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Evolution of Fragmented Mitochondrial Ribosomal RNA Genes in *Chlamydomonas*

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Abstract. The fragmented mitochondrial ribosomal RNAs (rRNAs) of the green algae *Chlamydomonas eugametos* and *Chlamydomonas reinhardtii* are discontinuously encoded in subgenic modules that are scrambled in order and interspersed with protein coding and tRNA genes. The mitochondrial rRNA genes of these two algae differ, however, in both the distribution and organization of rRNA coding information within their respective genomes. The objectives of this study were (1) to examine the phylogenetic relationships between the mitochondrial rRNA gene sequences of *C. eugametos* and *C. reinhardtii* and those of the conventional mitochondrial rRNA genes of the green alga, *Prototheca wickerhamii*, and land plants and (2) to attempt to deduce the evolutionary pathways that gave rise to the unusual mitochondrial rRNA gene structures in the genus *Chlamydomonas.* Although phylogenetic analysis revealed an affiliation between the mitochondrial rRNA gene sequences of the two *Chlamydomonas* taxa to the exclusion of all other mitochondrial rRNA gene sequences tested, no specific affiliation was noted between the *Chlamydomonas* sequences and *P. wickerhamii* or land plants. 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* mitochondrial rRNA genes, as well as a limited survey of the size of mitochondrial rRNAs in other members of the genus, lead us to propose that the last common ancestor of *Chlamydomonas* algae contained fragmented mitochondrial rRNA genes that were nearly co-linear with conventional rRNA genes.

Key words: Evolution — *Chlamydomonas* — Mitochondria — Ribosomal RNA genes

Introduction

There are many known examples of fragmented small subunit (SSU) and large subunit (LSU) rRNA molecules contained within eubacterial, organellar, and eukaryotic cytosolic ribosomes (Gray and Schnare 1995). The complexes that such rRNAs form in ribosomes are apparently structurally and functionally equivalent to the single continuous polynucleotide chains found in more conventional ribosomes. For all but a few of the genes that encode discontinuous rRNAs, the transcriptional order of the coding regions is in the standard 5′ to 3′ order of the homologous regions in continuous rRNA genes. Examples of discontinuous rRNA genes where the order of the gene pieces deviates from this convention are so far limited to the mitochondrial DNAs (mtDNA) of the ciliated protist, *Tetrahymena pyriformis* (Schnare et al. 1986; Heinonen et al. 1987), the green algae, *Chlamydomonas reinhardtii* and *Chlamydomonas eugametos* (Boer and Gray 1988; Denovan-Wright and Lee 1994), and the putative mtDNAs in the apicomplexans, *Plasmodium falciparum* (Vaidya et al. 1989; Feagin et al. 1992) and *Theileria parva* (Kairo et al. 1994). Although nothing is

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known about the processes that result in such discontinuous and scrambled rRNA coding regions, it does seem likely that they are secondarily derived from conventional continuous rRNA gene sequences (Gray and Schnare 1995).

The genus *Chlamydomonas* includes an evolutionarily diverse collection of readily available and easily cultured taxa whose phylogenetic relationships are becoming increasingly resolved. These features make the chlamydomonads useful organisms in which to study the evolution of discontinuous and scrambled mitochondrial rRNA genes. Molecular and morphological data suggest that most *Chlamydomonas* species are organized into two main sister lineages—one includes *C. eugametos* and the other includes *C. reinhardtii.* Data on chloroplast LSU and nuclear SSU rRNA sequence divergence suggest that the evolutionary distance between *C. eugametos* and *C. reinhardtii* is equivalent to or greater than that of vascular and nonvascular plants (Buchheim et al. 1990; Jupe et al. 1988; Turmel et al. 1993, 1995).

The rRNA coding regions of *C. eugametos* and *C. reinhardtii* mitochondrial DNA are highly fragmented, extensively scrambled, and interspersed with protein coding and transfer RNA genes (Boer and Gray 1988; Denovan-Wright and Lee 1992, 1994). Although the points of discontinuity in the mitochondrial rRNAs are confined to recognized variable regions of the SSU and LSU secondary structures, several variable regions are interrupted in one species but not the other. Furthermore, the order of the gene pieces is very different in the two taxa. Based on these results, it was proposed that the last common ancestor of *C. eugametos* and *C. reinhardtii* had discontinuous mitochondrial rRNA genes, although it was not possible to decide if the gene pieces in this ancestor were scrambled or in the conventional 5′ to 3′ order. Moreover, it was concluded that further division and the scrambling of these coding regions had occurred since the divergence of *C. eugametos* and *C. reinhardtii* (Denovan-Wright and Lee 1994). It was not possible on the basis of this previous work to estimate the specific number or order of events that led to the mitochondrial rRNA gene arrangements in these algae because of the considerable differences in their rRNA gene structure and organization.

