Fragmented and Scrambled Mitochondrial Ribosomal RNA Coding Regions Among Green Algae: A Model for Their Origin and Evolution

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Mitochondrial ribosomal RNA coding regions in the only three green algal taxa investigated to date are fundamentally different in that they are continuous in *Prototheca wickerhamii,* but highly fragmented and scrambled in *Chlamydomonas reinhardtii* and *Chlamydomonas eugametos.* To gain more insight into the mode of evolution of fragmented and scrambled mitochondrial ribosomal RNA (rRNA) genes within the green algal group, this work (1) provides additional information on fragmentation patterns of mitochondrial small- and large-subunit (SSU and LSU) rRNAs that strongly supports the concept of a gradual increase in the extent of discontinuity of mitochondrial rRNAs among chlorophycean green algae and (2) reports the first example of fragmented and scrambled mitochondrial LSU rRNA coding regions in a green algal taxon outside the *Chlamydomonas* group. The present study (1) suggests that the scrambling of the mitochondrial rRNA coding regions may have occurred early in the evolution of fragmented and scrambled mitochondrial rRNA genes within the chlorophycean green algal group, most likely in parallel with the fragmentation events, (2) proposes recombination as a possible mechanism involved in the evolution of these mitochondrial rRNA genes, and (3) presents a hypothetical pathway for converting continuous mitochondrial rRNA genes into the highly fragmented and scrambled rRNA coding regions of *Chlamydomonas* through a series of recombinatorial events between short repeated sequences.

Introduction

Ribosomal RNAs (rRNAs) are essential components for both the structure and function of ribosomes in all prokaryotic, eukaryotic, and organellar genetic systems. Most known large-subunit (LSU) and small subunit (SSU) rRNAs are rather strongly conserved in size and secondary structure within their respective type and evolutionary lineage (Gutell 1992). However, in some lineages, unconventional rRNAs have been described, including the rRNA complexes composed of split rather than single, covalently continuous polyribonucleotide chains. Such complexes have been identified among eubacteria as well as in the mitochondrial, chloroplast, and nucleocytosolic compartments of eukaryotes (Gray and Schnare 1996). The genes coding for the discontinuous rRNAs deviate from the conventional structure in that they are fragmented into coding modules that can be interspersed with either internal transcribed spacers, or protein-coding genes and/or transfer RNA (tRNA) genes. Moreover, in some mitochondrial genetic systems (e.g., *Tetrahymena pyriformis* [Heinonen et al. 1987], *Chlamydomonas reinhardtii* [Boer and Gray 1988], *Chlamydomonas eugametos* [Denovan-Wright and Lee 1994], *Plasmodium sp.* [Vaidya, Akella, and Suplick 1989; Feagin et al. 1992], and *Theileria parva* [Kairo et al. 1994]), the rRNA coding modules no longer follow the 5′–3′ transcriptional order of their counterparts within conventional continuous genes; rather, they are scrambled within the genome. In addition, in *Plasmodium falciparum* and *T. parva,* mitochondrial rRNA gene pieces are not coded on the same DNA strand.

Key words: fragmented and scrambled mitochondrial rRNA genes, recombination, repeated sequences, evolution, green algae.

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It has been proposed that all split rRNA genes are derived and evolved from continuous homologs (Gray and Schnare 1996). However, the processes responsible for the fragmentation of rRNA coding regions are not fully understood in any of the described genetic systems. Fragmented and scrambled mitochondrial rRNA genes have been reported in green algae, ciliates, and apicomplexans, and it seems likely they are the result of independent events that occurred in evolutionarily distant lineages.

The green algae represent a good study group with which to address questions about the origin and evolution of fragmented and scrambled mitochondrial rRNA coding regions. Two very distinct mitochondrial rRNA gene organizations have been described for the three green algal species investigated in this respect: conventional continuous rRNA genes in *Prototheca wickerhamii* (Wolff et al. 1994) and highly fragmented and scrambled rRNA coding regions in *C. reinhardtii* (Boer and Gray 1988) and *C. eugametos* (Denovan-Wright and Lee 1994).

The phylogeny of the green algal group is progressively being deciphered, and the new information gathered through molecular approaches will probably trigger the reconsideration of the traditional green algal systematics (Chapman and Buchheim 1991). Mattox and Stewart (1984) proposed a phylogenetic scenario in which they suggested the most important evolutionary events that occurred among some green flagellates and led to differences in the flagellate ancestors of the four advanced green algal groups, namely, the Charophyceae, Ulvophyceae, Pleurastrophyceae, and Chlorophyceae. All the extant green flagellate taxa that retained ancestral-like features were placed in a distinct group, i.e., the Micromonadophyceae. The authors assumed that in the earliest green flagellates the flagellar apparatus had only one of each kind of root. The swarmers of the Charophyceae are considered as a minimal change from that ancestral condition. The flagellates with a cruciate root

system with two of each kind of root (Ulvophyceae, Pleurastrophyceae, and Chlorophyceae) occurred by a doubling of one of the flagella (and the roots associated with it) of an early green flagellate. Flagellates of this type, with basal bodies in a counterclockwise orientation (CCW), gave rise to the Ulvophyceae with few additional changes. Among other flagellates with a cruciate system, the fusion or interweaving of scales generated a theca, and this resulted in selection toward the phycoplast and collapsing interzonal spindle. The green flagellates that retained the primitive counterclockwise orientation of the basal bodies gave rise to the Pleurastrophyceae. Other CCW flagellates gave rise to the Chlorophyceae by a further evolution to a directly opposed (DO) and clockwise (CW) orientation of the basal bodies in their flagellar apparatus. It was proposed that (1) *Hafniomonas montana* is a descendant of such CCW green flagellates, and (2) evolutionary intermediates can be found among chlorophycean taxa whose basal bodies are almost directly opposite each other (O'Kelly and Floyd 1984; O'Kelly, Watanabe, and Floyd 1994). Using a data set of ultrastructural and biochemical characters, Kantz et al. (1990) obtained a cladogram also indicating (1) the Pleurastrophyceae as a sister group to the Chlorophyceae; (2) the Ulvophyceae as a sister group to the Chloro/Pleurastrophyceae; and (3) the Charophyceae, the Ulvo-/Chloro/Pleurastrophyceae clade, and the micromonadophycean taxa emerging from an unresolved node.

The classification system of Mattox and Stewart (1984) divides the green algal group into five classes: Micromonadophyceae, Ulvophyceae, Pleurastrophyceae, Chlorophyceae, and Charophyceae. The authors placed all the vegetatively nonmotile chlorophycean species in one order, namely the Chlorococcales. In this view, the CW flagellate *Chlamydomonas* species and the autosporic *P. wickerhamii* belong to different orders, the Chlamydomonadales and the Chlorococcales, respectively, but they are both members of the same class, i.e., the Chlorophyceae. However, phylogenies based on nuclear rRNA sequences suggest that the Chlorococcales are not a monophyletic group: some taxa, such as *Scenedesmus obliquus* and some *Chlorella* species, affiliate with chlorophycean taxa with the DO flagellar configuration (e.g., *Neochloris aquatica*), whereas other taxa, such as *P. wickerhamii* and other *Chlorella* species, seem to be more closely related to members of the advanced pleurastrophycean lineages (*sensu* Mattox and Stewart 1984). In this context, it appears that although they are members of the green algal group, *Chlamydomonas* and *P. wickerhamii,* the two green algal lineages whose mitochondrial rRNA gene organizations appear unexpectedly different, may not be as closely related as previously thought; they are in fact members of two very distant evolutionary lineages, namely the CW and CCW, respectively, whose divergence is probably very old.

