (Fig. 11). Aliquots of RNA in each fraction of the sucrose gradient were denatured, applied to a nylon membrane and allowed to anneal with DNA probes that contained either the coding regions for the LSU rRNA species L_6 and the SSU rRNA species S_2 (probe III) or the coding regions for L_5 and S_3 (probe VI). Using the same hybridization conditions, probes III and VI specifically hybridized with *C. eugametos* mitochondrial rRNA in northern blot analysis of total cellular RNA (Fig. 6). The

Figure 11. Sucrose gradient fractionation of *C. eugametos* cellular ribosomes isolated in buffers containing 25 mM Mg^{2+} and identification of the mitochondrial rRNA-hybridizing fractions. Cellular ribosomes were layered on top of a sucrose gradient and subjected to centrifugation at 22,500 rpm in a Beckman SW28 rotor at 2^0C for 8 h. An aliquot of each fraction of the sucrose gradient was diluted and analyzed at 260 nm using a Beckman single-beam spectrophotometer (a) and another aliquot was applied to a nylon membrane in a slot blot apparatus and used in northern blot analysis with *C. eugametos* mitochondrial gene-specific probes (b). The gradient fractions are numbered in ascending order from the top of the gradient. The positions of the chloroplast (70S) and cytosolic (83S) ribosome UV-absorbing peaks are indicated. Restriction fragments of the *C. eugametos* mitochondrial genome containing most of the rRNA gene pieces for L_6 and S_2 (L_6/S_2; probe III) or L_5 and S_3 (L_5/S_3; probe IV) were radio-labelled and allowed to anneal with the RNA from each fraction of the sucrose gradient, as indicated to the left of each slot blot. RNA was extracted from fractions of the gradient labelled A through E and resolved by denaturing polyacrylamide gel electrophoresis (see Fig. 13).

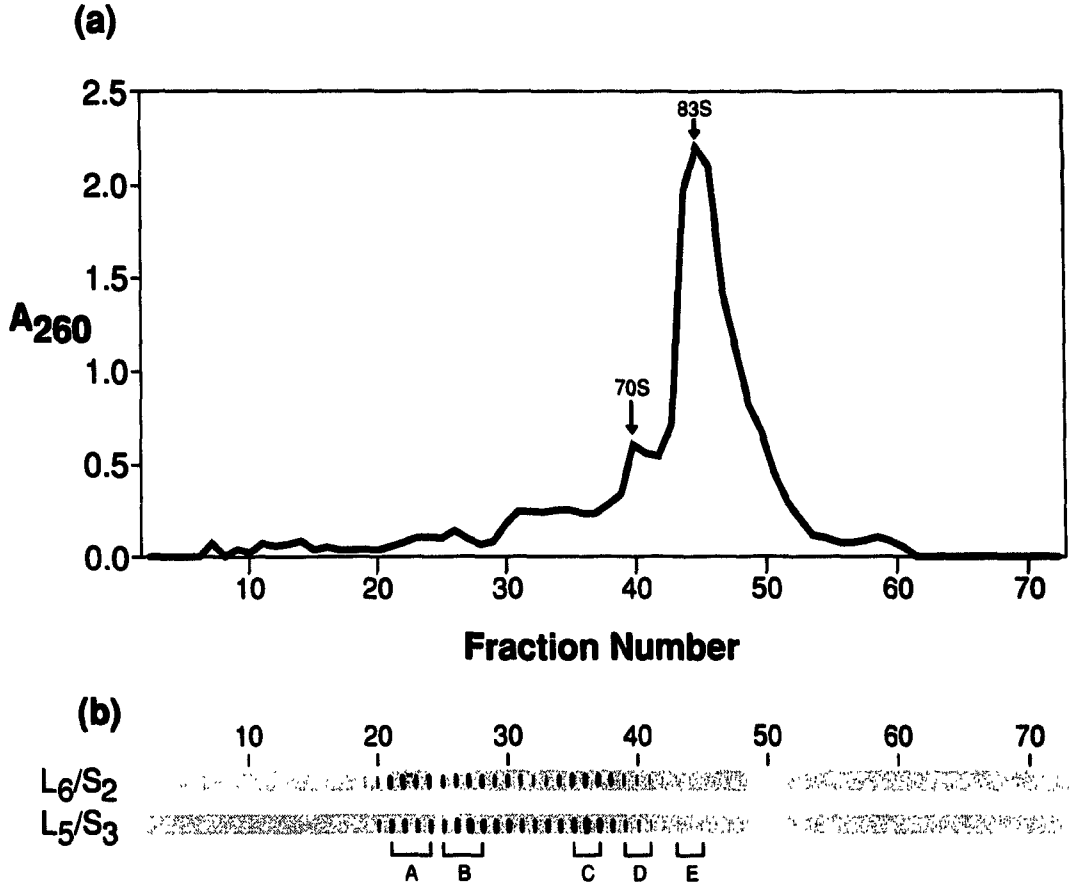

(a)
2.5
2.0
1.5
A_{260}
1.0
0.5
0.0
83S
70S
10
20
30
40
50
60
70
Fraction Number
(b)
10
20
30
40
50
60
70
L_6/S_2
L_5/S_3
A
B
C
D
E

Figure 11.

radio-labelled restriction fragments containing the coding regions for L_6/S_2 and L_5/S_3 each hybridized with RNA in fractions 20-40 of the sucrose gradient (Fig. 11). Due to differences in the intensity of hybridization of these probes to RNA within this region of the sucrose gradient, it appeared that there were three sets of hybridizing fractions, each of which had lower sedimentation values than either cytosolic or chloroplast monosomes. After the radio-labelled probes were removed from the membranes, end-labelled oligonucleotides specific to L_6, S_2 and S_1 were individually allowed to anneal to the same denatured RNA recovered from the 25 mM Mg^{2+} sucrose gradient fractions (Fig. 12). The oligonucleotide probe specific to L_6 hybridized to RNA in fractions 25-40. The oligonucleotide probes specific to S_1 and S_2 both hybridized with fractions 20-24 and 33-40. The S_2 probe weakly hybridized to RNA in fractions 25-28. The patterns of hybridization of the LSU- and SSU-specific oligonucleotides and the restriction fragments containing LSU and SSU rRNA coding regions suggest that the *C. eugametos* mitochondrial rRNA transcripts associate in ribonucleoprotein particles of three sedimentation classes corresponding to small (S, fractions 20-24) and large (L, fractions 25-31) ribosomal subunits and monosomes (M, fractions 35-40) as indicated in Figure 12. Judged on the relative hybridization signals, approximately 50% of the mitochondrial ribosomes are dissociated into ribosomal subunits in the presence of 25 mM Mg^{2+}.

RNA was extracted from fractions of the 25 mM Mg^{2+} sucrose gradient that hybridized with mitochondrial rRNA probes as well as from fractions identified by UV absorbance as the chloroplast and cytosolic monosomes. The RNA was 3' end labelled using pCp and RNA ligase and fractionated by polyacrylamide gel electrophoresis (Fig.13). The most intensely labelled and resolved bands correspond to small cytosolic and chloroplast rRNAs with the exception of an unidentified 100 nt band in lane A. Comparison of the different ribosome fractions revealed that the cytosolic 5.8S rRNA is present in both the cytosolic and chloroplast ribosome fractions and that the cytosolic 5S and 5.8S rRNAs and the chloroplast LSU 810-nt and 280-nt rRNAs are present in the putative mitochondrial monosome and large ribosomal

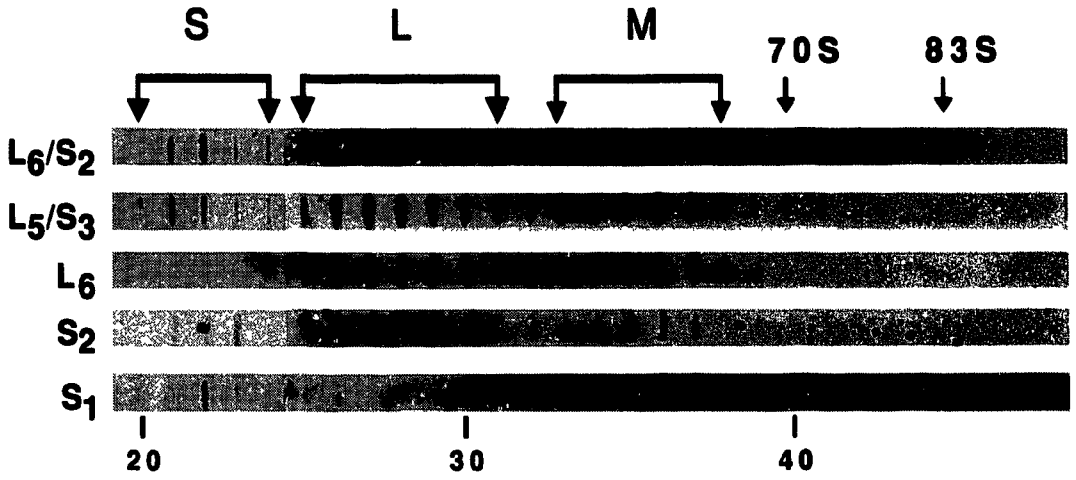


Figure 12. Northern hybridization analysis of *C. eugametos* ribosomes isolated and fractionated as described in Figure 11 using oligonucleotide probes specific to L_6, S_2 and S_1 and probes that contained the coding regions for L_6/S_2 (probe III) and L_5/S_3 (probe VI). The probes used in northern hybridization are indicated to the left of each series of slot blots. The fraction numbers at the bottom of the figure correspond to the UV absorbance profile presented in Figure 11. Fractions 20 through 48 are included in the figure. No hybridization to fractions 1-20 or 49-72 was observed. The positions of the chloroplast (70S) and cytosolic (83S) ribosomes are indicated. Putative mitochondrial small subunit, large subunit and monosome fractions are indicated as S, L and M, respectively (see text).

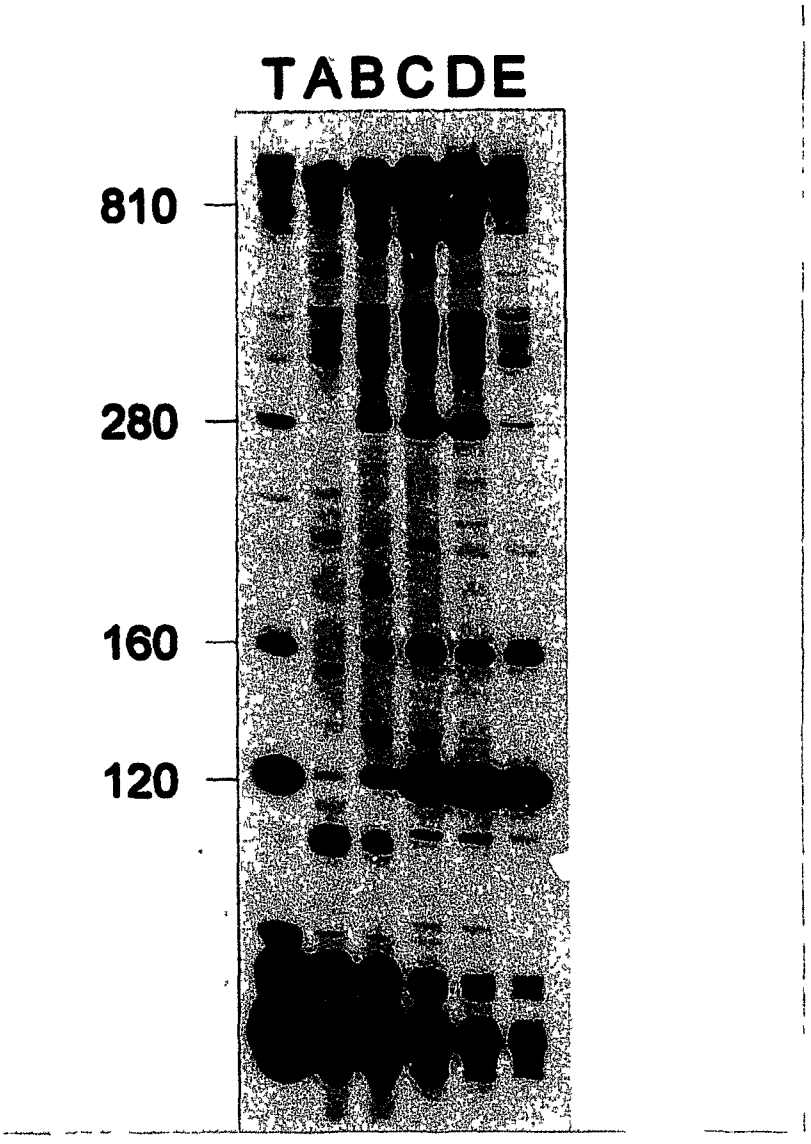


Figure 13. Radio-labelled *C. eugametos* RNA isolated from fractions of the 25 mM Mg^{2+} sucrose gradient and resolved by denaturing polyacrylamide gel electrophoresis. RNA was extracted from fractions of the sucrose gradient (Fig. 11) associated with mitochondrial rRNA hybridization and from fractions identifed as chloroplast and cytosolic monosomes. Fractions A through E correspond to those presented in Figure 11. The lane labelled T contains total cellular RNA. The sizes in nt of the most abundant small RNA species are indicated on the left and include the chloroplast and cytosolic 5S (120), cytosolic 5.8S (160), *α* fragment of the chloroplast LSU (280) and *γ* fragment of the chloroplast LSU (810) rRNAs.

subunit fractions. This observation is likely attributable to cross-contamination of ribosome fractions within the gradient by abundant chloroplast and cytosolic rRNAs, possibly due to mixing. Because many bands between 800 and 50 nt were detected in each fraction of the 25 mM Mg^{2+} gradient, the mitochondrial rRNAs of *C. eugametos* could not be identified by size in those fractions hybridizing with mitochondrial rRNA-specific probes.

Comparison of the sedimentation behaviour of ribosomes from *C. eugametos* and *C. reinhardtii*

C. eugametos and *C. reinhardtii* total cellular ribosomes were isolated using different concentrations of Mg^{2+} and simultaneously subjected to sucrose gradient fractionation to compare their apparent sedimentation coefficients. The centrifugation of the 40s fraction through a sucrose cushion was omitted during these and subsequent ribosome isolations to ensure that small mitochondrial rRNAs or ribonucleoprotein particles were not excluded from hybridization analysis. The spectrophotometric profile of total cellular ribosomes from *C. eugametos* and *C. reinhardtii* using 25, 5 and 0 mM Mg^{2+} are shown in Figure 14. At 25 and 5 mM Mg^{2+}, the cytosolic ribosomes from *C. eugametos* sedimented at the same rate as the 83S cytosolic ribosomes of *C. reinhardtii*. The 70S chloroplast ribosomes appear as a shoulder in front of the cytosolic ribosome peak. At 5 mM Mg^{2+}, a broad peak of between 50-60S appeared in the UV profile of each species. The UV profiles of *C. eugametos* and *C. reinhardtii* ribosomes fractionated from the 0 mM Mg^{2+} gradient show that the 83S cytosolic ribosome class is absent and that the majority of the UV-absorbing material is between 30S and 60S. Therefore, it appears that cytosolic and chloroplast ribosomes from *C. eugametos* and *C. reinhardtii* have similar sedimentation rates at different Mg^{2+} concentrations. Mitochondrial-specific UV-absorbing peaks were not observed in ribosome preparations from either alga.

Figure 14. Spectrophotometric analysis of *C. eugametos* and *C. reinhardtii* cellular ribosomes isolated and fractionated in sucrose gradients containing (a) 25, (b) 5 and (c) 0 mM Mg^{2+}. Equivalent amounts of total cellular ribosomes were layered on top of sucrose gradients and subjected to centrifugation at 22,500 rpm at 2^0C for 14 h in a Beckman SW28 rotor. The fractions are numbered in ascending order from the top of the gradient. The relative position of the cytosolic ribosomes (83S) is indicated on each graph. The thin and thick lines represent UV absorbance readings of the *C. eugametos* and *C. reinhardtii* total ribosome preparations, respectively.