The evolutionary association of the *C. eugametos* and *C. reinhardtii* mitochondrial genomes is supported by similarities in their mitochondrial genome coding functions, by similarities in their mitochondrial rRNA primary and secondary structures, and by their shared feature of discontinuous and scrambled mitochondrial rRNA coding regions (Boer and Gray 1988; Denovan-Wright and Lee 1992, 1994). However, Southern analysis of the *C. eugametos* and *C. reinhardtii* mitochondrial rRNA genes and northern analysis of their transcripts using probes derived from the two *Chlamydomonas* mtDNAs indicate that there has been considerable divergence between these sequences (Denovan-Wright and Lee 1992, 1994). In the present study, therefore, we investigated whether the mitochondrial SSU and LSU rRNA gene sequences of *C. eugametos* affiliate with those of *C. reinhardtii* in mitochondrial–eubacterial rRNA gene trees. Such an observation would support proposals for the derivation of the discontinuous and scrambled rRNA gene organization of both *C. eugametos* and *C. reinhardtii* from a common ancestor. The addition of the *C. eugametos* mitochondrial rRNA gene sequences to the SSU and LSU rRNA gene databases could also help resolve the apparent anomalous branching position of the *C. reinhardtii* mitochondrial rRNA genes in phylogenetic trees (Gray et al. 1989).

In an attempt to deduce the structure and organization of mitochondrial rRNA coding regions in the last common ancestor of *C. eugametos* and *C. reinhardtii*, we used the DERANGE program of Sankoff et al. (1992) to identify the midpoint region of the minimum rearrangement pathway necessary to interconvert the two gene piece orders. Using this same program, we also determined the minimum number of rearrangement steps necessary to generate the observed mitochondrial rRNA gene organizations of these taxa from a hypothetical ancestral rRNA gene structure that is co-linear with eubacterial rRNA genes. Using addition/deletion and gene rearrangement differences, the DERANGE program has been used to determine the evolutionary relationships between fungal and other eukaryotic mitochondrial genomes (Sankoff et al. 1992); such affiliations agree well with those determined by phylogenetic analysis of mitochondrial gene sequences. Therefore, it seemed reasonable that similar analyses could be used to formulate testable hypotheses about the organization of the ancestral mitochondrial rRNA genes of *C. eugametos* and *C. reinhardtii* and the evolutionary pathways that could have generated the extant rRNA gene piece arrangements in these algae.

As a complement to and preliminary test of models generated by the above approach, we estimated the position of discontinuities in the mitochondrial LSU rRNA in other evolutionarily diverse *Chlamydomonas* taxa by employing northern blot analysis of total cellular RNA with oligonucleotide probes complementary to conserved regions of the 3′ half of *Chlamydomonas* and *Scenedesmus* mitochondrial LSU rRNAs.

Materials and Methods

Strains and Culture Conditions. A wild-type strain of *Chlamydomonas eugametos* (UTEX 9) was 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 18.72) were obtained from Sammlung von Algenkulturen, Universität Göttingen. Wild-type *Chlamydomonas reinhardtii* mt^+ (CC-125) came from the Duke University culture collection. All liquid cultures were

grown in minimal medium as described by Lemieux et al. (1980) and the cells were harvested 6 h after the onset of the light period (L-6) when cell densities were approximately 4×10^6 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 (Denovan-Wright and Lee 1994). All solutions and glassware were treated with DEPC (diethylpyrocarbonate) to minimize RNase activity (Maniatis et al. 1982).