Nedelcu et al. (1996) showed that discontinuous mitochondrial LSU rRNAs are not confined to *Chlamydomonas* species but are, rather, a unifying feature for chlorophycean taxa with a CW or DO flagellar configuration as well as chlorococcalean taxa phylogenetically related to them. The authors also suggested a trend in the evolution of this trait, that is, a tendency toward an increase in the degree of discontinuity, from a continuous mitochondrial LSU rRNA in *H. montana* to highly fragmented mitochondrial LSU rRNAs in *C. eugametos* and *C. reinhardtii.*

Although mitochondrial rRNA genes are highly fragmented and scrambled in both *C. reinhardtii* and *C. eugametos,* the distributions of the coding information among their coding modules, as well the order of these modules within the genome, are different between the two species (Denovan-Wright and Lee 1994). Calculations of the minimal number of transpositions required to convert hypothetical ancestral rRNA gene organizations to the arrangements present in the two *Chlamydomonas* taxa, as well as a limited survey of the size of mitochondrial LSU rRNAs in other *Chlamydomonas* species, led Denovan-Wright et al. (1996) to propose that the last common ancestor of *Chlamydomonas* algae possessed fragmented mitochondrial rRNA genes whose coding modules were nearly colinear with their counterparts in conventional continuous rRNA genes. The authors presented a model in which the fragmentation and scrambling of the coding modules were assumed to be separate, consecutive events, and the rearrangement of the rRNA gene pieces was limited to transpositional events. However, no specific mechanism has been proposed to explain either the fragmentation or the scrambling of the resulting gene pieces.

This work provides additional information on fragmentation patterns of mitochondrial SSU and LSU rRNAs among chlorophycean lineages and sheds insight into the mode of evolution of fragmented and scrambled mitochondrial rRNA coding regions in this group. I have surveyed the mitochondrial LSU and SSU rRNAs in three taxa representing both the CW (i.e., *Chlamydomonas pulsatilla*) and DO (i.e., *N. aquatica* and *S. obliquus*) evolutionary lineages within the chlorophycean green algal group. This study (1) suggests a mechanism that may have been responsible for both the fragmentation and scrambling of the mitochondrial rRNA genes within the chlorophycean green algal group and (2) presents a hypothetical pathway for converting continuous mitochondrial rRNA genes to the highly fragmented and scrambled rRNA coding regions of *Chlamydomonas.*

Materials and Methods

Strains and Growing Conditions

The algal strains, sources, and growing media used were as follows: *Chlamydomonas reinhardtii* Dangeard (GC wt137c, Genetics Center at Duke University), minimal medium (Lemieux, Turmel, and Lee 1980); *Chlamydomonas eugametos* (UTEX 9, The Culture Collection of Algae at the University of Texas at Austin), minimal medium; *Chlamydomonas pulsatilla* Wollenweber (UTEX 2534, The Culture Collection of Algae at the University of Texas at Austin), artificial sea water (Starr and Zeikus 1993); *Neochloris aquatica* Starr (UTEX 138, The Culture Collection of Algae at the University

Table 1
Characteristics of Oligonucleotide Probes 1 to 8

Probe	Sequence (5′ to 3′)	Complementary to	*E. coli* Coordinates (Gutell, Schnare, and Gray 1992)
1.....	CGGGACTATCACCCTCTTTGGTTTCC (26mer)	5′-half LSU rRNA of *S. obliquus*	313–338
2.....	CACAGGACAACGGTGGCCCTTCTT (24mer)	5′-half LSU rRNA of *S. obliquus*	478–500
3.....	GACTCGCTCACTCATGTTGCAAAAGGC (27mer)	5′-half LSU rRNA of *S. obliquus*	563–589
4.....	CCGAACTTGATTGGCCTTTCACCCCTAGCCAC (32mer)	5′-half LSU rRNA of *C. reinhardtii*	768–799
5.....	GCTAGACCAGTGAGCTATTACGCTTTC (27mer)	5′-half LSU rRNA of *C. reinhardtii*	1,087–1,113
6.....	GCTGATAAACCTGTTATCCCTAGCGTA (27mer)	3′-half LSU rRNA of *S. obliquus*	2,438–2,464
7.....	AGGACGCGATGATCCAACATCGAGGTGCC (29mer)	3′-half LSU rRNA of *C. reinhardtii*	2,494–2,522
8.....	GGGTCTCTAATCCGGTTCGCTACCCA (26mer)	SSU rRNA of *C. reinhardtii*	772–797

of Texas at Austin), soil extract medium (Starr and Zeikus 1993); *Scenedesmus obliquus* (Turp.) Kutz (UTEX 78, The Culture Collection of Algae at the University of Texas at Austin), basal medium (Oh-Hama and Hase 1980); and *Prototheca wickerhamii* Soneda and Tubaki (UTEX 1533, The Culture Collection of Algae at the University of Texas at Austin), malt medium (Wolff and Kück 1990). Cultures were supplied with 1% CO_2 in air. Illumination was provided by cool-white fluorescent lamps (50–80 $\mu mol \cdot m^{-2} \cdot s^{-1}$) on a 12:12 h light–dark cycle. Cells were harvested when the culture density reached ca. 4×10^6 cells/ml.

Total RNA Extraction and Fractionation

Total RNA from six green algal species was extracted according to Rochaix and Malnoë (1982). Glyoxalated total RNA (ca. 10 μg) from each taxon was fractionated by agarose (1.5%) gel electrophoresis (Sambrook, Fritsch, and Maniatis 1989) and transferred to Hybond-N nylon membranes (Amersham) by vacuum blotting under the conditions recommended by the manufacturer (Pharmacia).

Northern Blot Hybridization

RNA blots were prehybridized at 37°C for 4 h in the hybridization buffer: 5 × SSPE (20 × SSPE = 3.6 M NaCl, 200 mM NaH_2Po_4, 20 mM EDTA, pH 7.4), 1 × BLOTTO (10 × BLOTTO = 5% instant milk, 10% sodium dodecyl sulphate [SDS], pH 7.8), 50% formamide. To detect putative mitochondrial rRNAs in the species investigated, synthetic oligodeoxynucleotide probes complementary to regions within the mitochondrial LSU and SSU rRNA of *C. reinhardtii* and *S. obliquus* were used. The characteristics of the probes are summarized in table 1, and the locations of their target regions are indicated in figure 2I*a* and II*a*. The oligonucleotide probes were 5′-end-labeled using [γ-^{32}P]ATP and polynucleotide kinase (Pharmacia) at 37°C for 1 h and then purified on Microspin Columns S-200 HR (Pharmacia). Hybridization reactions were carried out at 37°C for 21 h. The blots were washed twice for 15 min each time at room temperature, first in 2 × SSPE, 0.1% SDS, then in 0.5 × SSPE, 0.1% SDS.

DNA Sequence Similarity Analysis

The mitochondrial DNA sequence (EMBL X17375) of *S. obliquus* (KS3/2) was analyzed for sequence similarity using the BLAST algorithm (Altschul et al. 1990).

Results

Northern Blot Analyses

Total RNA was extracted from *C. reinhardtii, C. eugametos, C. pulsatilla, N. aquatica, S. obliquus,* and *P. wickerhamii.* Figure 1*A* shows the electrophoretic patterns of the green algal total RNAs fractionated in 1.5% agarose gels and stained with ethidium bromide (EtBr). The abundant cytosolic SSU (1,800 nt) and LSU rRNA (3,500 nt), as well as the chloroplast SSU (1,500 nt) and γ- and δ-LSU rRNA fragments (1,700 and 820 nt, respectively) of *C. reinhardtii* and *C. eugametos* served as references for the identification of the rRNA counterparts in the taxa investigated. Mitochondrial rRNAs are not visible by EtBr-staining due to their low abundance in total RNA preparations.