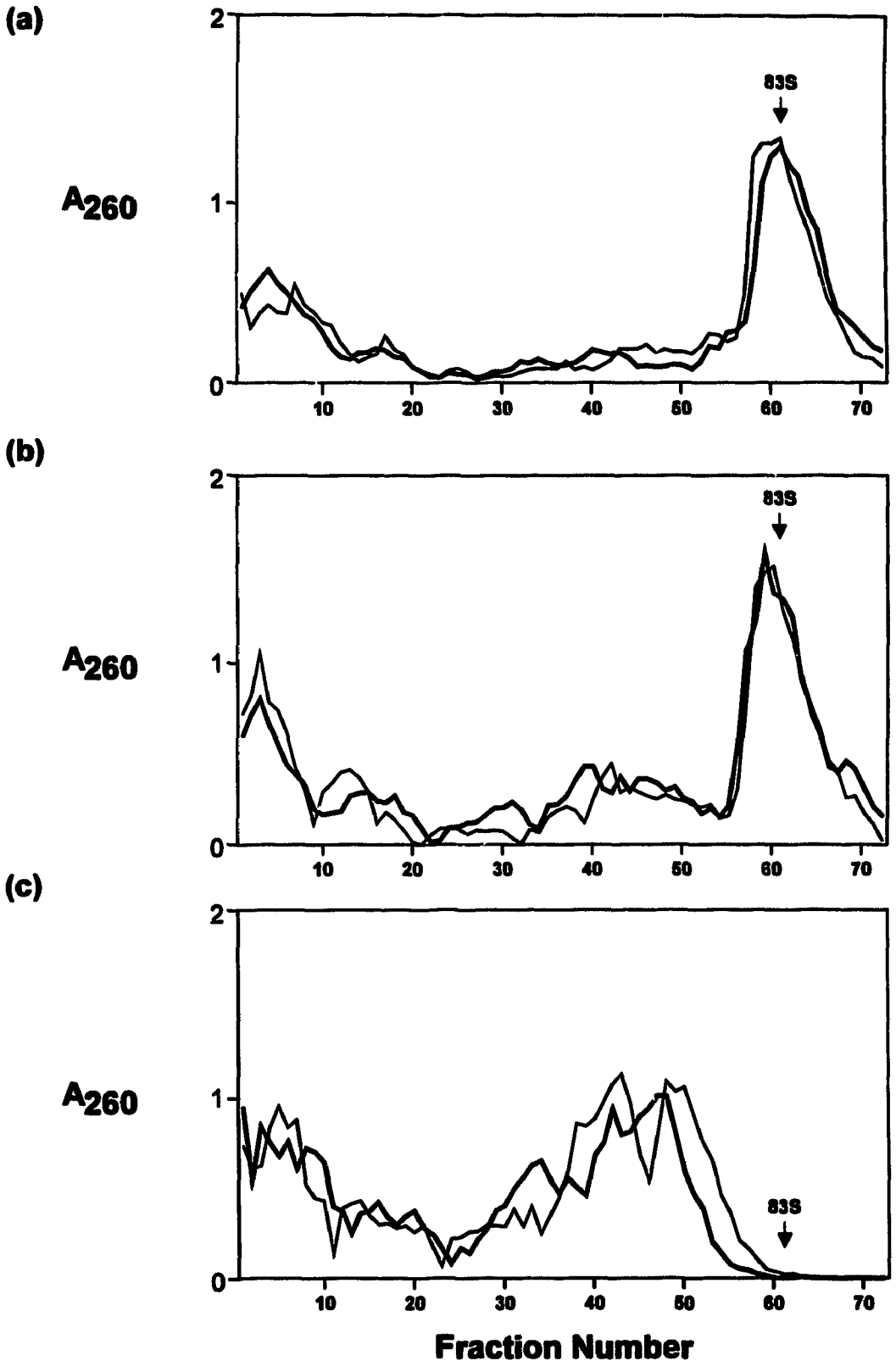

(a)
A260
2
1
0
83S
10
20
30
40
50
60
70
(b)
A260
2
1
0
83S
10
20
30
40
50
60
70
(c)
A260
2
1
0
83S
10
20
30
40
50
60
70
Fraction Number

Figure 14

The effect of Mg^{2+} concentration on the sedimentation of *C. eugametos* mitochondrial ribosomes

Slot blots of denatured *C. eugametos* RNA from aliquots of the gradients containing 5 and 0 mM Mg^{2+} were prepared and allowed to anneal to end-labelled oligonucleotides specific for the *C. eugametos* rRNAs L_6, S_2 and S_1. In gradients containing 5 or 0 mM Mg^{2+}, there was no difference in the sedimentation of the mitochondrial rRNA-hybridizing fractions, and the SSU rRNA-hybridizing fractions had a lower sedimentation value than did the LSU rRNA-hybridizing fractions (Fig. 15). Therefore, at the highest concentration of Mg^{2+} employed (25 mM), mitochondrial ribosomes are partially dissociated into ribosomal subunits and at lower concentrations of Mg^{2+} (5 and 0 mM), only small and large ribosomal subunits are present. Because small RNP particles and free rRNA were not removed by centrifugation through a sucrose cushion prior to sucrose gradient fractionation using the low Mg^{2+} concentrations, and because no hybridization was observed in the fractions isolated from the top of the sucrose gradient, it appears that there is no significant amount of mitochondrial rRNA within the cell that is not assembled into mitochondrial ribosomal subunits. Moreover, once the monosomes have dissociated into ribosomal subunits, the subunits do not further dissociate into smaller RNP particles, suggesting that there is not a reversible association between smaller RNP particles or individual mitochondrial rRNAs within these mitochondrial ribosomal subunits. Hybridization results of duplicate blots of the 25, 5 and 0 mM Mg^{2+} sucrose gradients using oligonucleotide probes specific to L_4 and L_5 gave identical profiles to those obtained by the hybridization of the probe specific to L_6 LSU rRNA (data not shown). By extrapolation, using the values of 70S and 83S for the chloroplast and cytosolic ribosomes, respectively, the *C. eugametos* mitochondrial ribosome monomers and large and small subunits have approximate sedimentation values of 60-68S, 40-47S and 30-37S, respectively. However, an accurate

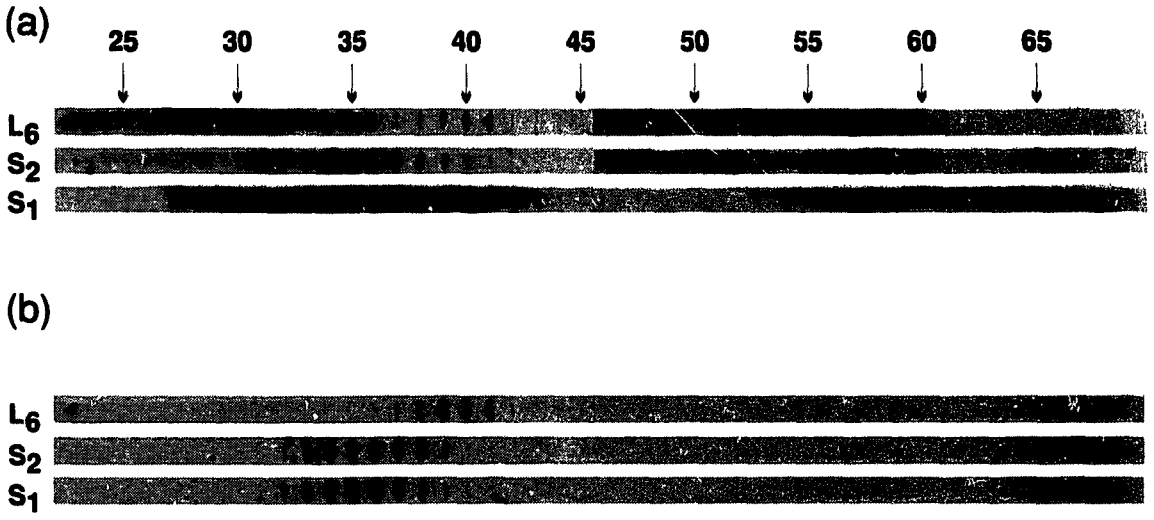


Figure 15. Northern hybridization anaiysis of *C. eugametos* ribosomes isolated and fractionated in gradients containing (a) 5 and (b) 0 mM Mg^{2+}. Aliquots of each fraction of the 5 and 0 mM Mg^{2+} gradients were applied to nylon membranes and allowed to anneal with radio-labelled oligonucleotide probes specific to L_6, S_2 and S_1 (indicated on the left of each panel). The fraction numbers indicated above the slot blots correspond to those in Figure 12 . The cytosolic ribosomes (83S) are associated with fraction number 61.

determination of the sedimentation coefficients of these mitochondrial ribosomes and subunits will require purification of this minor cellular component and analytical centrifugation.

Isolation of mitochondrial-enriched *C. eugametos* RNA

RNA isolated from a mitochondrial sub-cellular fraction of *C. eugametos* was enriched for RNAs that correspond to the size of the mitochondrial rRNAs predicted by sequencing and detected in northern blots of total cellular RNA (Fig.16). The yield of mitochondrial-enriched *C. eugametos* RNA was approximately 0.1% of the amount of total cellular RNA isolated from a similar number of cells. For isolation of a mitochondrial sub-cellular fraction, increasing the initial amount of harvested culture resulted in a lower abundance of mitochondrial rRNA relative to chloroplast and cytosolic rRNA. Hybridization of mitochondrial-enriched RNA with oligonucleotide probes specific to L_6 and S_2 confirmed that these RNA preparations were enriched for mitochondrial rRNAs (data not shown).

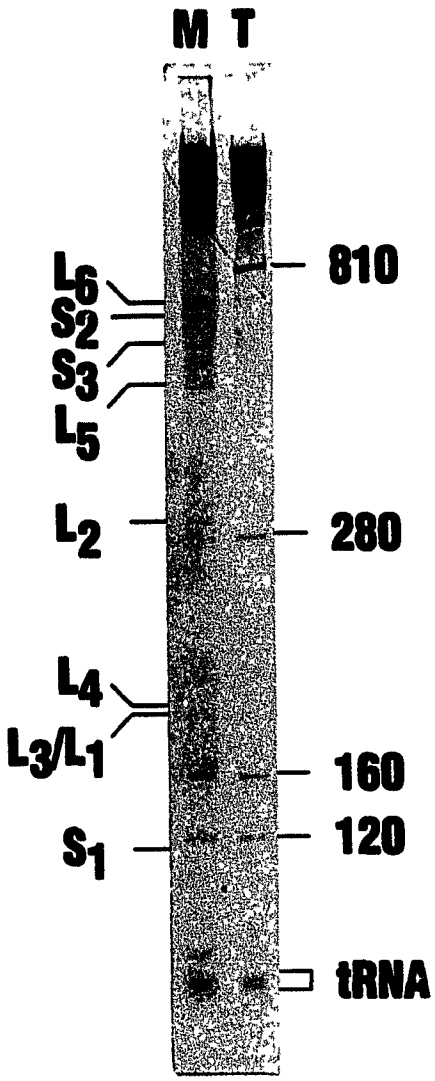


Figure 16. Gel electrophoresis and ethidium bromide staining of RNA isolated from a mitochondrial sub-cellular fraction of *C. eugametos*. Total cellular RNA (T) and mitochondrial-enriched (M) RNA (2 μg of each) was fractionated in denaturing polyacrylamide gels and stained with eithidium bromide. The positions of the cytosolic and chloroplast 5S rRNA (120 nt), cytosolic 5.8S rRNA (160 nt), chloroplast LSU rRNA α fragment (280 nt), chloroplast LSU rRNA γ fragment (810 nt) and tRNAs (60-80 nt) and the *C. eugametos* mitochondrial RNAs (S_1-S_3 and L_1-L_6) identified by size are indicated.

Discussion

Mitochondrial rRNA-hybridizing fractions were identified within sucrose gradients of total cellular ribosomes isolated from *C. eugametos*. The apparent sedimentation coefficients of the *C. eugametos* putative mitochondrial ribosomes and large and small ribosomal subunits are approximately 60-68S, 40-47S and 30-37S, respectively, when isolated in buffers containing 25 mM Mg^{2+}. It appears that the mitochondrial ribosomes of *C. eugametos* have a sedimentation coefficent that is intermediate between the 55-60S ribosomes isolated from animal mitochondria (O'Brien, 1971; Rabbits & Work, 1971) and the 70-80S mitochondrial ribosomes of fungi, ciliates and plants (Chi & Suyama, 1970; Vasconcelos & Bogorad, 1971; Avandhani & Buetow, 1972; Vignais *et al.*, 1972; Pinel *et al.*, 1986; Leaver & Harmey, 1973; Tait & Knowles, 1977). Spectrophotometric and hybridization analysis of sucrose gradients revealed that mitochondrial ribosomes are a very minor component of total cellular ribosomes in these algae.

C. eugametos mitochondrial ribosomes dissociate into ribosomal subunits at a higher concentration of Mg^{2+} than do cytosolic and chloroplast ribosomes. However, the ribosomal subunits do not appear to dissociate into smaller RNP particles, suggesting that there is not a reversible association between small rRNA or small RNP particles within the mitochondrial ribosomal subunits. Similarly, *C. reinhardtii* chloroplast large ribosomal subunits are stable in the absence of Mg^{2+} despite the demonstration that the chloroplast LSU rRNA is composed of four individual RNA species (Hoober & Blobel, 1969; Conde *et al.*, 1975; Rochaix & Darlix, 1982; Lemieux *et al.*, 1989). In this regard, it is tempting to speculate that the base-pairing between small rRNAs is established early in the biogenesis of ribosomal subunits containing discontinuous rRNAs, regardless of whether or not the rRNA gene pieces are transcribed in the same 5' to 3' order as homologous rRNA domains in continuous rRNAs, and that, once formed, these complexes of interacting small rRNAs are stable. However, the hybridization

results presented here indicate that mitochondrial ribosomal subunits and monosomes are a minor component of total steady-state cellular ribosomes and, therefore, it was unlikely that precursors of mitochondrial ribosomal subunits would be observed. Therefore, no evidence was collected to support either the hypothesis that mitochondrial ribosome biogenesis in *Chlamydomonas* proceeds by the association of small RNP particles composed of individual mitochondrial rRNAs and r-proteins (Boer & Gray, 1988a) or that a transcript(s) containing interspersed rRNA domains form(s) the ribosomal subunit cores through base-paring in larger RNP particles that are later processed to generate the mature mitochondrial rRNA termini (Chapter 2).

The individual mitochondrial rRNAs of *C. eugametos* were not identified following fractionation of RNA isolated from putative ribosomal subunits or monosomes. It appears that the RNA isolated from each ribosome class contains more small RNAs then would be expected for homogeneous preparations of either mitochondrial, cytosolic or chloroplast ribosomes. This observation suggests that, even though care was taken to minimize RNase activity, limited RNase cleavage may have occurred during the isolation procedure. Differences in the isolation of total cellular RNA and RNA from ribosomes may account for the additional fragmentation of the rRNA from ribosomes compared to the rRNAs identified in total cellular RNA. Total cellular RNA was prepared by lysing cells directly into cold acid-phenol and SDS, which contrasts with the delay between cell lysis, ribosome fractionation and RNA extraction from the ribosomal fractions of the sucrose gradients. This delay may have allowed cellular RNAses that are normally sequestered away from the rRNAs to catalyze limited digestion of rRNA in ribosomes. In the future, ribonuclease inhibitors should be added before lysis of the cells for ribosome isolation. Such a change in the isolation and fractionation protocol may allow the isolation of intact mitochondrial rRNA from ribosomes and ribosomal subunits and, therefore, a test of the hypothesis that each rRNA predicted to interact by base-pairing in the SSU and LSU rRNA secondary structure models is in fact associated in an RNP complex. The low yield of

mitochondrial rRNA isolated from wild-type *C. eugametos* indicates that it may be impractical to isolate mitochondrial ribosomes from a mitochondrial cell pellet in this algal strain. However, a wall-less mutant of *C. reinhardtii* is available and may be the organism of choice for isolating mitochondrial ribosomes.

If *Chlamydomonas* mitochondrial ribosomes can be purified in sufficient quantity, it would be of considerable interest to develop an *in vitro* translation system and to study the effect of antibiotics on the function of these mitochondrial ribosomes. Early studies of cell growth and inheritance of antibiotic resistance mutations in *C. reinhardtii* were inconclusive about the effect of antibiotics on mitochondrial translation. It was assumed that by examining the growth of wild-type and mutant cells in photo-, mixo- and heterotrophic conditions in the presence of antibiotics and by determining the inheritance pattern of antibiotic resistance markers, it would be possible to deduce the cellular compartment of antibiotic action. Most antibiotics tested, however, affect cell proliferation under all growth conditions. Surzycki & Gillham (1971) concluded that both the mitochondrial and chloroplast translation systems were equally affected by antibiotics that inhibited translation in chloroplasts. Boynton *et al.* (1973) and Conde *et al.* (1975) concluded that resistance to some antibiotics was conferred by mutations in ribosomal proteins encoded by the chloroplast DNA but utilized in both chloroplast and mitochondrial ribosomes. However, several of these resistance mutations have since been shown to alter chloroplast LSU or SSU rRNA directly (Harris *et al.*, 1989). Therefore, evidence for the apparent co-sensitivity and co-resistance of chloroplast and mitochondrial ribosomes in *C. reinhardtii* might reflect the requirement of translation products of the chloroplast for mitochondrial function rather than the sharing of common translational components. The determination of the antibiotic sensitivity or resistance of isolated mitochondrial ribosomes may make it possible to understand the interactions between chloroplast and mitochondrial function.

Chapter 4

Phylogenetic analysis of the mitochondrial rRNAs of *Chlamydomonas*

Introduction

Morphological, biochemical, genome organization and molecular phylogenetic analysis of nuclear and chloroplast-encoded rRNA genes indicate that the nuclear and chloroplast genomes of land plants (metaphytes) and green algae (chlorophytes) share a common evolutionary history (Pickett-Heaps, 1975; Chapman and Ragan, 1980; Kumazaki *et al.*, 1983; Gray *et al.*, 1984; Bold and Wynne, 1985; Palmer, 1985; Walker, 1985; Gunderson *et al.*, 1987; Weil, 1987; Huss & Sogin, 1990; Buchheim *et al.*, 1991; Chapman & Buchheim, 1992; Turmel *et al.*, 1993a). Based on differences in genome size, gene content and gene sequence, it is not clear whether the mitochondrial genomes of these two groups of photosynthetic organisms also share a single common evolutionary origin (Gray, 1988; Cedergren *et al.*, 1988; Gray *et al.*, 1989; Lonegran and Gray, 1994).