Figure 2 indicates (1) the location of the target regions for oligonucleotide probes 1 to 8 represented according to the *E. coli* LSU and SSU rRNA scale (fig. 2I*a* and II*a*), (2) the corresponding mitochondrial LSU and SSU rRNA fragments of *C. reinhardtii* and *C. eugametos* (fig. 2I*b* and II*b*), and (3) the putative mitochondrial rRNA counterparts identified in the species examined (fig. 2I*c* and II*c*). Information about the size of mitochondrial rRNAs hybridizing with oligonucleotide probes 1 to 8, as well as their locations within the mature rRNA complexes, i.e., SSU rRNA, 5′-half or 3′-half LSU rRNA, respectively, is summarized in table 2.

Under the hybridization conditions used, the probes annealed not only with mitochondrial rRNAs but also with their cytosolic and/or chloroplast counterparts. Hybridizing RNAs with no visible correspondents on EtBr-

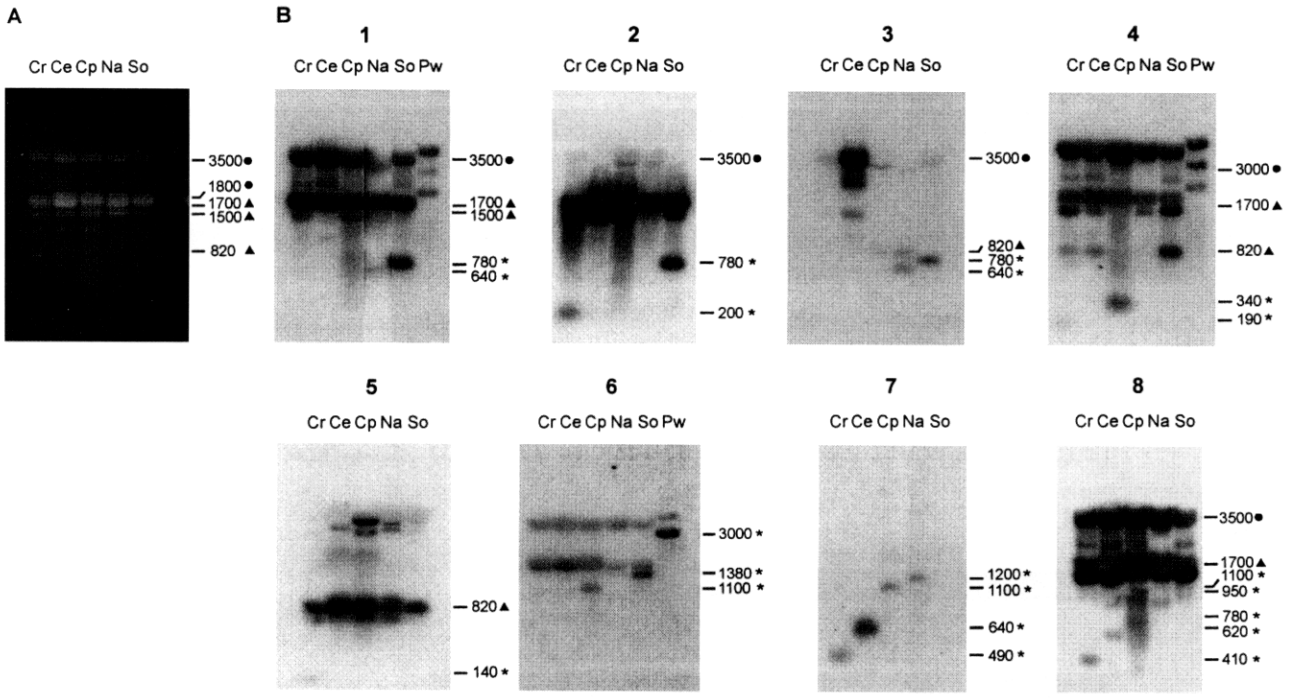

FIG. 1.—*A*, Electrophoretic patterns of green algal total RNA fractionated in 1.5% agarose gels. *Cr* = *Chlamydomonas reinhardtii, Ce* = *Chlamydomonas eugametos, Cp* = *Chlamydomonas pulsatilla, Na* = *Neochloris aquatica, So* = *Scenedesmus obliquus, Pw* = *Prototheca wickerhamii.* RNA molecular sizes are expressed in nucleotides. *C. reinhardtii* rRNA size references indicated are: cytosolic LSU rRNA (3,500 nt), cytosolic SSU rRNA (1,800 nt), chloroplast LSU rRNA δ-fragment (1,700 nt), chloroplast SSU rRNA (1,500 nt), and chloroplast LSU rRNA γ-fragment (820 nt). *B*, Northern blot analyses of total RNA fractionated in 1.5% agarose gels and hybridized with oligonucleotide probes (1 to 8). Abbreviations as in panel *A*. Only data for informative taxa shown. RNA molecular sizes are expressed in nucleotides. Stars, triangles, and circles denote rRNAs of mitochondrial, chloroplast, and cytosolic origin, respectively.

stained gels and no hybridizing counterparts among the cytosolic and chloroplast rRNAs of *C. reinhardtii* or *C. eugametos* are most likely of mitochondrial origin (fig. 1*B*). The oligonucleotide probes directed to regions within the 5′-half LSU rRNA identified small RNAs of about 340, 640, and 780 nt in *C. pulsatilla, N. aquatica,* and *S. obliquus,* respectively. The 200-, 190-, and 140-nt rRNAs corresponding to the *C. reinhardtii* mitochondrial LSU rRNA fragments L_1, L_{3b}, and L_5, respectively, as well as the 3,000-nt rRNA transcript in *P. wickerhamii,* are presented as positive controls. The two oligonucleotide probes targeted to the peptidyl-transferase center within the 3′-half LSU rRNA hybridized with RNAs of about 1,100, 1,200, and 1,380 nt in *C. pulsatilla, N. aquatica,* and *S. obliquus,* respectively. The 490- and 640-nt RNAs correspond to the *C. reinhardtii* and *C. eugametos* mitochondrial LSU rRNA L_8 and L_6 fragments, respectively, and the 3,000-nt RNA represents the large continuous mitochondrial LSU rRNA of *P. wickerhamii.* The oligonucleotide probe directed to the SSU rRNA detected RNAs of about 780, 950, and 1,100 nt in *C. pulsatilla, N. aquatica,* and *S. obliquus,* respectively. Positive controls are the S_3 SSU rRNA fragment in *C. reinhardtii* (410 nt) and the S_2 SSU rRNA fragment in *C. eugametos* (640 nt).