Plant mitochondrial rRNA genes show striking structural and sequence similarities to the rRNA genes of members of the α-proteobacteria, supporting hypotheses regarding the endosymbiotic origin of mitochondria (Bonen *et al.*, 1977; Spencer *et al.*, 1984; Yang *et al.*, 1985; Gray *et al.*, 1989; Van de Peer *et al.*, 1990). From analyses of SSU rRNA genes of *Prototheca wickerhamii*, a colourless *Chlorella*-like chlorophycean alga (Huss & Sogin, 1990), and *Acanthamoeba castellanii*, an ameoboid protozoan that is basal to the chlorophyte-metaphyte divergence in nuclear rRNA gene trees (Cedergren *et al.*, 1988; Hendriks *et al.*, 1991), it is clear that these organisms also have eubacterial/plant-like mitochondrial rRNA gene features (Wolff & Kück, 1990; Lonergran & Gray, 1994). In contrast, the mitochondrial rRNA genes of *C. reinhardtii* show an apparent rapid rate of sequence divergence, in comparison to plants, resulting in long branch lengths in phylogenetic trees and no obvious connection between the rRNA genes of *C. reinhardtii* and those of land plants (Cedegren *et al.*, 1988; Gray *et al.*, 1989). From this limited data set, it appears that *C. reinhardtii* differs from other chlorophytes and metaphytes in that its nuclear, chloroplast and mitochondrial rRNA

gene trees are not congruent. It has been suggested that the mitochondrial rRNA genes of *C. reinhardtii* were either acquired in a different endosymbiotic event than those of land plants, *A. castellanii* and *P. wickerhamii* or that the mitochondrial rRNA genes of *C. reinhardtii* have changed radically since the divergence of the *Chlamydomonas*- and *Chlorella*-like algae (Gray *et al.*, 1989; Lonergran & Gray, 1994). However, the sparsity of rRNA gene sequences makes it difficult to establish whether the mitochondrial rRNA gene structure and sequences of *C. reinhardtii* or *P. wickerhamii* are representative of chlorophycean green algae.

Southern analysis of mtDNA and northern analysis of mitochondrial rRNA indicate that there has been considerable sequence divergence between the mitochondrial rRNA genes of *C. eugametos* and *C. reinhardtii*. Therefore, I asked whether the mitochondrial rRNA gene sequences from a distantly related member of the genus *Chlamydomonas* affiliate with those of *C. reinhardtii* in mitochondrial rRNA gene trees. Such an observation would support hypotheses regarding the derivation of the scrambled rRNA genes in both *C. eugametos* and *C. reinhardtii* from a common ancestral gene. In addition, it was thought possible that adding additional *Chlamydomonas* taxa to databases used for phylogenetic tree construction would alter the apparent affiliations within mitochondrial rRNA phylogenetic trees.

Both distance- and parsimony-based phylogenetic analysis consistently showed that the mitochondrial rRNA genes of *C. eugametos* and *C. reinhardtii* shared a common ancestor with each other more recently than with those of other organisms. The long branch lengths separating *C. eugametos* and *C.reinhardtii* and separating these algae from other green algae and the land plants suggest that there has been a more rapid rate of nucleotide substitution in mitochondrial rRNA genes of the genus *Chlamydomonas* compared to other green algae and plants. Such long branch lengths can obscure the true evolutionary history of these algae. For this reason, it appears that additional sequences must be obtained from other chlorophycean green algae before the discrepancy between the nuclear and mitochondrial rRNA phylogenies can be resolved with confidence.

Methods

SSU and LSU rRNA gene sequences (Tables 5 and 6) were aligned by secondary structure models and used in pair-wise comparisons and phylogenetic treeing analyses. The database for the SSU and LSU rRNA alignments included 541 and 1093 homologous nucleotide positions, respectively. Phylogenetic trees based on distance for SSU and LSU rRNA gene sequences were obtained using Seqboot, DNADist, Neighbor, Consense and Draw programs of PHYLIP version 3.53c (Felsenstein, 1993). Parsimony analysis was performed using the DNAPars and Draw program of PHYLIP, version 3.53c (Felsenstein, 1993). The rRNA gene sequence of *Thermus thermotoga* was chosen as an outgroup for each analysis.

Table 5

GenBank accession numbers of the SSU rRNA gene sequences used in phylogenetic analysis.

Eubacteria	GenBank Accession Number
Planctomyces staleyi	M34126
Xylella fastidiosa	M26601
Vitreoscilla stercoraria	M22519
Spirochaeta aurantia	M57740
Treponema bryantii	M57737
Anaplasma marginale	M60313
Frankia sp.	M55343
Chlamydia trachomatis	M59178
Campylobacter hyointestinalis	M65009
Clavibacter xyli	M60935
Cytophaga lytica	M62796
Ancylobacter aquaticus	M62790
Borrelia hermsii	M60968
Flexibacter aurantiacus	M62792
Pasteurella multocida	M35018
Propionibacterium acnes	X53218
Flexistipes sinusarabici	M59231
Flavobacterium uliginosum	M62799
Runella slithyformis	M62786
Vagococcus fluvialis	X54258
Fervidobacterium islandicum	M59176
Flectobacillus marinus	M62788
Rickettsia prowazekii	M21789
Rickettsia rickettsii	M21293
Escherichia coli	J01695
Agrobacterium tumefaciens	M11223
Brucella abortus	X13695
Thermus thermophilus	X07998
Thermotoga maritima	M21774
Wolbachia persica	M21292
Streptomyces ambofaciens	M27245
Deinococcus radiodurans	M21413
Chromobacterium violaceum	M22510
Alcaligenes faecalis	M22508
Anacystis nidulans	X03538
Rhodocyclus purpureus	M34132
Rochalimaea quintana	M11927
Ehrlichia risticii	M21290
Mycoplasma hyopneumoniae	Y00149
Bacillus subtilis	K00637
Bacteroides fragilis	M11656
Heliobacterium chlorum	M11212
Chlamydia psittaci	M13769

Table 5 (continued)

Rhodopseudomonas acidophila	M34128
Rhodomicrobium vannielii	M34127
Vibrio anguillarum	X16895
Mycobacterium kansasii	X15916
Lactobacillus confusus	X52567
Flavobacterium halmophilum	M59153
Pseudomonas aeruginosa	X06684
Listeria monocytogenes	M58822
Anabaena sp.	X59559
Pirellula marina	X62912
Bifidobacterium adolescentis	M58729
Kurthia zopfii	M58800
Spirillum volutans	M34131
Clostridium innocuum	M23732
Leptospira interrogans	X17547
Acholeplasma laidlawii	M23932
Helicobacter mustelae	M35048
Desulfovibrio desulfuricans	M34113
Thermomicrobium roseum	M34115
Neisseria gonorrhoeae	X07714
Rhodobacter capsulatus	M34129
Myxococcus xanthus	M34114
Leuconostoc cremoris	M23034
Lactobacillus halotolerans	M23037
Megasphaera elsdenii	M26493
Proteus vulgaris	J01874
Acinetobacter calcoaceticus	M34139
Arthrobacter globiformis	M23411
Arhodomonas oleiferhydrans	M26631
Streptococcus pleomorphus	M23730
Coxiella burnetii	M21291
Oceanospirillum linum	M22365
Spiroplasma mirum	M24662
Erysipelothrix rhusiopathiae	M23728
Desulfosarcina variabilis	M26632
Chlorobium limicola	M31769
Kingella denitrificans	M22516
Pseudomonas cepacia	M22518
Pseudomonas testosteroni	M11224

Mitochondria

Triticum aestivum	K01229
Saccharomyces cerevisiae	M27607
Podospora anserina	X54864
Aspergillus nidulans	V00653
Schizosaccharomyces pombe	X54866
Tetrahymena pyriformis	X56171
Prototheca wickerhamii	X56099
Chlamydomonas reinhardtii	M32703
Paramecium tetraurelia	X03772

Acanthamoeba castellanii	M13435
Marchantia polymorpha	M68929
Glycine max	M16859
Chlamydomonas eugametos	L28929-L28931

Chloroplast

Pylaiella littoralis	X14873
Cyanidium caldarium	X52985
Euglena gracilis	M12677
Astasia longa	X14386
Chlamydomonas eugametos	NoAcc[1]
Glycine max	X06428
Marchantia polymorpha	X04465
Chlorella vulgaris	X16579
Nicotiana tabacum	V00165
Zea mays	Z00028
Chlamydomonas reinhardtii	X03269

[1] Durocher *et al.*, 1989.

Table 6

GenBank accession numbers of the LSU rRNA gene sequences used in phylogenetic analysis.

Eubacteria	GenBank Accession Number
Rhodobacter capsulatus	X06485
Pseudomonas aeruginosa	Y00432
Pseudomonas cepacia	X16368
Escherichia coli	J01695
Anacystis nidulans	X00512
Piruellula marina	X07408
Streptomyces ambofaciens	M27245
Micrococcus luteus	X06484
Thermus thermophilus	X12612
Bacillus subtilis	K00637,M10606,X00007
Mitochondria	
Acanthamoeba castellanii	U03732
Triticum aestivum	Z11889
Prototheca wickerhamii	X68722
Chlamydomonas reinhardtii	M25123-M25129
Chlamydomonas eugametos	L28929, L28930, L2L28931
Tetrahymena pyriformis	M58011
Neurospora crassa	X55443
Saccharomyces cerevisiae	J01527
Podospora anserina	X14735
Schizosaccharomyces pombe	X06597
Paramecium tetraurelia	K01749
Zea mays	K01868
Oenothera berteriana	X02559
Marchantia polymorpha	M68929
Chloroplast	
Chlamydomonas reinhardtii	X16686,X16687

Results and Discussion

Phylogenetic analysis of mitochondrial rRNA genes

Comparisons of secondary structure models of the *C. eugametos, C. reinhardtii* and plant mitochondrial rRNAs indicate that, although the *Chlamydomonas* rRNAs are smaller than those of plants and are composed of several individual rRNA transcripts (Chapter 2), the majority of the core rRNA structure is shared by these organisms. Therefore, the *Chlamydomonas* mitochondrial rRNAs have a comparable number of phylogenetically informative sites compared to plants and other algae.

The primary sequences of the mitochondrial rRNA genes of *C. eugametos* were included in multiple alignments of mitochondrial, eubacterial and plastid rRNA gene sequences based on conserved positions within secondary structure models. From these data sets, both distance- (Figs. 17 and 18) and parsimony-based (Figs.19 and 20) phylogenetic trees were constructed.

The mitochondrial SSU and LSU rRNA gene sequences of *C. eugametos* and *C. reinhardtii* consistently affiliate with each other to the exclusion of other mitochondrial or eubacterial rRNA genes using all of the treeing methods. Bootstrap analysis of the trees based on distance matrices indicate that this affiliation is statistically significant. Therefore, despite differences in rRNA gene structure and organization and the considerable primary sequence divergence between these two species of *Chlamydomonas*, their mitochondrial rRNA genes are clearly related by descent from a common ancestor.

In all of the trees constructed, the available mitochondrial rRNA genes form a single clade. Distance- and parsimony-based phylogenetic trees constructed using the larger SSU rRNA database show that the mitochondrial rRNAs are derived from within the α-proteobacteria (Figs. 17 and 19). The specific affiliations between the *Chlamydomonas*

Figure 17. A portion of the neighbor-joining distance matrix phylogenetic tree based on eubacterial, chloroplast and mitochondrial SSU rRNA gene sequences showing the relative branching pattern of mitochondrial and α-proteobacterial SSU rRNA gene sequences. The accession numbers for the 106 SSU rRNA sequences used to construct the original distance tree are listed in Table 5. Conserved regions of the SSU rRNA sequences were aligned based on secondary structure models and analyzed using PHYLIP, version, 3.53c (Felsenstein, 1993). The horizontal branch lengths are proportional to the calculated number of nucleotide substitutions observed between gene sequences and the scale bar indicates 10 substitutions per 100 nt. The mitochondrial (mt) and α-proteobacterial (α) sequences are bracketed. The number at the nodes connecting the branches indicates the number of times the group consisting of the taxa to the right of the fork occurred among 100 trees used in bootstrap analysis.

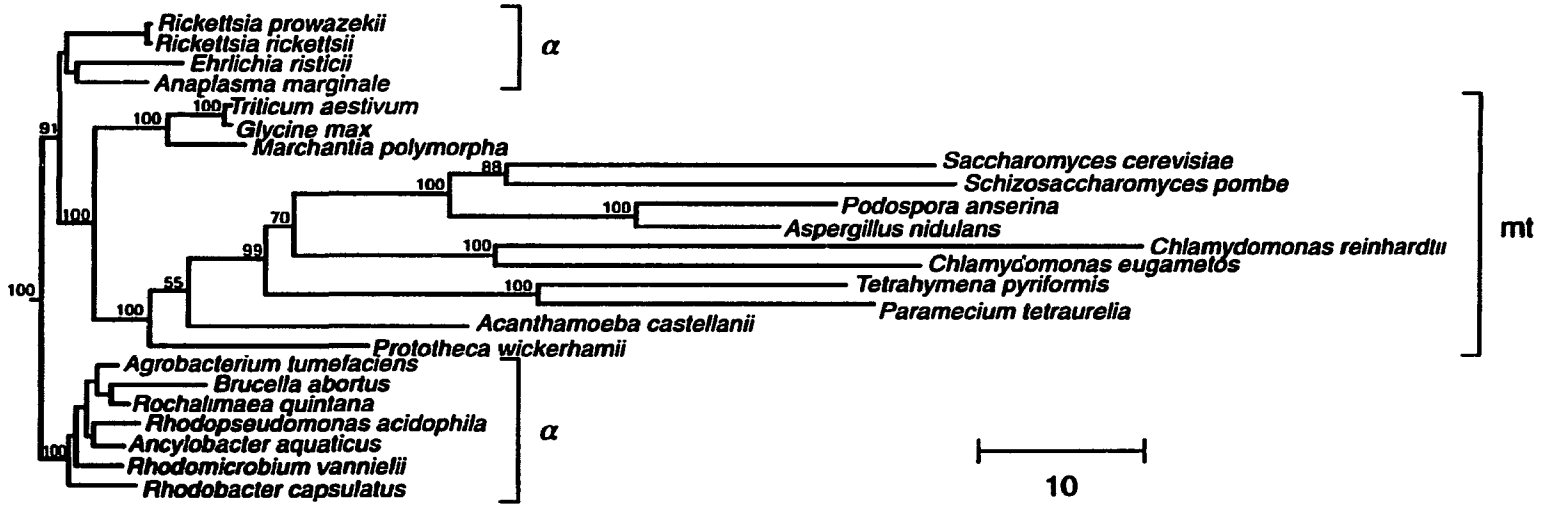

Rickettsia prowazekii
Rickettsia rickettsii
Ehrlichia risticii
Anaplasma marginale
α
Triticum aestivum
Glycine max
Marchantia polymorpha
Saccharomyces cerevisiae
Schizosaccharomyces pombe
Podospora anserina
Aspergillus nidulans
Chlamydomonas reinhardtii
Chlamydomonas eugametos
Tetrahymena pyriformis
Paramecium tetraurelia
Acanthamoeba castellanii
Prototheca wickerhamii
mt
Agrobacterium tumefaciens
Brucella abortus
Rochalimaea quintana
Rhodopseudomonas acidophila
Ancylobacter aquaticus
Rhodomicrobium vannielii
Rhodobacter capsulatus
α
10

Figure 17

Figure 18. Neighbor-joining distance matrix phylogenetic tree based on eubacterial, chloroplast and mitochondrial LSU rRNA gene sequences. Conserved regions of the LSU rRNA sequences were aligned according to secondary structure models and analyzed using PHYLIP, version 3.53c (Felsenstein, 1993). The accession numbers for the LSU rRNA sequences are listed in Table 6. The horizontal branch lengths are proportional to the number of nucleotide substitutions observed between gene sequences and the scale bar indicates 10 substitutions per 100 nt. Mitochondrial (mt), chloroplast (ct), α-, β- and γ-proteobacterial LSU rRNA gene sequences are indicated. All other sequences used in this analysis were eubacterial. The numbers at the nodes connecting the branches indicate the number of times the group consisting of the taxa to the right of the fork occurred among 100 trees used in bootstrap analysis.

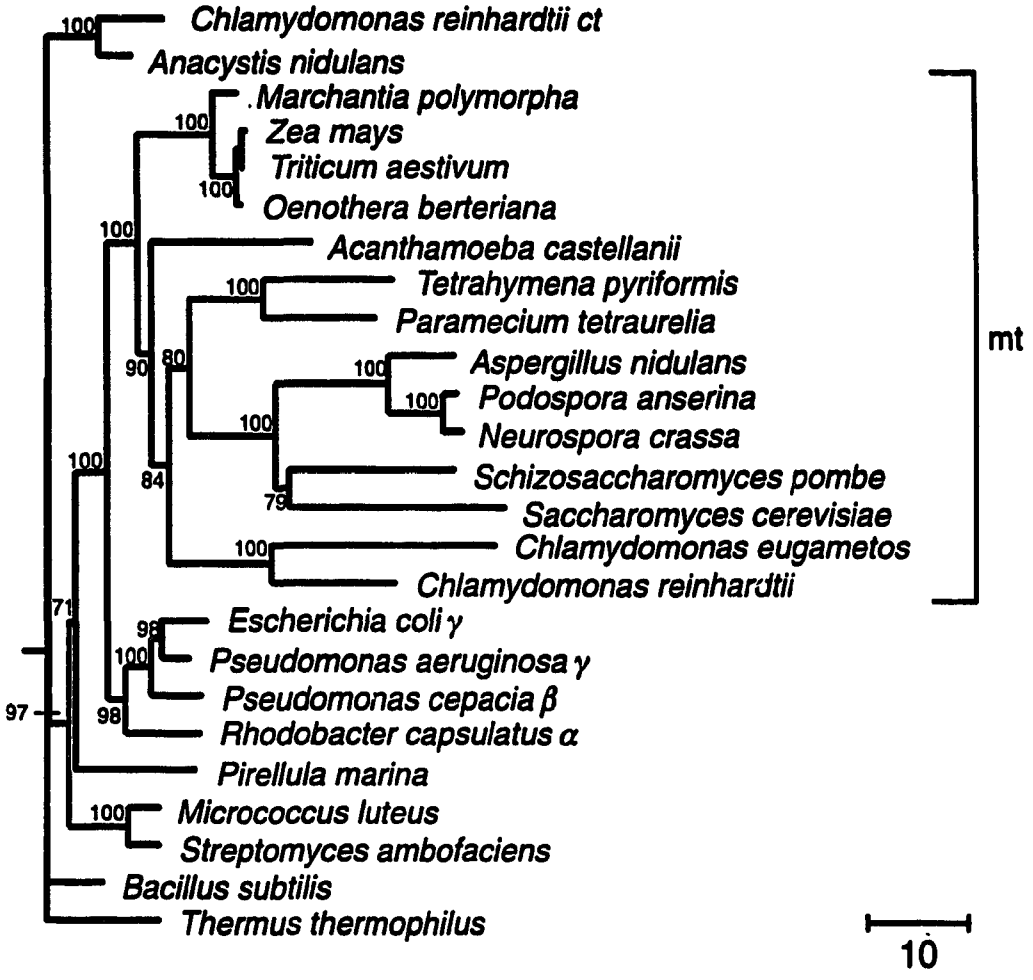

Chlamydomonas reinhardtii ct
Anacystis nidulans
Marchantia polymorpha
Zea mays
Triticum aestivum
Oenothera berteriana
Acanthamoeba castellanii
Tetrahymena pyriformis
Paramecium tetraurelia
Aspergillus nidulans
Podospora anserina
Neurospora crassa
Schizosaccharomyces pombe
Saccharomyces cerevisiae
Chlamydomonas eugametos
Chlamydomonas reinhardtii
Escherichia coli γ
Pseudomonas aeruginosa γ
Pseudomonas cepacia β
Rhodobacter capsulatus α
Pirellula marina
Micrococcus luteus
Streptomyces ambofaciens
Bacillus subtilis
Thermus thermophilus
mt
10

Figure 18

mitochondrial rRNA genes and those of fungi, yeast, ciliates and plants, however, are not consistent. The two *Chlamydomonas* species appear as a sister group to yeast and fungi in SSU distance- and SSU parsimony-based phylogenetic trees (Figs. 17 and 19). In these trees, the ciliates, *Tetrahymena pyriformis* and *Paramecium tetraurelia*, appear as the earliest diverging branch within the *Chlamydomonas*/fungal/yeast/ciliate group. In LSU distance and parsimony trees (Figs. 18 and 20), the *Chlamydomonas* algae appear to have diverged before the separation of the lineages leading to yeast, fungi and ciliates. However, low bootstrap values are associated with the node connecting the *Chlamydomonas* rRNA gene sequences to those of other taxa within the yeast/fungal/ciliate group in both SSU and LSU rRNA neighbor-joining trees (Figs. 17 and 18), suggesting that the specific branching order is sensitive to random sequence variation. *A. castellanii* rRNA genes have a position that is between the lineages leading to *Chlamydomonas* and plants in SSU and LSU distance- and SSU parsimony-based trees, although in the LSU parsimony tree, the *Chlamydomonas* algae appear as the earliest diverging lineage within the group composed of yeast, fungi, ciliates and *A. castellanii*. The *P. wickerhamii* mitochondrial SSU rRNA gene sequence does not specifically affiliate with those of either the plants or *Chlamydomonas* algae in either distance- or parsimony-based phylogenies (the LSU rRNA gene sequence of *P. wickerhamii* was not included in these analyses as it was not available at the time). The relative branching order of the mitochondrial rRNA genes of *A. castellanii* and *P. wickerhamii* is uncertain due to the small number of green algal gene sequences available and because low bootstrap values were observed at the nodes connecting these gene sequences to those of other lineages in SSU rRNA neighbor-joining trees. In summary, the addition of the *C. eugametos* mitochondrial rRNA gene sequences did not significantly alter the apparent branching patterns within the mitochondrial/eubacterial rRNA gene trees that had been observed previously.

There are considerable differences in the branch lengths separating different mitochondrial rRNA genes from the node connecting these genes with those of the *α*-

proteobacteria in trees based on distances between nucleotide sequences. Ciliates, fungi, yeast and *Chlamydomonas* mitochondrial SSU rRNA genes have corrected substitution rates of between 40 and 65 substitutions per 100 nt. In contrast, the mitochondrial SSU rRNA gene sequences of metaphytes show much lower levels of nucleotide substitution (approximately 8 substitutions per 100 nt). Similarly, the corrected substitution rates of *Chlamydomonas* and fungal LSU rRNA genes sequences from the node that connects all mitochondrial rRNA gene sequences are between 27 and 40 substitutions per 100 nt while those of metaphytes are about 14 substitutions per 100 nt. The average similarity among the sequences used for phylogenetic analysis of mitochondrial and α-proteobacterial SSU rRNAs was calculated to be 0.690 with a variance of 0.011; these calculations were based on pairwise sequence similarities between the 13 mitochondrial SSU rRNA sequences listed in Table 5 and *R. prowowazekii*, *R. rickettsii* and *A. tumefaciens*. In contrast, pairwise comparisons of 11 chloroplast SSU rRNAs (Table 5) and *Anacystis nidulans* (cyanobacteria) SSU rRNA show that the average similarity between these sequences is 0.886 with a variance of 5.6×10^{-4}, suggesting that the rate of chloroplast SSU rRNA divergence has been fairly constant since the time that chloroplasts were derived from a cyanobacterial ancestor (Gray, 1992). Because the variance in the calculation of the average sequence similarity between mitochondrial and α-proteobacterial SSU rRNA gene sequences is larger than that observed in the same comparison of chloroplast and cyanobacterial SSU rRNAs, it appears that the rate of mitochondrial SSU rRNA evolution has been variable in different mitochondrial lineages. Although all mitochondrial rRNA gene sequences consistently form a single clade that radiates from within the α-proteobacteria, it is possible that the mitochondrial rRNAs in different lineages were contributed by different endosymbionts and that representatives of the ancestral proteobacteria have not yet been characterized.

To test whether the fungal, yeast, ciliate and *Chlamydomonas* rRNA genes were being artificially grouped due to their common long branch lengths, neighbor-joining trees were

Figure 19. A portion of the SSU rRNA phylogenetic tree based on parsimony, indicating the relative affiliations between the mitochondrial and *α*-proteobacterial SSU rRNA gene sequences. The 105 mitochondrial, chloroplast and eubacterial SSU rRNA gene sequences (Table 5) in the complete database were aligned based on secondary structure models and analyzed using the DNAPars program of PHYLIP, version 3.53c (Felsenstein, 1993). The branch lengths are not proportional to sequence divergence. The mitochondrial (mt) and *α*-proteobacterial (*α*) sequences are bracketed.

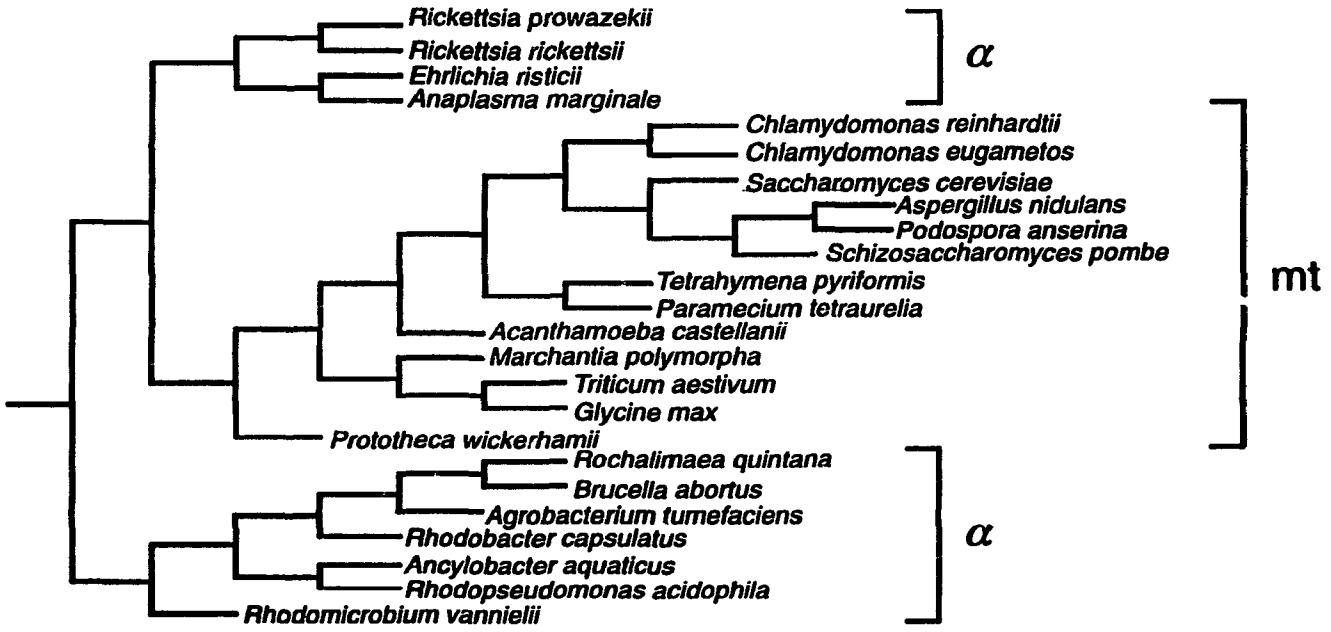

Rickettsia prowazekii
Rickettsia rickettsii
Ehrlichia risticii
Anaplasma marginale
α
Chlamydomonas reinhardtii
Chlamydomonas eugametos
Saccharomyces cerevisiae
Aspergillus nidulans
Podospora anserina
Schizosaccharomyces pombe
Tetrahymena pyriformis
Paramecium tetraurelia
Acanthamoeba castellanii
Marchantia polymorpha
Triticum aestivum
Glycine max
mt
Prototheca wickerhamii
Rochalimaea quintana
Brucella abortus
Agrobacterium tumefaciens
Rhodobacter capsulatus
Ancylobacter aquaticus
Rhodopseudomonas acidophila
α
Rhodomicrobium vannielii

Figure 19

Figure 20. LSU rRNA phylogenetic tree based on parsimony. Mitochondrial, chloroplast and eubacterial LSU rRNA gene sequences (Table 6) were aligned based on secondary structure models and analyzed using the DNAPars program of PHYLIP, version 3.53c (Felsenstein, 1993). The branch lengths are not proportional to sequence divergence. Mitochondrial (mt), chloroplast (ct), α-, β- and γ-proteobacterial LSU rRNA gene sequences are indicated. All other sequences used in this analysis were eubacterial rRNAs.

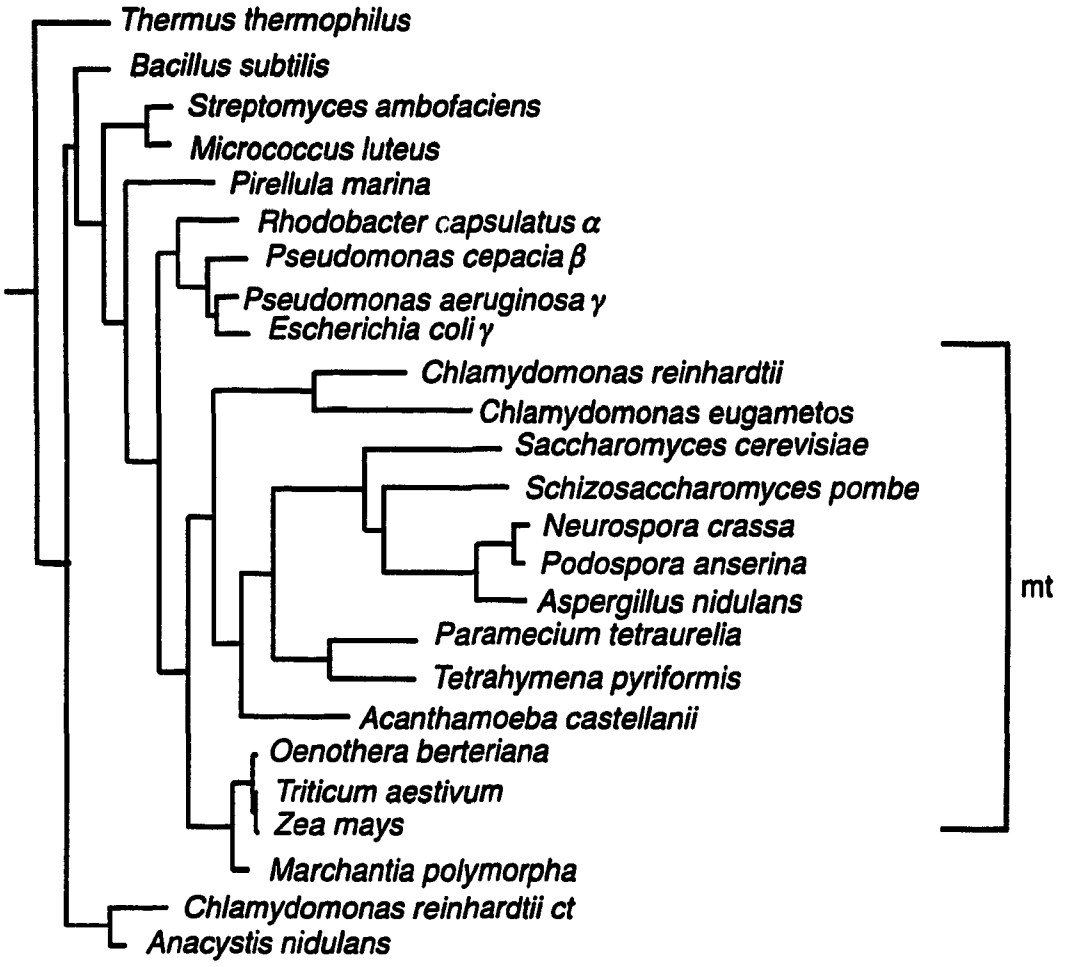

Thermus thermophilus
Bacillus subtilis
Streptomyces ambofaciens
Micrococcus luteus
Pirellula marina
Rhodobacter capsulatus α
Pseudomonas cepacia β
Pseudomonas aeruginosa γ
Escherichia coli γ
Chlamydomonas reinhardtii
Chlamydomonas eugametos
Saccharomyces cerevisiae
Schizosaccharomyces pombe
Neurospora crassa
Podospora anserina
Aspergillus nidulans
Paramecium tetraurelia
Tetrahymena pyriformis
Acanthamoeba castellanii
Oenothera berteriana
Triticum aestivum
Zea mays
Marchantia polymorpha
Chlamydomonas reinhardtii ct
Anacystis nidulans
mt

Figure 20

constructed using distance measurements based on transversions between gene sequences (Figs. 21 and 22). Transversion-based SSU and LSU distance trees were nearly identical in branching pattern to those based on nucleotide substitutions (Figs. 17 and 18) with the exception that the relative position of *A. castellanii* and *P. wickerhamii* changed within the two SSU distance-based trees. Earlier work using invariant analysis showed that the apparent affiliations between the rRNA gene sequences of *C. reinhardtii* and the fungal/yeast/ciliate lineages and separating *C. reinhardtii* from the metaphytes was not due to "long-branch length attracts" artifacts (Gray *et al.*, 1989).

The apparent affiliation between the *Chlamydomonas* and fungal lineages is not due to the rRNA genes of these taxa sharing a similar GC content that differs from those of plants and other algae (Table 7). *C. reinhardtii* mitochondrial SSU and LSU rRNAs have a GC content that is similar to those of plants, while the GC content of the *C. eugametos* homologue is more similar to the nucleotide composition of the mitochondrial counterparts in fungi, yeast and ciliates. The fact that the mitochondrial rRNA genes of these two species of *Chlamydomonas* do not share a similar GC bias could contribute to the long branch lengths observed between these algae and their last common ancestor.

Ideally, if two individual endosymbiotic events gave rise to the mitochondrial and chloroplast genomes, the phylogenetic trees based on nuclear, chloroplast and mitochondrial rRNA sequences should show the same affiliations between different taxa (Gray & Doolittle, 1982; Gray, 1989, 1992). However, the lack of consistency of these phylogenetic trees cannot be taken as proof that the mitochondrial rRNA genes of *Chlamydomonas* and other algae and plants were donated by different endosymbionts because phylogenetic affiliations based on distance and parsimony methods are each subject to systematic errors that obscure the true evolutionary history of taxa being examined. Treeing methodologies based on distance assume that all distance data used to link internal nodes are additive. Therefore, if no

Figure 21. A portion of the SSU rRNA neighbor-joining phylogenetic tree based on the number of transversions observed between gene sequences, showing the relative branching pattern of mitochondrial and *α*-proteobacterial SSU rRNA gene sequences. The SSU rRNA sequences used in the database for the neighbor-joining distance trees presented in Figure 15 were converted to a purine and pyrimidine code and then analyzed using the DNAdist and Neighbor programs of PHYLIP, version 3.53c (Felsenstein, 1993). The horizontal branches are proportional to the number of transversions observed. The scale bar indicate 5 transversions per 100 nt. Only the relative branching order and distance of the mitochondrial (mt) and *α*-proteobacterial (*α*) SSU rRNA gene sequences are shown.