S. obliquus Mitochondrial LSU rRNA Coding Region Analysis

A 3,885-bp mitochondrial DNA sequence (EMBL X17375) of a wild strain of *S. obliquus* was analyzed for sequence similarity using the BLAST algorithm (Altschul et al. 1990). New coding regions in addition to the 3′-half LSU rRNA coding region previously reported (Kück, Godenhardt, and Schmidt 1990) were identified. Figure 3 provides a diagrammatic representation of the 3′ region of the LSU rRNA gene of *S. obliquus* as described by Kück, Godenhardt, and Schmidt (1990), as well as the locations of the new tRNA and rRNA coding regions identified here (fig. 3*A*), and compares the mitochondrial LSU rRNA coding regions of *S. obliquus* to those of *P. wickerhamii* (fig. 3*B*). Within the first 480 nucleotides of the 3,885-bp *S. obliquus* mitochondrial DNA sequence, short regions showed 70%–89% similarity to sequences within the 5′-distal part of the 5′-half of eubacterial, mitochondrial, chloroplastic, and cytosolic LSU rRNAs. In addition, the region between coordinates 500 and 600 of the DNA sequence displayed 70%–85% sequence similarity to tRNA coding regions from various eubacterial, mitochondrial, and chloroplast genomes. Finally, starting around coordinate 700 of the *S. obliquus* mitochondrial DNA sequence, the first 40 nucleotides showed high sequence similarity with the very 3′-end of the 5′-half LSU rRNA and the following nucleotides with the adjacent 3′-half of various LSU rRNAs as proposed by Kück, Godenhardt, and Schmidt (1990). No sequence similarities with the central part of the 5′-half LSU rRNA domain were found, but the functional significance and its ubiquitous presence in most counterparts strongly suggest that the corresponding

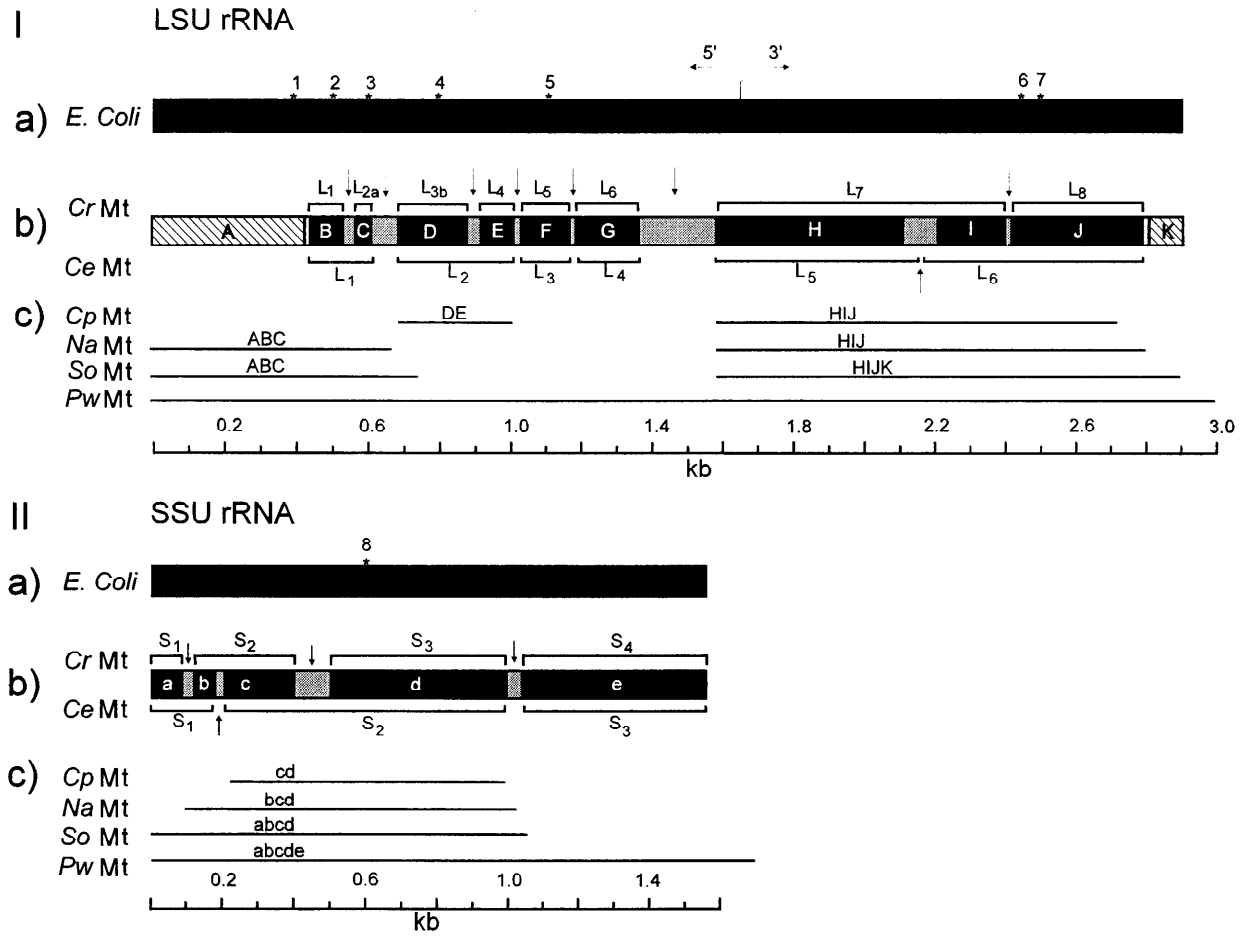

FIG. 2.—I*a* and II*a*, The location of the target regions for oligonucleotide probes 1 to 8 represented to the *E. coli* LSU and SSU rRNA scales, respectively. I*b* and II*b*, Fragmentation patterns within the mitochondrial (Mt) LSU and SSU rRNA, respectively, of *C. reinhardtii* (*Cr*) and *C. eugametos* (*Ce*); solid blocks indicate the smallest corresponding coding units that are continuous in the mitochondrial rRNAs of both *C. reinhardtii* and *C. eugametos*; stippled blocks indicate variable regions that are interrupted (arrows) in the two *Chlamydomonas* taxa; cross-hatched blocks indicate regions that are missing in both *Chlamydomonas* taxa. I*c* and II*c*, The size and suggested coding capacity of the putative mitochondrial rRNAs identified in the taxa investigated; abbreviations as in figure 1.

LSU rRNA coding region is present at a different location along the mitochondrial genome. On this evidence, I therefore conclude that in *S. obliquus*, the mitochondrial LSU rRNA gene is fragmented and the coding regions are scrambled. This is the first example of fragmented and scrambled mitochondrial LSU rRNA coding regions in a non-*Chlamydomonas* green algal taxon.

To determine if the observed sequence similarities of the new rRNA and tRNA coding regions identified in the *S. obliquus* mitochondrial DNA sequence are biologically meaningful, the DNA sequence was used to model the secondary structures of the potential transcripts (data not shown). The first 480 nt of the DNA sequence can be folded in a conventional eubacteria-like secondary structure corresponding to the region between *E. coli* coordinates 270 and 660. An additional 70-nt sequence is present around the 370 *E. coli* coordinate; in higher plant mitochondrial LSU rRNAs, a longer extra sequence is present 100 nt downstream of this lo-

Table 2
Size (in nucleotides) of the Hybridizing Mitochondrial rRNAs Detected by Oligonucleotide Probes 1 to 8

	Probe Directed to rRNA							
	5′-half LSU					3′-half LSU		SSU
Taxa	1	2	3	4	5	6	7	8
C. reinhardtii	—	200	—	190	140	—	490	410
C. eugametos	—	—	—	—	—	—	640	620
C. pulsatilla	—	—	—	340	—	1,100	1,100	780
N. aquatica	640	—	640	—	—	—	1,200	950
S. obliquus	780	780	780	—	—	1,380	—	1,100
P. wickerhamii	—	—	3,000	—	—	3,000	—	—

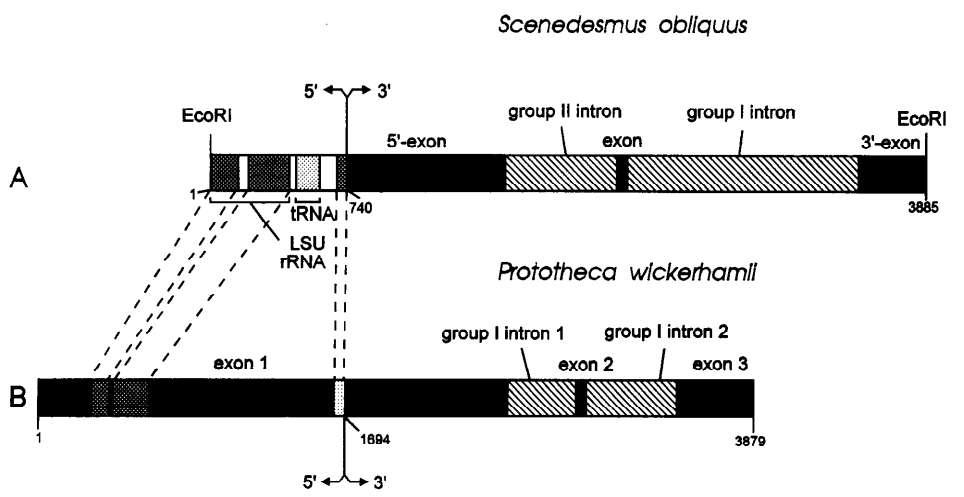

FIG. 3.—*A*, Diagrammatic representation of a mitochondrial LSU rDNA sequence of *S. obliquus* illustrating the 3′-half LSU rRNA coding region (solid blocks) and two introns (cross-hatched blocks) as reported by Kück, Godenhardt, and Schmidt (1990), as well the new coding regions identified within its 5′ region (stippled blocks). *B*, Comparison between the mitochondrial LSU rRNA gene of *P. wickerhamii* and that of *S. obliquus* indicating the locations of corresponding coding regions (stippled blocks) within the 5′-half of their LSU rRNA genes.

cation. The putative LSU rRNA fragment corresponds to the *C. reinhardtii* mitochondrial L_1 and L_{2a} LSU rRNA fragments and extends 180 nt into the first 450 nt of the 5′-end LSU rRNA, a region that is completely missing from both *C. reinhardtii* and *C. eugametos* mitochondrial LSU rRNAs. The tRNA-like DNA sequence can be folded in a conventional tRNA (Ala, UGC); such a tRNA is missing in both *Chlamydomonas* taxa investigated to date, but is present in the *P. wickerhamii* mitochondrial genome.

To rule out the possibility that the LSU rRNA coding region identified is a pseudogene and to determine if the coding region is transcribed as one single transcript, a series of Northern blot hybridizations were performed using total RNA and three synthetic deoxyoligonucleotide probes complementary to regions within the 5′-end LSU rRNA coding region of *S. obliquus*. All three oligonucleotide probes (i.e., 1, 2, and 3) directed to the potential 5′-end mitochondrial LSU rRNA transcript hybridized with a 780-nt RNA molecule (fig. 1*B*). The size of this transcript is larger than that of the rRNA predicted based on the coding region analyzed (480 nt), indicating that the coding region extends outside the 5′-end of the sequenced clone and is transcribed into a single transcript.

Discussion

Fragmented and Scrambled Mitochondrial rRNA Coding Regions Among Chlorophycean Green Algae

The Chlorophyceae is one of the five green algal classes (*sensu* Mattox and Stewart 1984) and consists of two evolutionarily distinct lineages with respect to the position of the basal bodies within the flagellar apparatus; these can be in either a DO or a CW orientation. It has been proposed that the CW configuration evolved from an ancestral CCW configuration, and the DO configuration represents an intermediate stage in the evolution of this trait (O'Kelly 1992). Taxa investigated in this study are representative of both CW and DO chlorophycean lineages. As a member of the *Chlamydomonas* genus, *C. pulsatilla* most likely belongs to the CW clade (no phylogenetic data are available for this taxon), whereas *N. aquatica* is a member of the DO clade. *S. obliquus* is an autosporic taxon (i.e., no flagellate stage in its life cycle) phylogenetically affiliated at the nuclear rRNA sequence level with taxa within the DO clade (Wilcox et al. 1992; Steinkötter et al. 1994).

In all known discontinuous rRNAs, the break points are confined to the variable regions of the rRNA molecules (Gray and Schnare 1996). Figure 2I*b* and II*b* indicates the variable regions of the mitochondrial LSU and SSU rRNAs that are interrupted in both or only one of the two *Chlamydomonas* taxa, namely *C. reinhardtii* and *C. eugametos*. Letter designations were assigned to the smallest corresponding coding units that are continuous or missing in the mitochondrial rRNAs of both *Chlamydomonas* taxa; a–e and A–K refer to the SSU and LSU rRNAs, respectively, in the 5′–3′ transcriptional order of their counterparts in conventional rRNAs. Among the variable regions of the *Chlamydomonas* mitochondrial rRNAs, five are interrupted in both taxa, and seven are unique to either *C. reinhardtii* or *C. eugametos*. Denovan-Wright and Lee (1994) suggested that (1) some or all of the common breakpoints in corresponding variable regions in the two *Chlamydomonas* taxa were inherited from their last common ancestor, and (2) the unique breakpoints were derived independently after the divergence of the lineages leading to *C. reinhardtii* and *C. eugametos*. The interruption of additional variable regions in *C. reinhardtii* mitochondrial rRNAs relative to their *C. eugametos* counterparts resulted in an increase in the degree of fragmentation and a decrease in the coding capacity of the corresponding coding modules in *C. reinhardtii*. For example, the *C. eugametos* L_1 LSU rRNA coding module incorporates two adjacent coding units, i.e., B and C, whereas the *C. reinhardtii* mitochondrial L_1 LSU rRNA coding module consists only of coding unit B; in other words, the cod-

ing modules are not necessarily homologous between taxa, but the coding units are.

Nedelcu et al. (1996) provided evidence that discontinuous mitochondrial LSU rRNA is not a feature unique to *Chlamydomonas* taxa but, rather, a unifying characteristic for the CW and DO green algal taxa. The authors suggested a trend in the evolution of the chlorophycean mitochondrial LSU rRNAs, i.e., a gradual increase in the extent of discontinuity, from a continuous mitochondrial LSU rRNA molecule in *H. montana* to six and eight LSU rRNA fragments in *C. eugametos* and *C. reinhardtii,* respectively. Moreover, the presence of continuous mitochondrial LSU rRNAs in *H. montana,* a taxon that retained ancestral-like features relative to other chlorophycean lineages (Ettl and Moestrup 1980; O'Kelly, Watanabe, and Floyd 1994), indicated that discontinuous mitochondrial LSU rRNAs may have developed at or near the base of the Chlorophyceae.