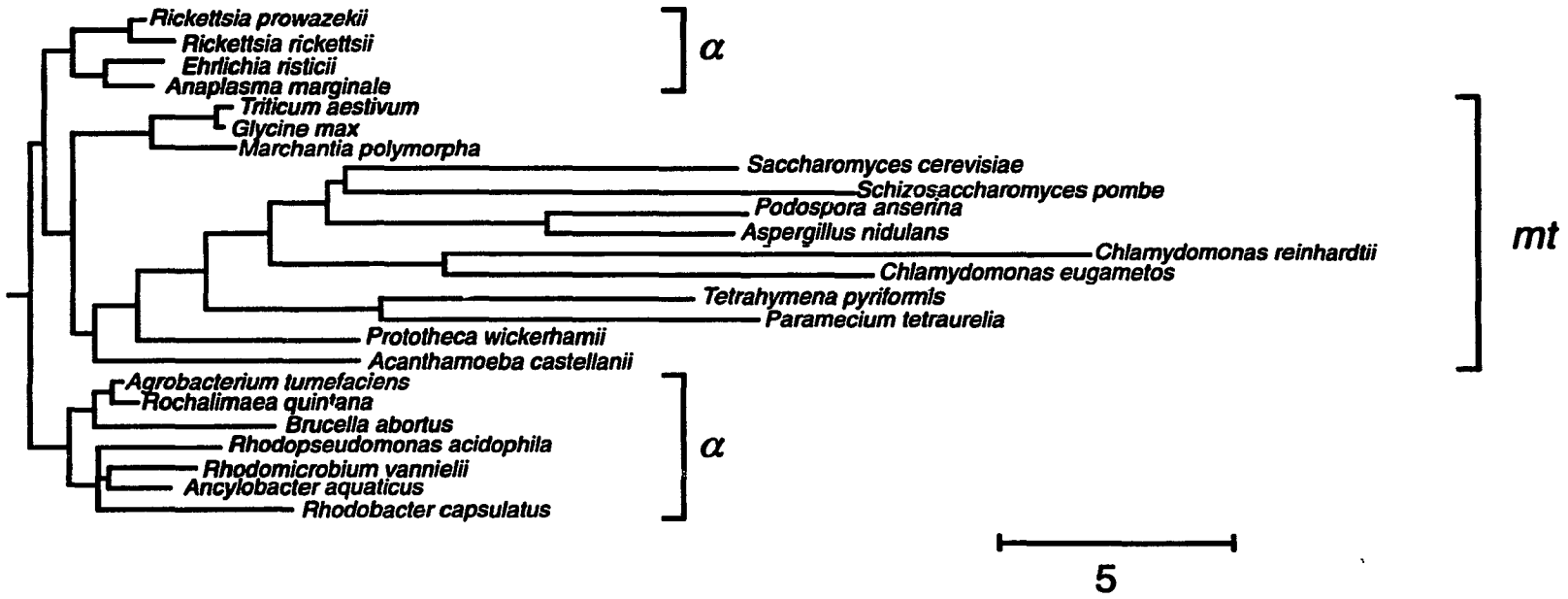

Rickettsia prowazekii
Rickettsia rickettsii
Ehrlichia risticii
Anaplasma marginale
α
Triticum aestivum
Glycine max
Marchantia polymorpha
Saccharomyces cerevisiae
Schizosaccharomyces pombe
Podospora anserina
Aspergillus nidulans
Chlamydomonas reinhardtii
Chlamydomonas eugametos
Tetrahymena pyriformis
Paramecium tetraurelia
Prototheca wickerhamii
Acanthamoeba castellanii
mt
Agrobacterium tumefaciens
Rochalimaea quintana
Brucella abortus
Rhodopseudomonas acidophila
Rhodomicrobium vannielii
Ancylobacter aquaticus
Rhodobacter capsulatus
α
5

Figure 21

Figure 22. LSU rRNA neighbor-joining phylogenetic tree based on the number of transversions observed between gene sequences. The LSU rRNA sequences used in the database for the neighbor-joining distance trees presented in Figure 16 were converted to a purine and pyrimidine code and then analyzed using the DNAdist and Neighbor programs of PHYLIP, version 3.53c (Felsenstein, 1993). The horizontal branches are proportional to the number of transversions observed. The scale bar indicates 10 transversions per 100 nt. Mitochondrial (mt), chloroplast (ct), α-, β- and γ-proteobacterial LSU rRNA gene sequences are indicated. All other sequences used in this analysis are eubacterial rRNAs.

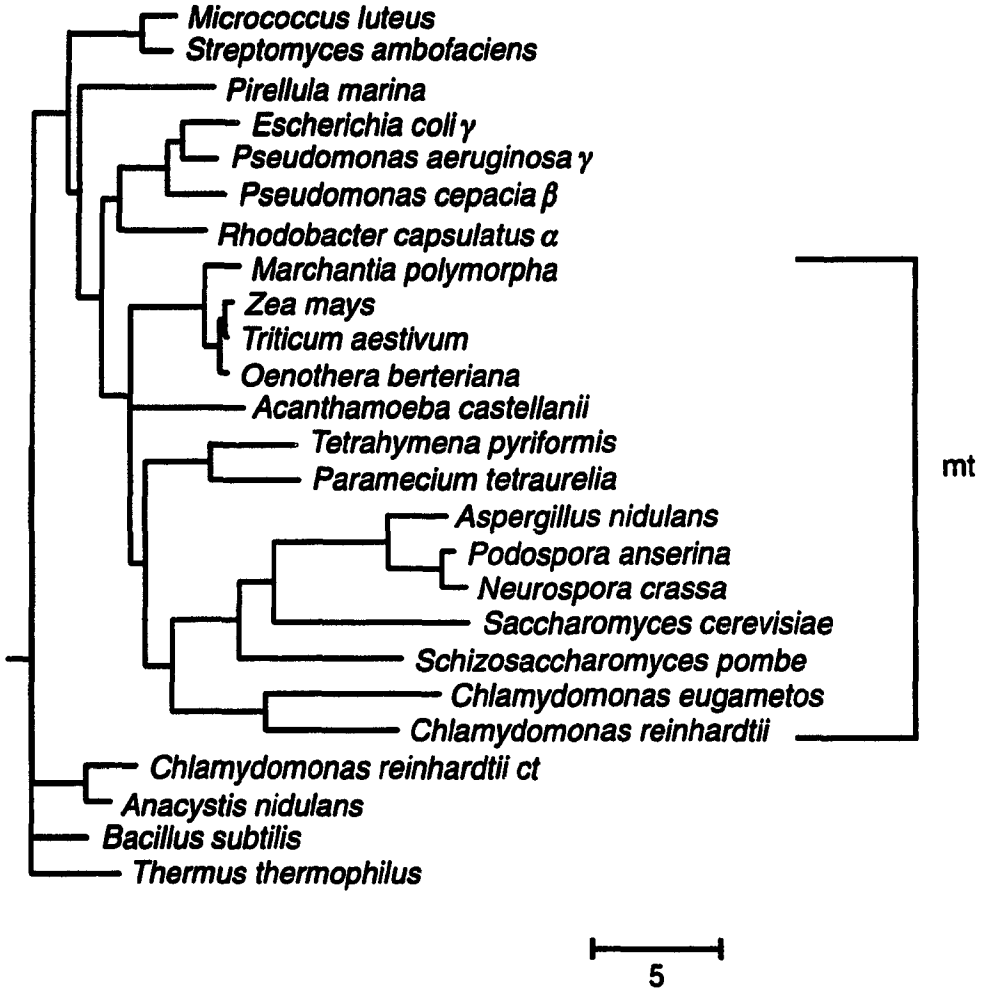

Micrococcus luteus
Streptomyces ambofaciens
Pirellula marina
Escherichia coli γ
Pseudomonas aeruginosa γ
Pseudomonas cepacia β
Rhodobacter capsulatus α
Marchantia polymorpha
Zea mays
Triticum aestivum
Oenothera berteriana
Acanthamoeba castellanii
Tetrahymena pyriformis
Paramecium tetraurelia
Aspergillus nidulans
Podospora anserina
Neurospora crassa
Saccharomyces cerevisiae
Schizosaccharomyces pombe
Chlamydomonas eugametos
Chlamydomonas reinhardtii
Chlamydomonas reinhardtii ct
Anacystis nidulans
Bacillus subtilis
Thermus thermophilus
mt
5

Figure 22

Table 7

GC content (%) of mitochondrial SSU and LSU rRNA gene sequences used in phylogenetic analysis.

Organism	SSU rRNA	LSU rRNA
Chlamydomonas eugametos	39.9	43.5
Chlamydomonas reinhardtii	49.7	50.5
Prototheca wickerhamii	47.3	–
Acanthamoeba castellanii	42.9	44.6
Saccharomyces cerevisiae	32.1	37.3
Schizosaccharomyces pombe	36.4	40.9
Neurospora crassa	–	43.2
Podospora anserina	41.4	–
Aspergillus nidulans	41.0	43.8
Tetrahymena pyriformis	38.1	40.0
Paramecium tetraurelia	42.8	43.8
Marchantia polymorpha	52.7	52.2
Zea mays	–	53.4
Oenothera berteriana	–	53.4
Triticum aestivum	55.7	53.8
Glycine max	55.7	–

distance data are available for sister taxa of those organisms at the end of long branches, the specific affiliations between organisms are uncertain (Swofford & Olsen, 1990). Similarly, long unbranched lineages mask multiple substitutions in parsimony analysis (Swofford & Olsen, 1990). In each type of phylogenetic analysis performed, long branches were observed between the two *Chlamydomonas* species and between these algae and all other taxa. For this reason, and because the rate of mitochondrial rRNA gene sequence divergence radically differs between plants and *Chlamydomonas* algae, additional mitochondrial rRNA gene sequences from other members of the Chlorophyta must be added to the databases used to construct phylogenetic trees before the apparent discrepancy between the nuclear and mitochondrial rRNA gene trees can be resolved.

Chapter 5

Evolution of fragmented mitochondrial rRNAs in *Chlamydomonas*.

Introduction

Comparison of the structural features and phylogenetic affiliation of the mitochondrial rRNA genes of *C. eugametos* and *C. reinhardtii* supports the hypothesis that these rRNA genes were inherited from a common ancestor which had fragmented and possibly scrambled mitochondrial rRNA genes (Chapters 2 and 4). However, it was not possible to speculate about the specific number or order of events that led to the mitochondrial rRNA gene arrangements in these algae because of the considerable number of differences in their rRNA gene structure and organization. I favour the simplest explanation that the differences in the structure and organization of the mitochondrial rRNA genes of *C. eugametos* and *C. reinhardtii* have resulted from the further division and movement of rRNA gene pieces present in the mitochondria of the last common ancestor of these algae. Nevertheless, it is possible that the differences in the fragmentation pattern of the *C. eugametos* and *C. reinhardtii* rRNA gene pieces are the net result of both disruption and reassembly of the pieces. Therefore, I felt it might be informative to calculate the minimum number of gene rearrangements necessary to generate the observed mitochondrial rRNA gene organizations of *C. eugametos* and *C. reinhardtii* from a hypothetical ancestral rRNA gene structure that was intermediate between the structures observed in these algae and from a hypothetical rRNA gene structure that is co-linear with eubacterial rRNA genes. Analysis of the minimum number of addition/deletion differences and gene rearrangements has been used to determine the evolutionary relationships between mitochondrial genomes; such affiliations agree well with those determined by phylogenetic analysis of mitochondrial gene sequences (Sankoff *et al.*, 1992). Therefore, it seemed reasonable that similar analyses could be used to formulate testable hypotheses about the organization of the ancestral mitochondrial rRNA genes in *Chlamydomonas* algae and the evolutionary pathways that could have generated these rRNA genes in pieces.

As a complement to and preliminary test of models generated by the above approach, I chose to examine the mitochondrial rRNA of several green algal species. Specifically, I wished to determine whether the fragmented mitochondrial LSU rRNAs detected in *C. reinhardtii* and *C. eugametos* are a feature shared by other members of the genus *Chlamydomonas* (this work extends that presented by Fenety, 1993) and to test whether algae closely related to *C. eugametos* and *C. reinhardtii* share any of the unique fragmentation sites in their mitochondrial LSU rRNA. Phylogenetic studies of the green algae, based on sequences of chloroplast-encoded LSU (Turmel *et al.*, 1991; 1993a) and nucleus-encoded SSU (Buchheim *et al.*, 1990; Buchheim and Chapman,1992) rRNA genes have revealed that the algal pairs *C. eugametos*/*Chlamydomonas humicola* and *C. reinhardtii*/*Chlamydomonas frankii* are early diverging representatives of each of the major lineages within the *Chlamydomonas* genus (Turmel *et al.*, 1993a). Molecular phylogenetic and morphological studies support a more recent connection between *C. eugametos* and *Chlamydomonas pitschmanii* and between *C. reinhardtii* and *Chlamydomonas iyengarii*. Therefore, a survey of the 3' half of the mitochondrial LSU rRNA in each of these *Chlamydomonas* species was initiated to determine whether differences observed between the mitochondrial rRNAs of *C. eugametos* and *C. reinhardtii* were lineage-specific. In addition, experiments were performed to determine whether the continuous mitochondrial LSU rRNAs observed in *Prototheca wickerhamii* (Wolff *et al.*, 1994) were present in other chlorophycean algae, including *Chlorella vulgaris* and *Uronema belkae*, as well as in the pleurastrophycean alga *Pleurastrum terrestre*. A representative of the class Pleurastrophyceae was selected because this class is believed to be the sister class of the chlorophycean algae (Mattox and Stewart, 1984).

The minimum number of mitochondrial rRNA gene rearrangements necessary to generate the observed rRNA gene organization in *C. eugametos* and *C. reinhardtii* was calculated from two hypothetical ancestral rRNA gene structures, one intermediate between the structures observed presently in these algae and the other co-linear with continuous rRNAs.

Northern hybridization analysis of total RNA extracts from several green algal species was conducted with oligonucleotide probes complementary to the peptidyl transferase centre of either *C. reinhardtii* and *C. eugametos* or *Scenedesmus obliquus*. These studies revealed that all *Chlamydomonas* taxa tested have discontinuous mitochondrial LSU rRNAs but that *Chlorella vulgaris*, *Uronema belkae* and *Pleurastrum terrestre* have continuous mitochondrial LSU rRNAs about 3000 nt in length. Some of the sites of discontinuity within the mitochondrial LSU rRNA of *Chlamydomonas* appear to be common to all members of the genus, while other breaks are lineage-specific. Overall, the hybridization analysis tends to favour the model whereby rRNA genes have been derived from ancestral mitochondrial rRNA genes that were co-linear with conventional rRNA genes.

Materials and Methods

Calculation of minimal mutational distance between mitochondrial rRNA gene orders

Homologous rRNA coding regions of *C. eugametos* and *C. reinhardtii* were defined by the position of ends of the rRNA gene pieces in either or both mitochondrial DNAs (Table 4). The computer program DERANGE (Sankoff *et al.*, 1992) was used to calculate the minimum evolutionary distance between the *C. eugametos* and *C. reinhardtii* mitochondrial rRNA gene arrangements. For this analysis, addition/deletion differences were ignored and genomic rearrangements were limited to transpositional events (see results).

Strains and culture conditions

Wild-type strains of *Chlamydomonas eugametos* (UTEX 9), *Chlorella vulgaris* (UTEX 259), *Pleurastrum terrestre* (UTEX 333) and *Uronema belkae* (UTEX 1179) were obtained from the University of Texas at Austin culture collection. Wild-type strains of *Chlamydomonas humicola* (SAG 11.9), *Chlamydomonas pitschmanii* (SAG 14.73), *Chlamydomonas iyengarii* (SAG 25.72) and *Chlamydomonas frankii* (SAG 19.72) were obtained from Sammlung von Algenkulturen, Universität Göttingen. Wild-type *Chlamydomonas reinhardtii* (strain 137c, mt^+) came from the Duke University culture collection.

All liquid cultures were grown in minimal media as described by Lemieux and Lee (1980) and the cells were harvested six h after the onset of the light period (L=6) when cell densities were approximately $4x10^6$ cells/ml .

RNA isolation and gel electrophoresis

Total cellular RNA was isolated as described by Turmel *et al.* (1988). Denaturing polyacrylamide and agarose gel electrophoresis conditions and the transfer of fractionated RNA to nylon membranes have been described previously (Chapter 2). All solutions and glassware were treated to minimize RNase activity (Maniatis *et al.*, 1982).

Northern hybridization

Three synthetic oligonucleotide probes were employed in this study. Probe 1 is a 29-nt oligomer (5' AGG ACG CGA TGA TCC AAC ATC GAG GTG CC 3') and probe 2 is an 18-nt oligomer (5' CAT AGG GTC TCT TCG TCC 3'); both of these probes are complementary to different portions of the peptidyl transferase centre of both *C. eugametos* and *C. reinhardtii* mitochondrial LSU rRNA. Using the numbering of the *Escherichia coli* 23S rRNA, probe 1 and probe 2 are complementary to nucleotides 2494-2522 and 2052-2069, respectively. Probe 3 is a 27-nt oligomer (5' GCT GAT AAA CCT GTT ATC CCT AGC GTA 3') that is complementary to a portion of the peptidyl transferase centre of the mitochondrial LSU rRNA of *Scenedesmus obliquus* (Kück *et al.*, 1990); the *E. coli* co-ordinates of this region are 2438-2513. The relative positions of these peptidyl transferase centre-specific probes are shown in Figure 23. Filters of immobilized total cellular RNA from each species within the study group were pre-hybridized in 5X SSPE (1X SSPE = 180 mM NaCl, 10 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.4), 5X Denhardt's solution (1X Denhardt's solution = 0.1% w/v polyvinylpyrollidone, 0.1% w/v bovine serum albumin, 0.1% w/v Ficoll), 0.1 mg/ml denatured salmon sperm DNA for 12 h. The hybridizations were allowed to proceed for 24 h at room temperature following the addition of oligonucleotide probe that was 5' end-labelled with [γ-^{32}P]ATP(6000 Ci/mMol) and T4 polynucleotide kinase (Pharmacia). The hybridized filters were washed twice in 5X SSPE for

15 min, twice in 2X SSPE for 15 min and once in 2X SSPE/0.1% SDS for 30 min; washes were performed at room temperature.