The present study provides additional data supporting a gradual increase in the degree of fragmentation of the mitochondrial LSU as well as SSU rRNA coding regions among chlorophycean green algae. The oligonucleotide probes used here are complementary to highly conserved regions within the mitochondrial rRNA coding units A, B, C, D, F, J, and d as designated in figure 2I*b* and II*b*. Extending the suggestion made by Denovan-Wright and Lee (1994), i.e., that the common breakpoints in the mitochondrial rRNAs of *C. reinhardtii* and *C. eugametos* were inherited from their last common ancestor, I consider it most likely that the variable regions interrupted in both *Chlamydomonas* taxa as well as in *S. obliquus* were already interrupted in their most recent common ancestor. Note that the DO clade including *N. aquatica* and *S. obliquus* shares a common ancestor with the CW clade containing the *Chlamydomonas* taxa (Steinkötter et al. 1994). Consequently, the variable regions that are interrupted in both *C. reinhardtii* and *C. eugametos* as well as in *S. obliquus* mitochondrial rRNAs would most likely also be interrupted in *C. pulsatilla* and *N. aquatica.* Based on the size of the hybridizing mitochondrial rRNAs, the corresponding and the adjacent coding units in *C. reinhardtii* and *C. eugametos,* and the locations of variable regions likely to be interrupted, I suggest in figure 2I*c* and II*c* the coding units corresponding to the rRNA fragments identified in the species investigated. However, more detailed analyses have to be done to fully confirm these inferences. The identification of mitochondrial rRNA fragments corresponding to coding modules containing a higher number of coding units in *S. obliquus* (abcd, ABC, HIJK), *N. aquatica* (bcd, ABC, HIJ), and *C. pulsatilla* (cd, DE, HIJ) relative to *C. eugametos* (ab, cd, e, BC, DE, F, G, H, IJ) and *C. reinhardtii* (a, bc, d, e, B, C, D, E, F, G, HI, J) definitely suggests an evolutionary trend toward an increase in the degree of rRNA-coding-module fragmentation within the chlorophycean green algal group. The lower degree of fragmentation of mitochondrial rRNAs in *C. pulsatilla* compared to those of *C. reinhardtii* or *C. eugametos* (e.g., HIJ in *C. pulsatilla* compared to HI/J or H/IJ in *C. reinhardtii* or *C. eugametos,* respectively) indicates that this lineage may have diverged earlier than the most recent common ancestor of *C. reinhardtii* and *C. eugametos.*

The results presented here also suggest that some of the breakpoints (i.e., d/e, C/D, G/H) shared by the three *Chlamydomonas* taxa are older than the most recent common ancestor of this group. This observation contradicts the previous suggestion of Denovan-Wright and Lee (1994) that the processes of mitochondrial rRNA fragmentation began in the *Chlamydomonas* lineage after its divergence from other chlorophycean species with conventional rRNAs but before the last common ancestor of *C. reinhardtii* and *C. eugametos.* The analysis of the 3,885-nt mitochondrial DNA sequence of *S. obliquus* revealed that the mitochondrial LSU rRNA gene in this species is not only fragmented, as previously suggested (Nedelcu et al. 1996), but also scrambled. The two rRNA LSU coding regions identified through nucleotide sequence and secondary structure modeling comparisons consist of the coding modules ABC and HIJ, respectively, and are separated by a tRNA coding region. The internal part of the LSU rRNA gene corresponding to the D, E, F, and G coding units, which is highly conserved, functionally important, and ubiquitously present in various counterparts, is presumably present in the genome at another location. Both of the breakpoints within the mitochondrial LSU rRNA coding region of *S. obliquus* (i.e., C/D and G/H) are also shared by all three *Chlamydomonas* taxa. On the other hand, the mitochondrial LSU rRNA gene of *S. obliquus* contains the two coding units A and K, which are missing in *Chlamydomonas* but are present in *P. wickerhamii* and land plants. This finding suggests that these two coding units were present in the mitochondrial LSU rRNA gene in the common ancestor of the DO and CW lineages.

Discontinuous Then Scrambled Versus Discontinuous and Scrambled Mitochondrial rRNA Coding Regions

The mitochondrial rRNA genes of *C. reinhardtii* (Boer and Gray 1988) and *C. eugametos* (Denovan-Wright and Lee 1994) show different degrees and patterns of fragmentation and scrambling of the mitochondrial rRNA coding regions. Denovan-Wright et al. (1996) used the DERANGE program (Sankoff et al. 1992) to deduce the structure and organization of the mitochondrial rRNA coding regions in the last common ancestor of *C. reinhardtii* and *C. eugametos.* Denovan-Wright et al. assumed that the mitochondrial rRNA coding regions were altered to produce individual transcripts prior to the gene piece rearrangements that were limited to transpositional events. The model presented by the authors suggests that (1) in the last common ancestor of *C. reinhardtii* and *C. eugametos,* the mitochondrial rRNA coding regions were fragmented in gene pieces colinear with their counterparts in continuous rRNA genes with the exception of a small coding module situated upstream of the SSU rRNA gene; and (2) the fragmented and scrambled mitochondrial rRNA coding regions in this ancestor had evolved from already fairly fragmented but not scrambled rRNA coding regions in a single evolutionary step (i.e., one transpo-

sitional event). This model predicts that in taxa basal to the *Chlamydomonas* group, mitochondrial rRNA genes should be fragmented but not scrambled.

The lower degree of fragmentation of the mitochondrial rRNAs in *C. pulsatilla* relative to *C. reinhardtii* and *C. eugametos* suggests that *C. pulsatilla* might have diverged before the most recent common ancestor of these two *Chlamydomonas* species. Therefore, it would be of interest to find out whether the mitochondrial rRNA coding regions of *C. pulsatilla* are colinear with their counterparts in continuous genes or scrambled within the genome. However, the presence of fragmented and scrambled mitochondrial LSU rRNA coding regions in *S. obliquus,* an autosporic species phylogenetically related to zoosporic DO taxa (Steinkötter et al. 1994), indicates that scrambling may have already been present in the most recent common ancestor of the DO and CW lineages, much earlier than previously proposed (Denovan-Wright et al. 1996). The suggested low degree of fragmentation of the *S. obliquus* mitochondrial LSU rRNA gene, as well as the presence of features that are absent in *Chlamydomonas* but present in *P. wickerhamii* mitochondria (e.g., the 5′-end and 3′-end LSU rRNA regions), indicates that the mitochondrial LSU rRNA gene in this taxon represents an early stage in the evolution of discontinuous and scrambled mitochondrial rRNA genes within Chlorophyceae. It appears, therefore, that the mitochondrial rRNA genes in the chlorophycean green algae may have undergone rearrangements before they became highly fragmented. The presence of a tRNA gene between the two mitochondrial LSU rRNA coding regions in *S. obliquus* may be a consequence of a rearrangement event. Similar observations regarding the presence of tRNA or tRNA-like genes in the proximity of the endpoints of rearranged sequences in land plant and *Chlamydomonas* chloroplast genomes have led to the hypothesis that tRNA gene sequences may be implicated in gene shuffling (Boudreau and Turmel 1996 and references therein). In conclusion, the scrambling of the mitochondrial rRNA coding regions may have developed at an early stage in the evolution of chlorophycean mitochondrial rRNA genes, most likely in parallel with the fragmentation events.

Recombination as a Possible Mechanism Responsible for the Mitochondrial rRNA Gene Rearrangements Within Chlorophyceae

The mechanisms responsible for either the fragmentation or the scrambling of the mitochondrial rRNA coding regions in *Chlamydomonas* are not known yet, although several suggestions have been made. The GC-rich repeat clusters identified in *C. reinhardtii* mitochondrial DNA were suspected to have contributed to the extensive rRNA gene arrangements through a mechanism analogous to bacterial transposition (Boer and Gray 1991). Denovan-Wright and Lee (1994), however, assumed that the unusual gene structure in *Chlamydomonas* mitochondria arose from conventional, continuous rRNA genes by two separate, consecutive processes: the introduction of processing signals and the scrambling of coding regions defined by these signals. The absence of a reverse-transcriptase-like open reading frame in *C. eugametos* mitochondrial DNA led the authors to favor the view that the mitochondrial rRNA coding regions in *Chlamydomonas* became scrambled by recombination between nonhomologous regions of mitochondrial DNA molecules such as the dispersed repeated elements found in *C. reinhardtii* (Boer and Gray 1991) and *C. eugametos* (their unpublished results) rather than by reverse transcription (Boer and Gray 1988).