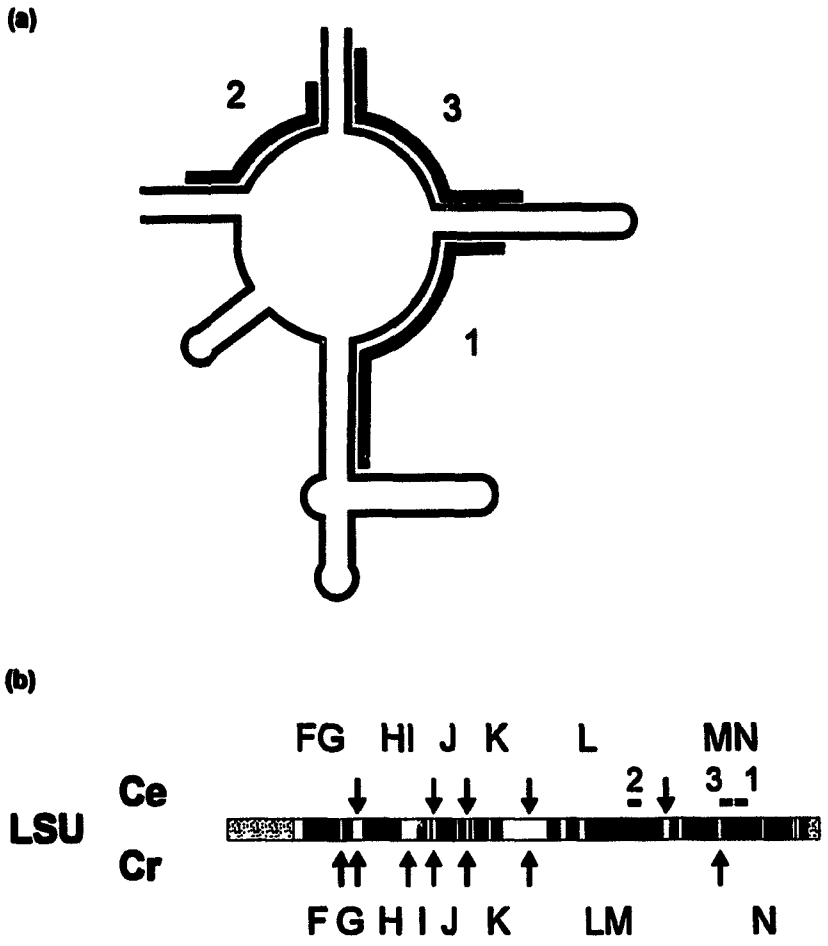


Figure 23. Relative positions of the peptidyl transferase centre-specific oligonucleotide probes used to detect mitochondrial rRNAs. A skeleton drawing of the peptidyl transferase centre within the 3' half of LSU rRNA secondary structure showing the relative positions of probes 1, 2 and 3 is presented in part a. The relative positions of the oligonucleotide probes within a linear representation of the *E. coli* LSU rRNA are indicated by dashes in part b. The positions of the domain breakpoints observed in the *C. eugametos* and *C. reinhardtii* LSU rRNA gene pieces are indicated by arrows using the conventions described in Figure 10, and homologous coding regions (Table 4) are indicated by letters.

Results

Calculation of the minimal mutation distance between mitochondrial rRNA gene piece orders in *C. eugametos* and *C. reinhardtii*.

The mitochondrial rRNA genes in the last common ancestor of *Chlamydomonas* algae could have had either of two different gene arrangements, given the assumptions that the highly fragmented and scrambled mitochondrial rRNA genes in *C. eugametos* and *C. reinhardtii* are functional and that all rRNA genes are related by descent from continuous rRNA genes. The last common ancestor of *C. eugametos* and *C. reinhardtii* could have had a mitochondrial rRNA gene structure that was intermediate between the scrambled gene piece organization observed in *C. eugametos* and *C. reinhardtii*, or one that was co-linear with conventional rRNA genes. Regardless of type, the ancestral mitochondrial rRNA genes likely contained many of the shared breakpoints observed in *C. eugametos* and *C. reinhardtii* (Appendix).

The DERANGE program of Sankoff *et al.* (1992) was employed to determine the minimum mutational distance between the rRNA gene piece organization in *C. eugametos* and *C. reinhardtii*. Homologous sections of the mitochondrial rRNA were designated by a letter indicating the point of interruption in either or both algae; A through E and F through N are the SSU and LSU rRNA coding regions, respectively (see Table 4). Based on the observations that the *C. reinhardtii* and *C. eugametos* rRNAs are all encoded from the same DNA strand and that there are no duplications of rRNA gene pieces within these genomes, the movement of rRNA gene pieces was limited to transpositional events. Because there are no reported examples where conserved rRNA sequences are not in the usual 5' to 3' order in a covalently continuous rRNA species, it was assumed that intermediates in which the rRNA gene pieces became juxtaposed from their usual order represent individual rRNA transcripts. The

introduction of a new break point within SSU or LSU rRNA coding regions were assumed to be compatible with function because the resulting individual transcripts could function in composite rRNA structures. Finally, this analysis was simplified by linearizing the circular *C. eugametos* mitochondrial gene map (see Sankoff *et al.*, 1990).

In calculating the number of transpositional events that would change the rRNA gene order of *C. reinhardtii* to that of *C. eugametos*, the shortest deduced pathway involved seven transpositions (Model 1, Fig. 24). Assuming that the midpoint in this pathway represents the rRNA gene piece order of the last common ancestor, the hypothetical ancestral mitochondrial rRNA genes would have been more fragmented and scrambled than is the case in either alga presently. Moreover, such a pathway would require sub-division of some rRNA gene pieces and the reassembling of others into larger rRNA gene pieces during the evolution of the lineages leading to *C. eugametos* and *C. reinhardtii.*

The minimum mutational distance from a conventional rRNA gene order in which the SSU was transcriptionally upstream of the LSU rRNA coding information (Gray and Schnare, 1994) was also calculated and a pathway of four steps from this hypothetical ancestor to the pattern observed in *C. eugametos* or the one in *C. reinhardtii* was found (Model 2, Fig. 25). However, LSU rRNA gene piece K is immediately upstream of SSU rRNA gene piece A in the mitochondrial genomes of both *C. eugametos* and *C. reinhardtii*, raising the possibility that the common ancestor of these two algae also possessed this rRNA gene piece arrangement. If the ancestral *Chlamydomonas* mitochondrial genome did have such a rRNA gene arrangement, the total number of transpositional events required to generate the *C. eugametos* and *C. reinhardtii* rRNA gene order would be reduced to seven. In this evolutionary pathway, the changes in the rRNA gene structures involve only division of rRNA gene pieces into smaller coding regions.

Figure 24. A model for the movement of mitochondrial rRNA gene pieces from an ancestral pattern that was intermediate between the rRNA gene piece organizations observed in *C. eugametos* and *C. reinhardtii*. The calculation is based on minimum mutational distance. Homologous sections of the mitochondrial rRNA genes were designated as a letter indicating the point of interruption in either or both algae; A through E and F through N are the SSU and LSU rRNA coding regions, respectively (see Table 4). Gene pieces that encode single transcripts are represented by individual letters or closely spaced groups of letters. Gene pieces involved in transpositional events are boxed and arrows indicate the final position of the coding information following the event. "Break" refers to the division of a gene piece into two separate gene pieces that each encodes a separate transcript. "Join" refers to the juxtaposition of previously separated gene pieces into larger gene pieces that are then transcribed as a single rRNA species. These models represent the minimum mutational distance between the observed gene orders; alternate pathways involving more or a different order of transpositional events are possible.

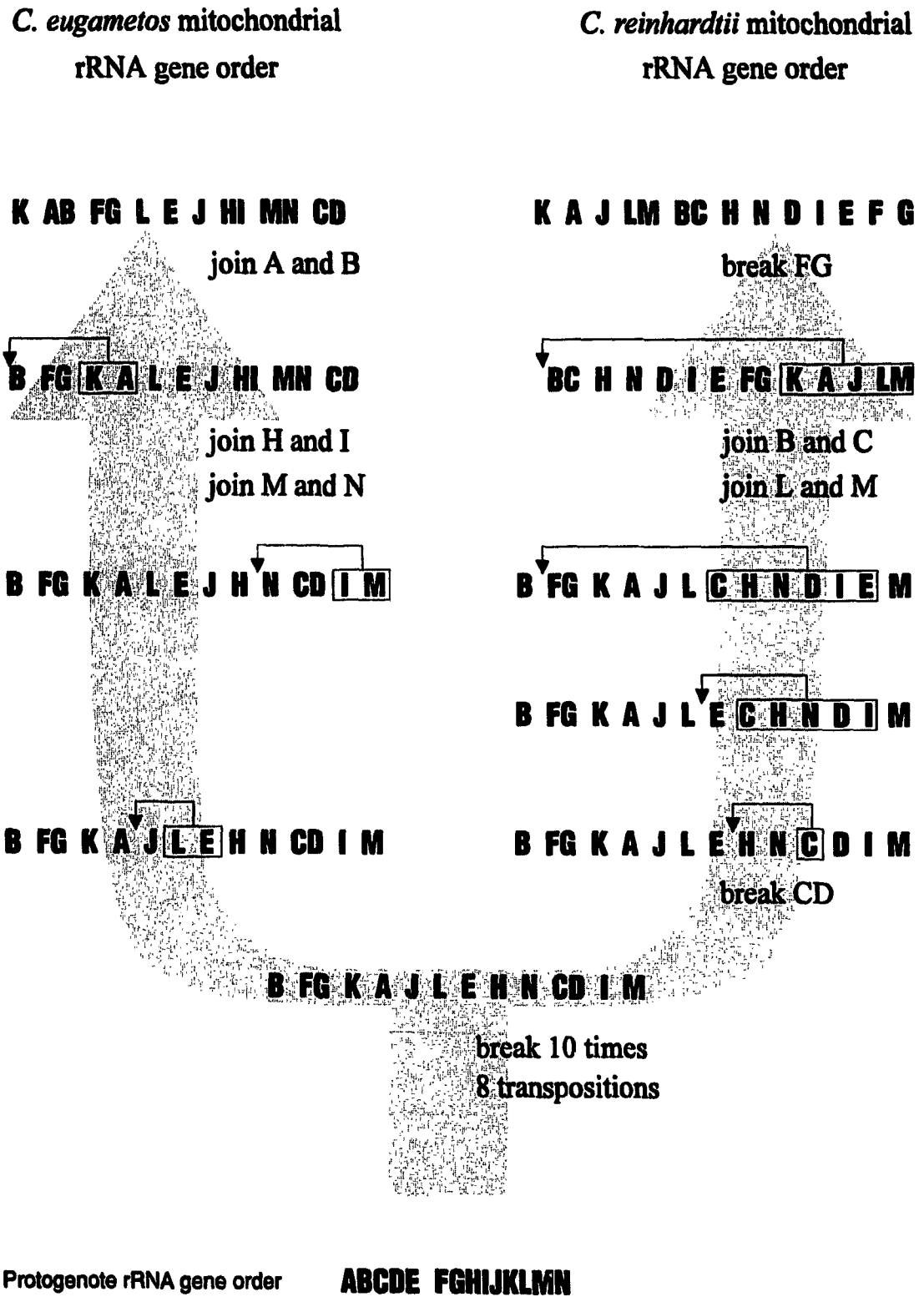

C. eugametos mitochondrial rRNA gene order
C. reinhardtii mitochondrial rRNA gene order
K AB FG L E J HI MN CD
join A and B
B FG K A L E J HI MN CD
join H and I
join M and N
B FG K A L E J H N CD I M
B FG K A J L E H N CD I M
K A J LM BC H N D I E F G
break FG
BC H N D I E FG K A J LM
join B and C
join L and M
B FG K A J L C H N D I E M
B FG K A J L E C H N D I M
B FG K A J L E H N C D I M
break CD
B FG K A J L E H N CD I M
break 10 times
8 transpositions
Protogenote rRNA gene order
ABCDE FGHIJKLMN

Figure 24

Figure 25. A model for the movement of mitochondrial rRNA gene pieces from an ancestral rRNA gene pattern where homologous rRNA coding regions are co-linear with *E. coli* rRNA genes and the SSU rRNA gene is encoded upstream of the LSU rRNA gene. The designation of homologous rRNA coding information and the conventions used are described in Figure 22.

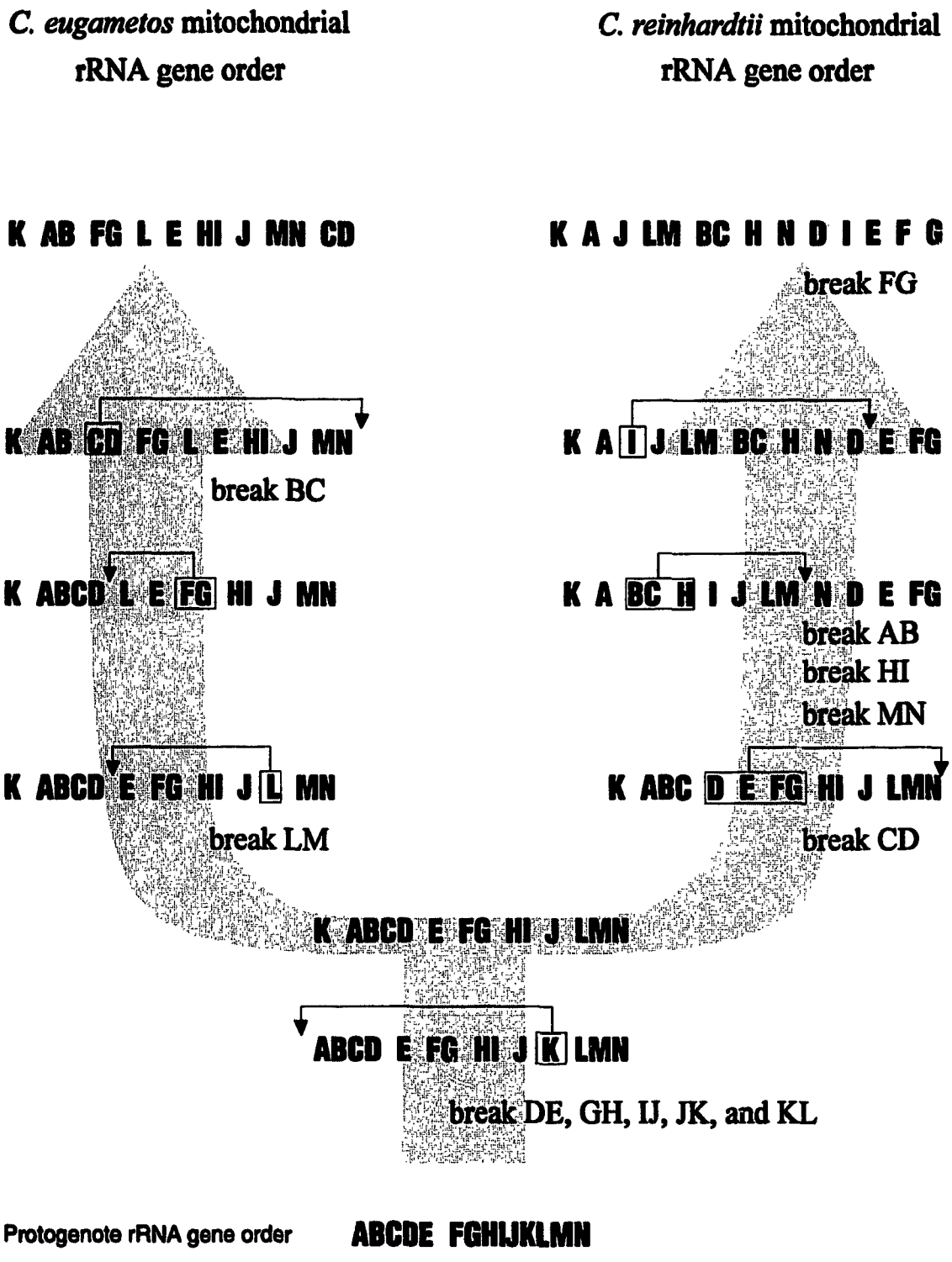

C. eugametos mitochondrial rRNA gene order
C. reinhardtii mitochondrial rRNA gene order
K AB FG L E HI J MN CD
K A J LM BC H N D I E F G
break FG
K AB CD FG L E HI J MN
break BC
K A I J LM BC H N D E FG
K ABCD L E FG HI J MN
K A BC H I J LM N D E FG
break AB
break HI
break MN
K ABCD E FG HI J L MN
break LM
K ABC D E FG HI J LMN
break CD
K ABCD E FG HI J LMN
ABCD E FG HI J K LMN
break DE, GH, IJ, JK, and KL
Protogenote rRNA gene order
ABCDE FGHIJKLMN

Figure 25

Identification of mitochondrial LSU rRNAs in *Chlamydomonas* and other green algae

As a preliminary test of these two hypotheses, I chose to determine the sizes of the transcripts that contribute to the peptidyl transferase centre of the mitochondrial LSU rRNAs in several green algal species. Both *C. eugametos* and *C. reinhardtii* share a common site of rRNA discontinuity that separates the 5' and 3' halves of the mitochondrial LSU rRNA; this common site separates coding regions K and L as designated in Table 4. The 3' half of the molecule is composed of two separate rRNAs in each species, but the division of the *C. eugametos* and *C. reinhardtii* mitochondrial LSU rRNA gene pieces corresponds to sites 16 and 17 (as defined by Gray and Schnare, 1995), respectively (see Fig. 23). Therefore, using the convention presented in Figures 24 and 25, gene piece L is separate from MN in *C. eugametos* while gene piece LM is separate from N in *C. reinhardtii*. In both cases, two rRNAs reconstitute the 3' half of the mitochondrial LSU rRNA; together they form structural domains IV, V and VI (defined by Noller, 1984) and make up the peptidyl transferase centre of these mitochondrial LSU rRNAs. However, the small rRNAs that are complementary to probe 1 are 493 and 640 nt in *C. reinhardtii* and *C. eugametos*, respectively, while the mitochondrial rRNAs complementary to probe 2 in *C. reinhardtii* and *C. eugametos* are 680 and 480 nt, respectively (Fig. 23). The total size of the 3' half of the mitochondrial LSU rRNA is 1120 nt in *C. eugametos* and 1170 nt in *C. reinhardtii*; the overall size difference is the result of length differences in several variable regions within the 3' half of the LSU rRNA.

Very similar ethidium bromide staining patterns were observed for total RNA isolated from six species of *Chlamydomonas* and from the non-*Chlamydomonas* green algae, *C. vulgaris*, *U. belkae* and *P. terrestre* after fractionatation by denaturing agarose (Fig. 26) and polyacrylamide (data not shown) gel electrophoresis. Cytosolic LSU (3.5 kb), chloroplast SSU (1.5 kb), 5.8S (160 nt) and 5S (120 nt) rRNAs were identified in each total RNA sample, based

on abundance and mobility relative to RNA molecular size standards. As was observed previously (Fenety, 1993), abundant RNAs of 280 (*α*) and 810 (*γ*) nt that co-migrated with *Chlamydomonas* chloroplast LSU rRNA fragments were observed in the non-*Chlamydomonas* RNAs fractionated by polyacrylamide gel electrophoresis and stained with ethidium bromide (data not shown) with the exception of *P. terrestre*, which lacked the chloroplast 810-nt RNA species and instead contained a 2500-nt abundant RNA (Fig. 26). The 1720-nt chloroplast LSU and 1800-nt cytosolic SSU rRNAs co-migrated as a broad, intensely staining band in both 1.2% and 2.5% agarose gels.

Northern blot hybridizations of electrophoretically separated total RNA from the study group taxa were carried out with probes complementary to the mitochondrial LSU rRNAs of *C. reinhardtii* and *C. eugametos* (probes 1 and 2, respectively). These experiments identified two RNA molecules of less than 700-nt within each of the six *Chlamydomonas* species that did not co-migrate with the chloroplast or cytosolic rRNAs (Fig. 26 and 27). The hybridization of these probes to putative mitochondrial rRNAs was approximately equal in each species of *Chlamydomonas*, with the exception that *C. humicola* RNA hybridized only weakly with each probe. The northern hybridization signals using probes 1 and 2 were less intense for the RNA fractionated by agarose (Fig. 26) as opposed to polyacrylamide (Fig. 27) gel electrophoresis. This difference is likely due to the lower amount of total *C. humicola* RNA on the agarose gel relative to the amount of RNA in the other samples as judged by the ethidium bromide staining pattern (Fig. 26). Probe 1 hybridized with RNA molecules of 3000 nt, 2900 nt and 3100 nt within total RNA of *C. vulgaris*, *U. belkae* and *P. terrestre*, respectively (Fig. 26). These hybridizing RNAs are most likely mitochondrial LSU rRNAs based on their low abundance in total RNA. Probe 1 also annealed with RNAs of 1720 nt in *C. reinhardtii* and *C. humicola*. Probe 2 hybridized with 3000 nt and 2900 nt RNAs in *C. vulgaris* and *U. belkae*, respectively, but hybridization of this probe to the 3100 nt RNA of *P. terrestre* was obscured by other hybridization signals. The most intense hybridization signals generated by probe 2

Figure 26. Ethidium bromide staining pattern of a 1.2% denaturing agarose gel containing 5 μg total RNA from each of the study group taxa and northern hybridization analysis using peptidyl transferase centre-specific oligonucleotide probes 1, 2 and 3. The lettering above each lane indicates the origin of each RNA sample as follows: Cp, *C. pitschmanii*; Ce, *C. eugametos*; Cr, *C. reinhardtii*; Ci, *C. iyengarii*; Cf, *C. frankii*; Ch, *C. humicola*; Cv, *C. vulgaris*; Ub, *U. belkae*; Pt, *P. terrestre*. The panel on the left is the ethidium-bromide stained gel and the oligonucleotide probes are indicated above each northern blot. The sizes of the cytosolic and chloroplast rRNAs are indicated: cytosolic LSU rRNA, 3.5 kb (Hoober & Blobel, 1969); cytosolic SSU rRNA, 1.8 kb (Bourque *et al.*, 1971); chloroplast LSU δ fragment, 1.7 kb (Rochaix & Darlix, 1982; Lemieux *et al.*, 1989; Turmel *et al.*, 1993a); chloroplast SSU rRNA, 1.5 kb (Dron *et al.*, 1982; Durocher *et al.*, 1989); chloroplast LSU γ fragment, 0.8 kb (Turmel *et al.*, 1993a).

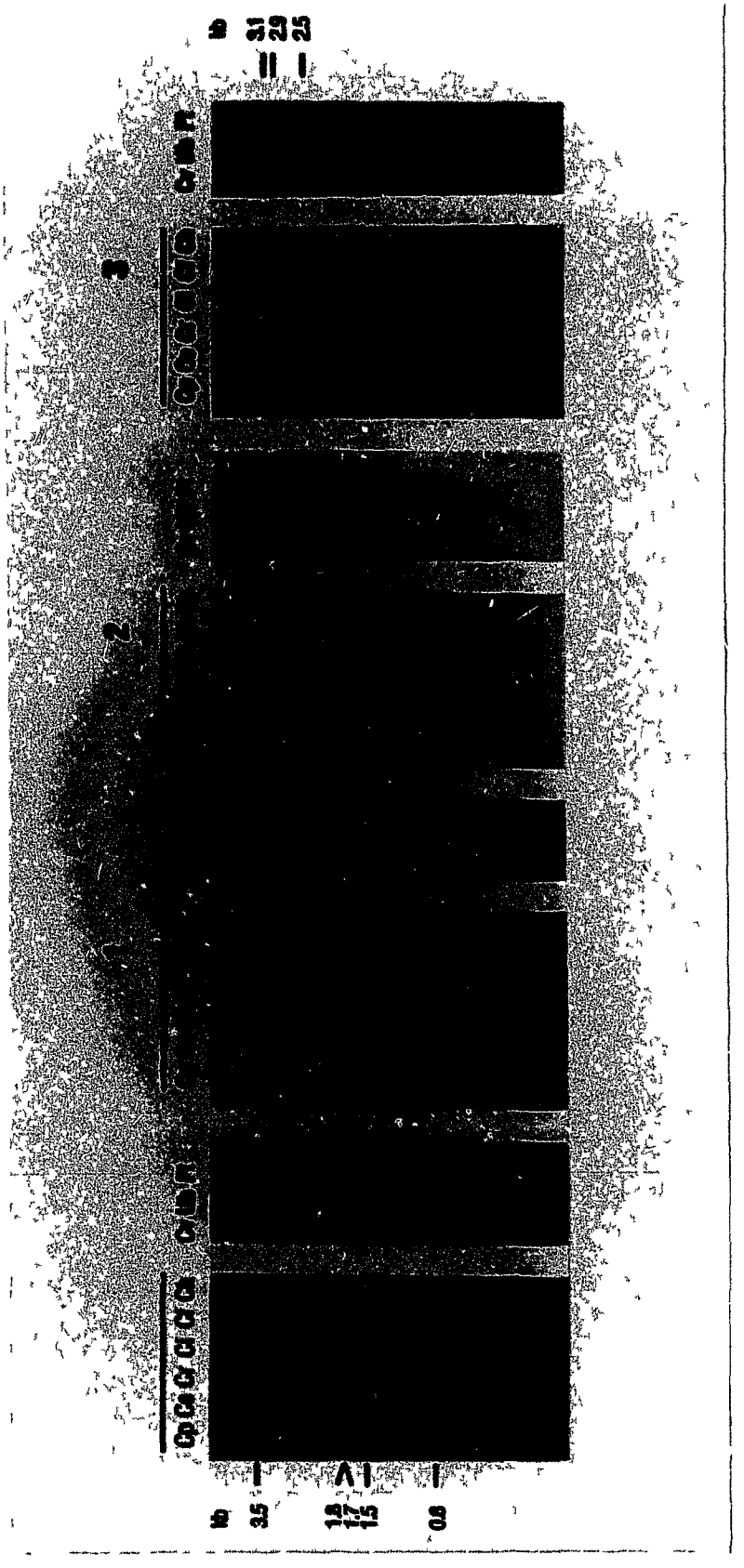


Figure 26.

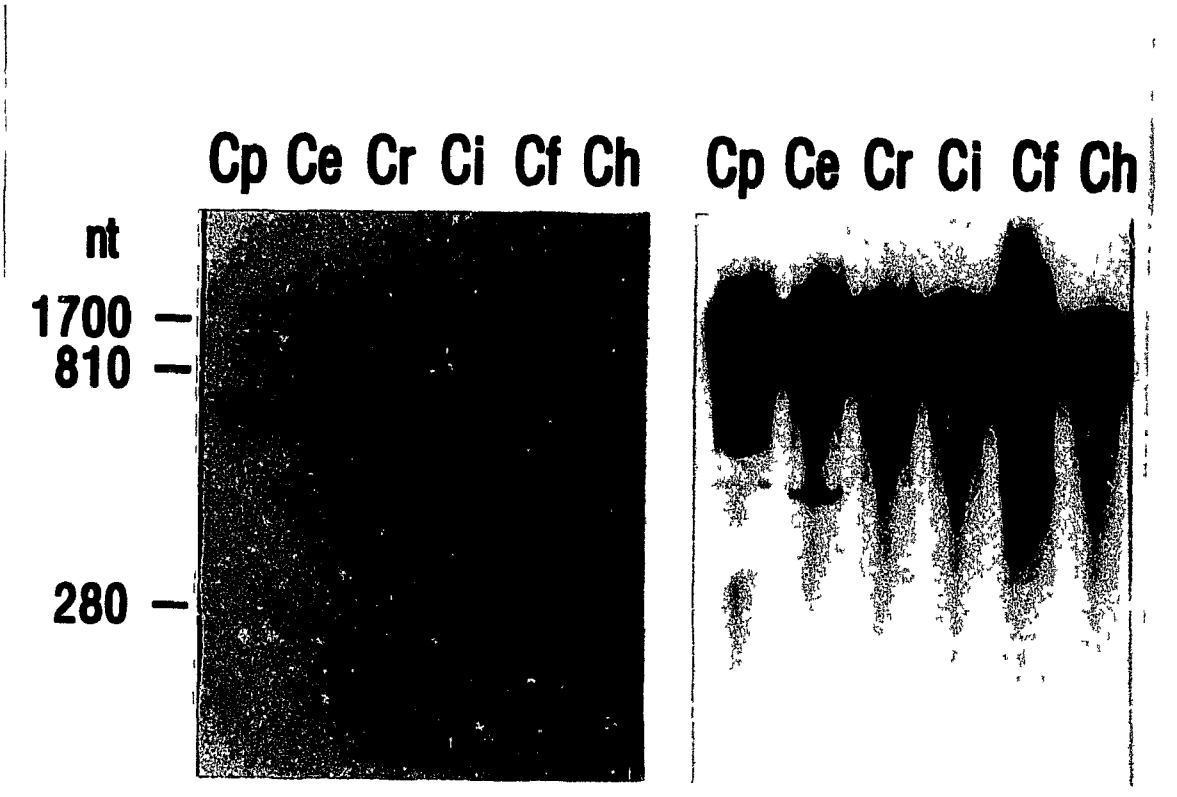


Figure 27. Northern hybridization analysis of RNA isolated from six species of *Chlamydomonas* and fractionated by polyacrylamide gel electrophoresis using probes 1 (left panel) and 2 (right panel). The labelling of RNA samples is described in Figure 24. The sizes of the identified abundant RNAs are indicated as follows: chloroplast LSU *δ* fragment, 1700 nt; chloroplast LSU *γ* fragment, 810 nt; chloroplast LSU *α* fragment, 280 nt (Turmel *et al.*, 1993a).

corresponded to the chloroplast LSU rRNAs (1720 nt) in each alga (except *P. terrestre)* and with a 2500 nt RNA in *P. terrestre*. Previous studies have indicated that the chloroplast LSU rRNAs of the *Chlamydomonas* species in this study group are composed of four interacting rRNA molecules (Turmel et al., 1993a). Similarly, the chloroplast LSU rRNA gene of *Chlorella ellipsoidea* is thought to have an ITS in the same position as the *Chlamydomonas* algae (Yamada and Shimaji, 1987; Gray and Schnare, 1995). *P. terrestre* apparently lacks the ITS that would separate its 2500-nt transcript into two chloroplast rRNAs of 810 and 1720 nt (Fenety, 1993). Moreover, probe 2 annealed with the cytosolic LSU rRNA of *C. frankii* and *P. terrestre* and to 2500-nt RNAs of unknown function in *C. frankii* and *C. vulgaris*. The hybridization to both chloroplast and cytosolic LSU rRNAs of oligonucleotide probes complementary to highly conserved regions of the *Chlamydomonas* mitochondrial rRNA is not surprising considering both the abundance of these rRNAs within total RNA and the conservation of the primary sequence of this region of the LSU rRNA in all major evolutionary lineages (Noller, 1991). It is also not surprising that such short probes anneal with non-rRNA transcripts during low stringency hybridization.

The weak intensity of hybridization of probes 1 and 2 and the stronger intensity of hybridization of probe 3 to the *C. humicola* mitochondrial rRNAs relative to the hybridization pattern of these probes to the other *Chlamydomonas* mitochondrial rRNAs suggests that sequence differences in highly conserved rRNAs exist between *C. humicola* and the other *Chlamydomonas*. Such sequence divergence would be consistent with the distant relationship observed between *C. humicola* and both *C. eugametos* and *C. reinhardtii* in nuclear SSU and chloroplast LSU rRNA phylogenetic analyses (Buchheim *et al.*, 1990; Turmel *et al.*, 1993a).

Northern blot hybridizations of total RNA extracts with a probe complementary to the mitochondrial LSU rRNA of *S. obliquus* (probe 3) detected large RNAs of 3000 and 3100 nt in total RNA extracts of *C. vulgaris* and *P. terrestre*, as well as the cytosolic LSU and chloroplast-encoded LSU (*δ*- fragment) rRNAs in all RNA samples (Fig. 26). At least two of the three

mitochondrial peptidyl transferase centre-specific probes detected the same sized RNA in each of *C. vulgaris*, *U. belkae* and *P. terrestre*. Probe 3 weakly hybridized with the small mitochondrial rRNAs detected by probe 1 in *C. eugametos* and *C. reinhardtii* and hybridized more intensely than probe 1 to the 630-nt mitochondrial rRNA in *C. humicola*. Several other RNAs of unknown function annealed with probe 3, including a 1500-nt RNA in *C. pitschmanii*, 1500-nt and 1900-nt RNAs in *P. terrestre* and a 2500-nt RNA in *C. vulgaris*.

The sizes of the putative mitochondrial RNA species for each of the study group taxa were estimated (Table 7). The sizes of the mitochondrial rRNA species complementary to probes 1 and 2 in *C. reinhardtii* (Boer and Gray, 1988) and *C. eugametos* (Chapter 2) agree well with these estimates. Within the *Chlamydomonas* algal RNA, two size classes of mitochondrial rRNA complementary to probe 1 were observed. Probe 1 detected RNAs of between 620 and 640 nt in *C. pitschmanii*, *C. humicola* and *C. eugametos* and hybridized with RNAs between 430 and 490 nt in *C. frankii*, *C. iyengarii* and *C. reinhardtii*. Probe 2 annealed with RNAs of 480 nt each in *C. pitschmanii* and *C. eugametos* and RNAs of 680 nt each in *C. reinhardtii* and *C. iyengarii*. RNAs complementary to probe 2 in *C. humicola* and *C. frankii* were 580 and 540 nt, respectively. No small RNAs in *C. vulgaris*, *U. belkae* or *P. terrestre* RNA annealed with probes 1, 2 or 3.

Table 8

Sizes of RNAs that hybridized with mitochondrial peptidyl transferase centre-specific probes in northern blots of total cellular RNA isolated from *Chlamydmonas* and other green algae.

Taxon	Autolysin group[1]	Morphological Group[2]	Size of the mitochondrial LSU detected[3]		
			1	2	3
C. pitschmanii	13	Chlorogoniella	620	480	-
C. eugametos	12	Chlamydella	640	480	640
C. humicola	7	Chlorogoniella	630	580	630
C. reinhardtii	1	Euchlamydomonas	490	680	490
C. iyengarii	5	Euchlamydomonas	460	680	-
C. frankii	9	Euchlamydomonas	430	540	-
C. vulgaris	NA	NA	3000	3000	3000
U. belkae	NA	NA	2900	2900	-
P. terrestre	NA	NA	3100	-	3100

[1] Schlösser (1984); NA, not applicable.

[2] Ettl (1976); NA, not applicable.

[3] Sizes of the RNAs (distinct from the chloroplast and cytosolic LSU rRNAs) that hybridized with probe 1 (1), probe 2 (2) and probe 3 (3) in northern blot analysis of total cellular RNA.