Recombination has also been invoked to explain the genomic rearrangements accounting for the complex mitochondrial genome structures of higher plants. It has been proposed that the frequency of homologous recombination is related to the presence of recombinogenic repeated sequences (Palmer and Shields 1984). Fauron et al. (1995) presented a multi-recombination model to illustrate the multipartite structure and the evolution of the maize mitochondrial genome through inter- and intramolecular recombination between two- or three-copy direct and inverted repeats. Intramolecular recombination between the members of a two-copy inverted repeat generates two isomeric forms that differ from each other only by the orientation of the sequence located between the repeats, whereas recombination within a two-copy direct repeat gives rise to two subgenomic circular DNA molecules, each of which contains one of the repeats and one of the single-copy regions between the repeats (fig. 4*A*).

The presence of scrambled but not highly fragmented mitochondrial LSU rRNA coding regions in *S. obliquus* suggests that scrambling may have developed at an early stage in the evolution of discontinuous and scrambled rRNA genes, probably complementing the fragmentation events. Considering this possibility, I propose a recombination event that would disrupt and scramble a coding region in a single step. This model is an extension of ones presented by Fauron et al. (1995) for the evolution of the maize mitochondrial genome and involves an intramolecular homologous recombinatorial event between two sets of two-copy inverted repeats. Generally, such an event would result in an interexchange of the sequences situated between the two sets of inverted repeats (fig. 4*B*). Figure 4*C* illustrates how recombination events such as the one suggested above, as well as those proposed by Fauron et al. (1995), could have been involved in the evolution of chlorophycean mitochondrial rRNA genes. The model requires that short inverted repeat sequences be present within the variable regions of an rRNA gene as well as flanking a non-rRNA coding region; in addition, the two coding regions to be exchanged must be present in oppositely oriented transcriptional units. In this way the rRNA gene becomes fragmented and scrambled simultaneously, and the exchanged coding regions could be incorporated within the opposite transcriptional unit and transcribed accordingly. The rRNA coding module transferred into a new transcriptional unit could be released from the new polycistronic transcript following the processing of the adjacent protein-coding or tRNA sequences from the transcript, and could subsequently interact by intermo-

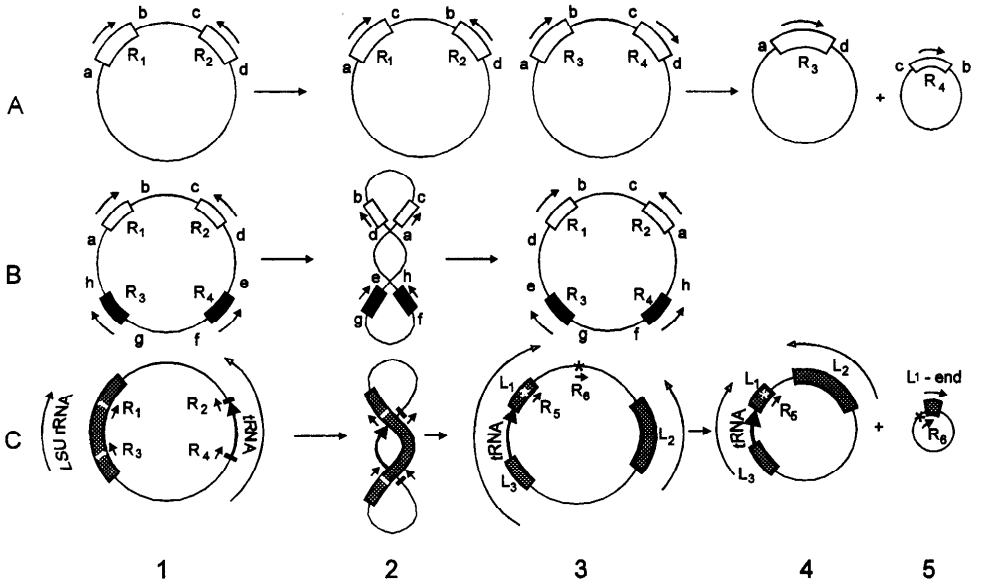

FIG. 4.—*A*, Recombination events between two-copy inverted (R_1/R_2) and direct (R_3/R_4) repeats, respectively, as proposed by Fauron et al. (1995); a, b, c, and d are sequences flanking the repeats. *B*, Recombination event between two sets of two-copy inverted repeats (R_1/R_2 and R_3/R_4) resulting in an interexchange of the sequences between the repeats; a, b, c, d, e, f, g, and h are sequences flanking the repeats. *C*, Hypothetical recombination events responsible for the fragmentation, scrambling, and excision of mitochondrial rRNA coding regions within a chlorophycean mitochondrial genome. Two sets of short inverted repeats, i.e., R_1/R_2 and R_3/R_4 (small blocks), present within the variable regions of an LSU rRNA gene (grey block) and flanking a tRNA gene (solid arrow), respectively (step 1), recombine simultaneously (step 2) and generate a new molecule in which the sequences between the two sets of repeats are interexchanged (step 3). Note that the LSU rRNA and tRNA genes belong to transcription units transcribed in opposite directions. A recombination event between a two-copy direct repeat, i.e., R_5 and R_6 (stars), one of which is situated within the L_1 LSU rRNA coding region (step 4), would result in the excision of the sequences between the repeats, including a small LSU rRNA coding region (step 5).

lecular base pairing with the other two rRNA fragments to restore the conserved rRNA secondary structure.

Such a scenario has the advantage that an rRNA gene would become fragmented and scrambled as a result of a single rearrangement event; there is no need to suggest two distinct mechanisms to explain the observed rearrangements. In addition, by proposing recombination as a mechanism involved in the evolution of mitochondrial rRNA coding regions within the chlorophycean group, not only the fragmentation and scrambling of the rRNA coding region but also the loss of rRNA and non-rRNA coding regions during the evolution of the mitochondrial genome of green algae could be explained.

Short direct repeated sequences have been reported in the plant mitochondrial genome and shown to be involved in intramolecular recombination events (Hartman et al. 1994 and references therein; Benslimane et al. 1996). Short GC-rich repeat clusters have already been described in the *C. reinhardtii* mitochondrial genome and suggested to be reminiscent of the *Pst* I palindromes in *Neurospora crassa* mitochondrial DNA or analogous to the GC clusters in *Saccharomyces cerevisiae* mitochondrial DNA (Boer and Gray 1991 and references therein). I have analyzed the mitochondrial DNA sequence of *C. eugametos* mitochondrial DNA (E. M. Denovan-Wright, personal communication) and identified a similar but larger and more complex set of GC-rich direct and inverted repeats within spacer regions as well as introns (data to be presented and discussed elsewhere). Likewise, in the intergenic spacers between the tRNA gene and the LSU rRNA coding regions of the *S. obliquus* mitochondrial DNA sequence analyzed, I have identified remnants of the complex AT-rich repetitive motifs flanking the tRNA genes in the mitochondrial genome of *P. wickerhamii* (Wolff et al. 1994), as well as GC-rich short inverted repeats similar to those present in the mitochondrial genome of *C. reinhardtii*. Comparisons among the locations of these elements within the mitochondrial genome of *C. reinhardtii, C. eugametos* and the available sequence of *S. obliquus* revealed similarities regarding the positions of these repeats relative to the rRNA coding units within the respective genomes (see fig. 5). I therefore suspect that the fragmented and scrambled mitochondrial rRNA coding regions in the chlorophycean green algal group may have been generated through multiple recombination events triggered by the accumulation of short repeated sequences within the variable regions of the rRNA genes and the intergenic spacers of these mitochondrial genomes. Recombination events between short dispersed repeats have also been proposed to account for the various rearrangements described in the *Chlamydomonas* chloroplast genome (discussed by Boudreau and Turmel 1996).