Discussion

The minimal mutation distances between mitochondrial rRNA gene piece orders in *C. eugametos* and *C. reinhardtii* were calculated from a hypothetical rRNA gene structure that was intermediate between the structures observed in these algae (Model 1) and from a hypothetical rRNA gene structure that is co-linear with eubacterial rRNA genes (Model 2). Although there was not a significant difference in the number of transpositional events invoked for either model, I favour the later hypothesis because any ancestral *Chlamydomonas* rRNA gene structure was ultimately derived from the protogenote-type continuous rRNA genes and, in total, far fewer transpositional events are necessary to generate the *C. eugametos* or *C. reinhardtii* mitochondrial rRNA gene organization from a hypothetical ancestor that had rRNA genes that were discontinuous but co-linear with conventional rRNA genes than from the hypothetical ancestor evoked for Model 1. Therefore, the simplest explanation suggests that the *Chlamydomonas* rRNA gene structures were derived from conventional but discontinuous rRNA gene structures that have become further differentially fragmented and scrambled in the lineages leading to *C. eugametos* and *C. reinhardtii*.

Predictions about the structure and organization of the mitochondrial rRNA genes in other members of the *Chlamydomonas* genus can be made on the basis of the potential evolutionary intermediates evoked for each hypothesis. If the common ancestor of the *Chlamydomonas* algae had more individual rRNA gene pieces than either *C. eugametos* or *C. reinhardtii* (Model 1), then early diverging representatives of each of the major lineages could also have had smaller gene pieces and need not necessarily have maintained or inherited unique sites of rRNA discontinuity. Moreover, this model would predict that rRNA gene pieces could have become rejoined and differentially disrupted in each *Chlamydomonas* representative and that the organization of the gene pieces would be highly variable in inter-specific comparisons. If the common ancestor of the *Chlamydomonas* algae had rRNA genes

that were co-linear with conventional rRNA gene pieces (Model 2), then one might expect that the common sites of rRNA discontinuity observed in *C. eugametos* and *C. reinhardtii* would be present in all extant members of the genus and that the unique sites of rRNA discontinuity may have been maintained in representatives of the major lineages.

Northern hybridization analysis of total RNA from representatives of the major *Chlamydomonas* lineages supports the hypothesis that the mitochondrial LSU rRNAs of all *Chlamydomonas* algae are fragmented (Chapter 2). These results reveal that *C. eugametos* and *C. pitschmanii* have similar sized rRNAs that hybridize with probes 1 and 2 and that these rRNAs are different than those that hybridize with probes 1 and 2 in *C. reinhardtii* and *C. iyengarii*. The mitochondrial rRNAs of *C. frankii* that hybridized with probes 1 and 2 are shorter than, but similar in size to, those of *C. reinhardtii*. The *C. humicola* mitochondrial rRNAs that hybridize with probe 1 are nearly identical in size to those of *C. eugametos* and *C. pitschmanii* , whereas the *C. humicola* mitochondrial rRNAs that hybridize with probe 2 are comparable to but larger than those of *C. eugametos*. There is a range in the size of the LSU rRNA 3' half of between 970 (*C. frankii*) to 1210 (*C. humicola*) nt and the total size of the 3' half of all of the *Chlamydomonas* mitochondrial rRNAs examined are larger than the minimum size of the core of this molecule excluding variable domains (approximately 910 nt). This observation in conjunction with the size differences observed between the LSU rRNA 3' half of closely related algal pairs such as *C. eugametos* and *C. pitschmanii* suggests that the length differences are within variable regions and are not due to further subdivision of the 3' half of the molecule into separate rRNAs. I interpret these results to suggest that the common domain breakpoint separating the 5' and 3' halves of the LSU rRNA observed in *C. eugametos* and *C. reinhardtii* is common to all *Chlamydomonas* mitochondrial rRNAs, which would support the hypothesis that the ancestral mitochondrial rRNA of these algae was also fragmented at this position. In addition, it appears that algae closely allied with *C. eugametos* and *C. reinhardtii* possess the unique fragmentation sites observed previously within the 3' half of the

LSU rRNA. Moreover, these results are consistent with the unique fragmentation sites being shared by more distantly related algal pairs such as *C. eugametos/C. humicola* and *C. reinhardtii/C. frankii*, although it is not yet possible to eliminate the possibility that other variable regions, apart from site 16 and 17, within the 3' half of the LSU rRNA are disrupted in these *Chlamydomonas* species. Overall, the observation that algae within the two major *Chlamydomonas* lineages appear to have maintained similar sites of rRNA discontinuity tends to favour the model of rRNA gene evolution involving only the division of rRNA genes (Model 2). It does not appear that any of the algae tested have sustained the division between both coding regions L/M and M/N, as would be predicted in the first model.

Mitochondrial rRNA gene sequencing will be required to test other predictions of the models presented here. For example, a common arrangement of gene pieces K and A in all members of the genus would suggest that fragmentation and scrambling arose early within the evolution of the genus. Moreover, it would be of interest to find simple rearrangements of homologous mitochondrial rRNA gene pieces in algae that are more closely related to either *C. eugametos* and *C. reinhardtii* than these algae are to each other and to examine the sequences flanking the rRNA gene pieces. For example, both the mitochondrial and chloroplast genomes of *C. eugametos* and *C. reinhardtii* are extensively rearranged (Turmel *et al.*, 1990), obscuring the events that led to genome reorganization. Comparison of the chloroplast genomes of *C. eugametos* and *C. pitschmanii*, however, shows that these ctDNA differ by only two inversions, each involving a number of genes, within one of the ctDNA single copy regions (Turmel *et al.*, 1990). Because the results presented in this work suggest that *C. eugametos* and *C. pitschmanii* share a similar distribution of mitochondrial rRNA coding information, at least in the 3' half of the LSU rRNA, it will be of interest to compare the mitochondrial rRNA gene orders in these algae. Such analysis may reveal simpler mitochondrial genomic rearrangements between closely related pairs than were observed

between the *C. eugametos* and *C. reinhardtii* mtDNAs and may illuminate the mechanism by which these rRNA gene pieces became scrambled.

The putative mitochondrial LSU rRNAs of *C. vulgaris*, *U. belkae* and *P. terrestre* are molecules of approximately 3000, 2900 and 3100 nt, respectively. The mitochondrial LSU rRNAs of these chlorophycean and pleurastrophycean algae, like those of *P. wickerhamii*, land plants and the vast majority of LSU rRNAs examined are large continuous molecules (Gray and Schnare, 1994). It will be of interest to determine to what extent discontinuous mitochondrial rRNAs are shared with other green algae or whether this structure is confined solely to the clade of *Chlamydomonas* algae.

Conclusions and Future work

Hybridization studies demonstrate that the mitochondrial gene maps of *C. eugametos* and *C. moewusii* are co-linear and that the *C. eugametos/C. moewusii* mitochondrial rRNA genes appear be disrupted and interspersed with protein-coding domains. However, the mitochondrial rRNA gene pieces and other mitochondrial protein-coding regions are extensively rearranged between the *C. eugametos/C. moewusii* and *C. reinhardtii/C.smithii* algal pairs. A detailed comparison of the mitochondrial rRNA gene structure and organization in *C. eugametos* and *C. reinhardtii* was initiated because there were apparently sufficient differences in gene structure between, but not within, these algal pairs to suggest a possible evolutionary pathway of the unusual rRNA gene structure observed in *C. reinhardtii* (Boer & Gray, 1988a).

The mitochondrial rRNA genes of *C. eugametos* are divided into three SSU and six LSU rRNA gene pieces that are dispersed across 15 kb of the circular gene map. Although the *C. eugametos* and *C. reinhardtii* mitochondrial genomes both exhibit a discontinuous and scrambled rRNA gene structure, the rRNA coding information is differently distributed among separate gene pieces and the gene pieces are extensively rearranged when the two green algal mitochondrial genomes are compared. These differences in gene structure and organization support the hypothesis that the processes responsible for the division of rRNA into smaller gene pieces and the reorganization of the individual rRNA gene pieces have been ongoing since the divergence of the two major lineages within the genus. Therefore, it appears that the discontinuous and scrambled mitochondrial rRNA gene structure is a derived characteristic of these algae. This observation makes it unnecessary to invoke, but does not eliminate, alternate hypotheses regarding secondary endosymbiotic events or the lateral transfer of discontinuous and scrambled rRNA gene pieces.

Mitochondrial rRNA-hybridizing fractions were identified within sucrose gradients of total cellular *C. eugametos* ribosomes. This observation, in conjunction with the rRNA

secondary structure models and northern blot analysis, supports the hypothesis that the mitochondrial rRNA gene pieces in *Chlamydomonas* encode functional rRNAs that associate in mitochondrial ribosomal subunits. However, it remains to be demonstrated that each of the SSU and LSU mitochondrial rRNA transcripts predicted to base-pair in secondary structure models are, in fact, specifically associated in mitochondrial ribosomal subunits and that these mitochondrial ribosomes are capable of protein synthesis.

Phylogenetic analysis of the mitochondrial rRNAs of *C. eugametos* and *C. reinhardtii* and other mitochondrial, chloroplast and eubacterial rRNAs has shown that the *C. eugametos* and *C. reinhardtii* mitochondrial rRNA genes, despite considerable sequence divergence and differences in structure and organization, affiliate with each other to the exclusion of all other rRNA gene sequences tested, thus supporting their common ancestry. There does not appear to be a specific relationship between discontinuous and scrambled mitochondrial rRNA genes and an increased rate of nucleotide substitution; both slowly (plant) and rapidly (yeast/fungi) evolving mitochondrial rRNA genes encode continuous rRNAs. The mitochondrial rRNA genes of these two *Chlamydomonas* algae do not specifically affiliate with those of plants or *P. wickerhamii*, the only other chlorophycean alga for which mitochondrial rRNA gene sequence is available. However, because few chlorophycean mitochondrial rRNA genes have been examined, it was not possible to determine whether the unusual position of the mitochondrial rRNA genes of *Chlamydomonas* algae relative to plants and other chlorophytes is due to i) an increased rate of nucleotide substitution in the mitochondrial rRNA genes of *Chlamydomonas*, ii) systematic errors in distance- and parsimony-based phylogenies, or iii) a divergent evolutionary history of the mitochondrial genomes of *Chlamydomonas* and plants, in contrast to the shared evolutionary histories of their chloroplast and nuclear genomes. Before the apparent inconsistencies between nuclear, chloroplast and mitochondrial rRNA gene trees can be resolved, additional mitochondrial rRNA gene sequences must be collected and used in phylogenetic analysis. If green algal mitochondrial rRNA gene sequences are found that

affiliate with those of *Chlamydomonas* but encode continuous mitochondrial rRNAs, it would support the view that the *Chlamydomonas* mitochondrial rRNA gene pieces are derived from a green algal ancestor.

Northern hybridization analysis of the 3' half of the mitochondrial LSU rRNAs of six species of *Chlamydomonas* and other chlorophycean and pleurastrophycean algae suggests that fragmented mitochondrial rRNA is limited to a small group of algae within the Chlorophyta, although it is not known if this mitochondrial rRNA gene organization extends to other green algae beyond the genus *Chlamydomonas*. Some of the sites of discontinuity within the mitochondrial LSU rRNA of *Chlamydomonas* appear to be common to all members of the genus, while other sites of discontinuity are lineage-specific, supporting the hypothesis that the last common ancestor of all *Chlamydomonas* species contained fragmented mitochondrial LSU rRNAs. *C. eugametos* and *C. pitschmanii* apparently have a similar distribution of coding information, at least for the 3' half of the LSU rRNA, but are less closely related than interfertile pairs such as *C. eugametos* and *C. moewusii*. It will be of interest to determine whether the rRNA coding information is in the same genomic context in algal pairs such as *C. eugametos* and *C. pitschmanii*. Such data would complement the comparison of mitochondrial rRNA gene structures presented in this work and may help to refine models about the evolution of this gene structure especially with regard to the sequences that flank rearranged rRNA coding modules and possible mechanisms for the movement of the gene pieces.

I favour the view that rRNA discontinuities evolve as the result of the expansion of variable regions. Expanded variable regions that protrude from the core of the ribosome would, in a specific cellular context, be susceptible to resident cellular or inter-organellar RNases; some systems may be predisposed to developing fragmented rRNAs depending on the diversity of RNases present. An equilibrium may exist between expansion and contraction of variable regions within rRNAs such that they may be converted to ITSs or return to being covalently continuous portions of the rRNA molecule. Scrambling of the resulting gene pieces

appears to be the result of separate evolutionary processes. It remains to be determined whether recombination between rRNA coding modules, retrotransposition or transposition contribute to scrambling of rRNA gene pieces.

To date, scrambled rRNA genes have been observed only in mitochondrial genomes although discontinuous rRNA genes have been observed in eubacterial, nuclear, chloroplast and mitochondrial genomes. It is possible that natural selection is ineffective at eliminating scrambled rRNA gene arrangements in mtDNA because ribosome biogenesis may proceed without a tight coupling between rRNA transcription and ribosome formation in mitochondria (Boer & Gray, 1988a). Models involving either the generation of ribosomal sub-modules and the later assembly of ribosomal subunits from such RNP particles (Boer & Gray, 1988a) or the association of scrambled rRNA sequences and ribosomal proteins in a larger primary transcript that are subsequently processed to liberate the ribosomal subunits (Chapter 2) would both be compatible with such an hypothesis. The isolation and characterization of precursors containing mitochondrial ribosomal RNA and protein could possibly indicate how ribosome biogenesis proceeds in these mitochondria. Furthermore, it may be possible to use mitochondrial transformation (Randolph-Anderson *et al.*, 1993) in these unicellular algae to introduce either further divided and/or scrambled mitochondrial rRNA genes and determine if and how such transformants assemble functional mitochondrial ribosomes. Alternatively, eubacterial, nuclear and chloroplast genomes may contain rRNA genes that are co-linear with conventional rRNA genes because the mechanisms that act to homogenize the multiple rRNA gene copies within these genomes may function to efficiently correct chance rearrangements of homologous rRNA coding information. Such mechanisms may include gene conversion, unequal crossing-over or transposition (Dover, 1982; Dover & Flavell, 1984). These copy correction mechanisms may be less efficient at promoting homogenization of rRNA genes between mitochondrial genomes before genomic rearrangements within mtDNA are partitioned into separate cells. It would be of interest to determine whether the addition of conventional

rRNA genes to *Chlamydomonas* mtDNA by transformation would lead to the generation of homoplasmic cells containing conventional mitochondrial rRNAs. Furthermore, one might be able to determine whether scrambled rRNA genes introduced into ctDNA by transformation (Boynton *et al.*, 1988) are stably maintained during the transmission of the chloroplast genome.

The number and dispersion of restriction fragment length polymorphisms between the mtDNAs of *C. eugametos* and *C. moewusii* make it possible to follow the transmission of these genomes in inter-specific crosses (Lee *et al.*, 1990; Cormier, 1993) and to determine whether mitochondrial DNA recombination occurs in these algae. Although an increased understanding of mtDNA recombination is important in the context of mtDNA evolution in general, it may help to confirm the hypothesis (Chapter 2) that the unusual organization of dispersed modular rRNA genes in the mitochondrial genomes of *C. reinhardtii* (Boer & Gray, 1988a) and *C. eugametos* evolved as a result of recombination between mitochondrial genomes initiated by the association of DNA sequences with limited sequence similarity, such as dispersed repeats, or whether other molecular mechanisms, such as transposition or retrotransposition, have contributed to mtDNA genome reorganization.

Finally, during the course of this work, I determined the DNA sequence of over 90% of the *C. eugametos* mitochondrial genome. Analysis of this sequence has confirmed that each of the protein-coding genes identified by Southern analysis (Chapter 1) is present in the *C. eugametos* mtDNA. In future, I wish to finish determining the DNA sequence of this genome and to complete a detailed comparison between the protein- and tRNA-coding genes, genome content, overall genome organization and expression of the mtDNAs of *C. eugametos* and *C. reinhardtii*. Such comparisons may reveal common features in genome organization and expression in *Chlamydomonas* algae and extend our knowledge regarding the structural and functional diversity of mitochondrial genomes in general.

Appendix

To determine the probability that the five corresponding mitochondrial rRNA variable regions that are interrupted in both *C. eugametos* and *C. reinhardtii* arose independently, we assumed that the seven and ten interrupted variable regions in the *C. eugametos* and *C. reinhardtii* mitochondrial rRNAs, respectively, arose at random among the 30 variable rRNA regions identified in each alga or only among 18 of the 30 variable rRNA regions that have been reported to be discontinuous in these and other fragmented rRNAs. Then let n = the number of variable positions that can be disrupted (30 or 18), m_1 = the number of positions that are discontinuous in *C. eugametos* (7), m_2 = the number of positions that are discontinuous in *C. reinhardtii* (10) and k = the number of sites that could be in the common discontinuous state in both species ($0 \leq k \leq 7$). The probability of having k common discontinuous sites (P_k) was calculated using the following combinatorial formula prepared for us by Professor E. Zouros (Dalhousie University).

$$P_k = \frac{\dfrac{m_1!}{k!\,(m_1 - k)!} * \dfrac{(n - m_1)!}{(m_2 - k)!\,[(n - m_1) - (m_2 - k)]!}}{\dfrac{n!}{m_2!\,(n - m_2)!}}$$

The cumulative probability (ΣP) that five or more variable rRNA regions are in the common discontinuous state in both species was calculated as follows: $\Sigma P = (P_{k=5} + P_{k=6} + P_{k=7})$

When n=30, P=0.024; and when n=18, P=0.278

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