To illustrate how recombination events similar to those presented here could be entirely responsible for the extensive mitochondrial rRNA gene rearrangements, a hypothetical pathway to gradually convert conventional continuous mitochondrial rRNA genes to the rRNA gene arrangement described in *C. eugametos* is presented in figure 5. Fragmentation patterns similar to those

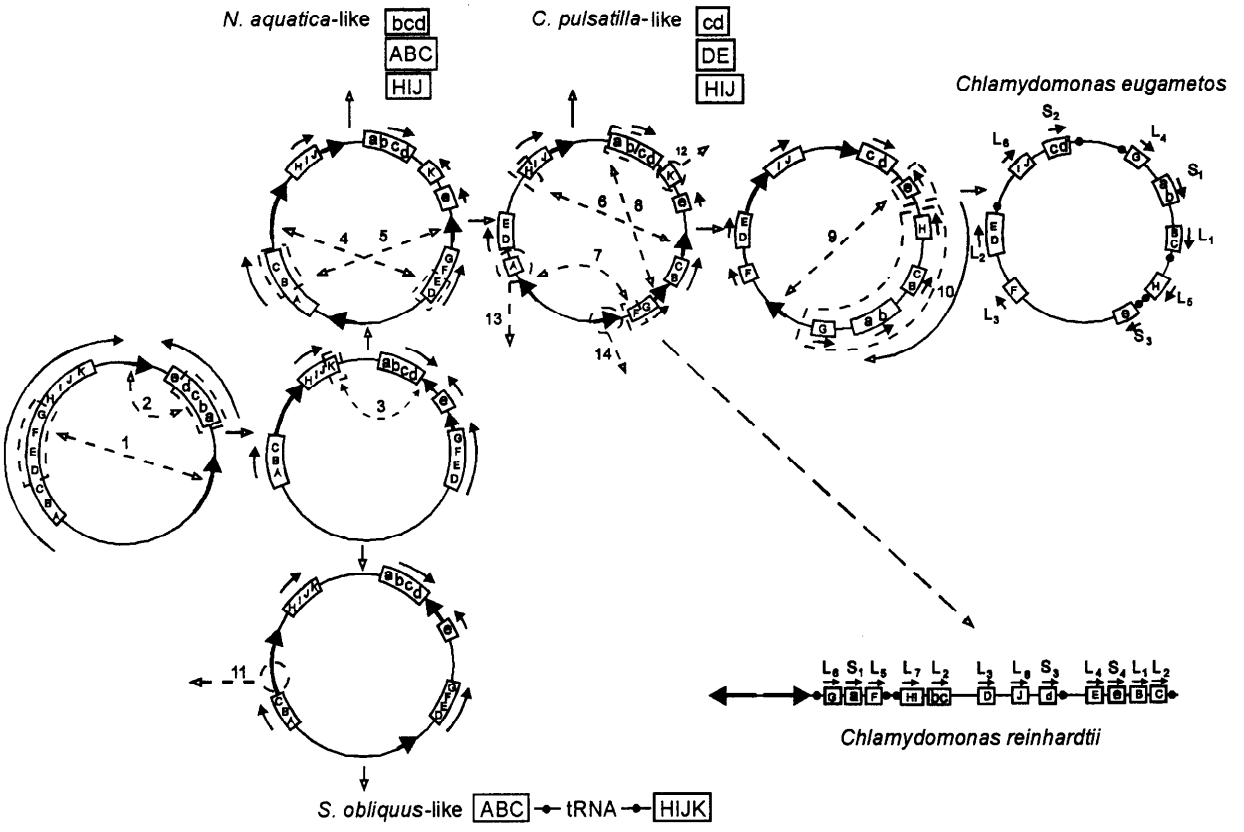

FIG. 5.—Hypothetical pathway from conventional continuous mitochondrial rRNA genes to the gene arrangement described in the *C. eugametos* mitochondrial genome, and the locations of short repeated sequences within the *C. eugametos, C. reinhardtii* and *S. obliquus* mitochondrial DNA. Diagrams are not drawn to scale. Letters indicate rRNA coding units (a–e refer to SSU rRNA, and A–K refer to LSU rRNA); blocked letters indicate a coding module. Thick solid arrows on the circles designate non-rDNA sequences (i.e., tRNA- or protein-coding genes) and indicate the transcriptional direction of that sequence. Thin solid arrows outside the circle indicate the transcriptional direction of that region. Interrupted arrows indicate recombination events as follows: 1 to 9 are recombination events between two sets of two-copy inverted repeats resulting in interexchanges of sequences from opposite transcriptional units; 10 is a recombination event between one set of a two-copy inverted repeat resulting in the changing of the transcriptional orientation of the regions flanked by the repeats; 11 to 14 are recombinational events between one set of two-copy direct repeats followed by the excision of small subgenomic circles containing the sequence situated between the repeats. Small solid circles on the *C. reinhardtii, C. eugametos,* and *S. obliquus* gene maps denote the position of intergenic clusters of repeats.

suggested for *S. obliquus, N. aquatica,* and *C. pulsatilla* were incorporated into the pathway. Note that the pathway is not necessarily the most parsimonious solution and is not intended to suggest the succession of events or the ancestral genomic organization of any of the taxa indicated at the termini of the pathway. The scenario also shows that recombination events could have been involved not only in the rRNA gene rearrangements but also in the removal of some rRNA as well as non-rRNA coding regions to the extent observed in the mitochondrial genomes of *C. reinhardtii* and *C. eugametos.*

The hypothetical pathway presented in figure 5 starts from a circular mitochondrial genome as described for all the green algal lineages investigated to date except *C. reinhardtii.* In addition, in this presumptive ancestral mitochondrial genome the genes are considered to have been organized in more than one transcriptional unit, as reported in the primitive prasynophyte green alga *Platymonas subcordiformis* (Kessler and Zetsche 1995), and the SSU and LSU rRNA genes are assumed to have been transcribed in opposite directions, as described in *P. wickerhamii* (Wolff et al. 1994). The rRNA-coding units and tRNA- or protein-coding genes to be interexchanged are always from opposite transcriptional units. The presence of more than one gene copy for the rRNA genes was not taken into account since all of the green algal mitochondrial genomes investigated to date encode single copies of all their genes. This scenario shows that, starting from a circular genome containing more than one transcriptional unit and continuous conventional LSU rRNA and SSU rRNA genes transcribed in opposite directions, a genome organization similar to that of *C. eugametos* (i.e., nine rRNA coding modules scrambled along the genome and transcribed in one direction) can be reached through a series of 12 recombination events between two sets of two-copy inverted repeats (9 events), one set of two-copy direct repeats (2 events), and one set of a two-copy inverted repeat (1 event).

The phylogenetic position, i.e., in both CW and DO chlorophycean lineages, of the taxa examined in this study allows one to speculate that evolutionary changes in rRNA gene organization started around the chlorophycean divergence from the pool of ancestral green fla-

gellate algae and have continued since. These changes may have involved both a gradual fragmentation and loss of mitochondrial rRNA coding regions as well as a decrease in size and coding capacity of the entire mitochondrial genome to the dramatic extents observed in *C. reinhardtii* and *C. eugametos*. To further argue for such a scenario, more data on mitochondrial genomes of other chlorophycean green algae have to become available.

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