Northern Hybridization. Three oligonucleotides were used as hybridization probes. Probe 1 was an 18-nt oligomer (5′ CAT AGG GTC TCT TCG TCC 3′) and probe 3 was a 29-nt oligomer (5′ AGG ACG CGA TGA TCC AAC ATC GAG GTG CC 3′); both of these probes are complementary to different portions of the peptidyl transferase center of both *C. eugametos* and *C. reinhardtii* mitochondrial LSU rRNA. Using the numbering of the *Escherichia coli* 23S rRNA, probe 1 and probe 3 are complementary to nucleotides 2052–2069 and 2494–2522, respectively. Probe 2 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 center of the mitochondrial LSU rRNA of *Scenedesmus obliquus* (Kück et al. 1990); the *E. coli* coordinates of this region are 2438–2464. The relative positions of these peptidyltransferase-center-specific probes are shown in Fig. 2. Filters of immobilized total cellular RNA from each species within the study group were prehybridized in 5× SSPE (1 × SSPE = 180 mM NaCl, 10 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.4), 5× Denhardt's solution (1× Denhardt's solution = 0.1% w/v polyvinylpyrrolidone, 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-labeled with [γ-^{32}P]ATP(6,000 Ci/mM) and T4 polynucleotide kinase (Pharmacia). The hybridized filters were washed twice in 5× SSPE for 15 min, twice in 2× SSPE for 15 min, and once in 2× SSPE/0.1% SDS for 30 min; washes were performed at room temperature.

Phylogenetic Analysis of rRNA Gene Sequences. SSU and LSU rRNA gene sequences were aligned using secondary-structure models as guides, and the resulting alignments, representing the conserved cores of the genes, were used in pair-wise comparisons and phylogenetic treeing analyses. The database for the SSU and LSU rRNA alignments included 1,002 and 1,093 nucleotide positions, respectively. The SSU and LSU databases were comprised of 108 and 25 taxa, respectively. The more comprehensive SSU rRNA databases included representatives of all eubacterial groups and all published nonmetazoan and nonkinetoplast mitochondria as well as several chloroplast SSU gene sequences. (A list of the specific taxa used is available—contact the authors). Bootstrapped distance trees (100 replicates) for SSU and LSU rRNA gene sequences were generated using the SEQBOOT, DNADIST (''ML'' option), NEIGHBOR, and CONSENSE programs from PHYLIP version 3.53c (Felsenstein 1993). Parsimony analysis was performed using the DNAPARS program from PHYLIP. The LSU data set was also analyzed using PHYLIP's DNAML with default parameters. The rRNA gene sequences of *Thermus thermotoga* was chosen as an arbitrary outgroup for each analysis.

Calculation of Minimal Transpositional 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 1). The computer program DERANGE (Sankoff et al. 1992) was used to calculate the minimum number of rearrangements necessary to convert one gene piece organization to another. For this analysis, there was no use of addition/deletion changes or of inversions; genomic rearrangements were limited to transpositional events.

Table 1. Distribution of homologous coding information in the *C. eugametos* and *C. reinhardtii* mitochondrial rRNA gene pieces

C. eugametos		*C. reinhardtii*	
Gene piece[a]	Coding information[b]	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

[a] The gene pieces of the SSU and LSU have been numbered sequentially in the 5′ to 3′ order in which homologous sequences appear in conventional rRNA genes (Boer and Gray 1988; Denovan-Wright and Lee 1994)

[b] The rRNA coding information has been designated by capital letters to indicate the information between any two breakpoints in comparisons of the mitochondrial rRNA of *C. eugametos* and *C. reinhardtii.* The lettered blocks of coding information using the coordinates of the *E. coli* 16S (A–E) and 23S rRNA (F–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

Results and Discussion

Phylogenetic Analysis of Mitochondrial rRNA Genes

A primary goal of the work presented here was to determine if the mitochondrial rRNA sequences of *C. eugametos* and *C. reinhardtii* affiliate with each other on phylogenetic trees. These sequences can both be fitted to standard secondary-structure models for rRNAs. The gaps in the sequences that represent the discontinuities within these rRNAs occur in variable regions which are not incorporated into the databases and, therefore, have no effect on the phylogenetic analysis. All methods of analysis employed show that, despite the considerable primary sequence divergence between them, *C. eugametos* and *C. reinhardtii* mitochondrial rRNA gene sequences consistently affiliate with each other to the exclusion of other mitochondrial or eubacterial rRNA genes. Bootstrap analyses of the distance trees (Fig. 1) imply that this affiliation is meaningful. Moreover, the affiliation of the mitochondrial rRNA genes of the two *Chlamydomonas* taxa is not strongly influenced by base composition, as the GC contents of the *C. reinhardtii*

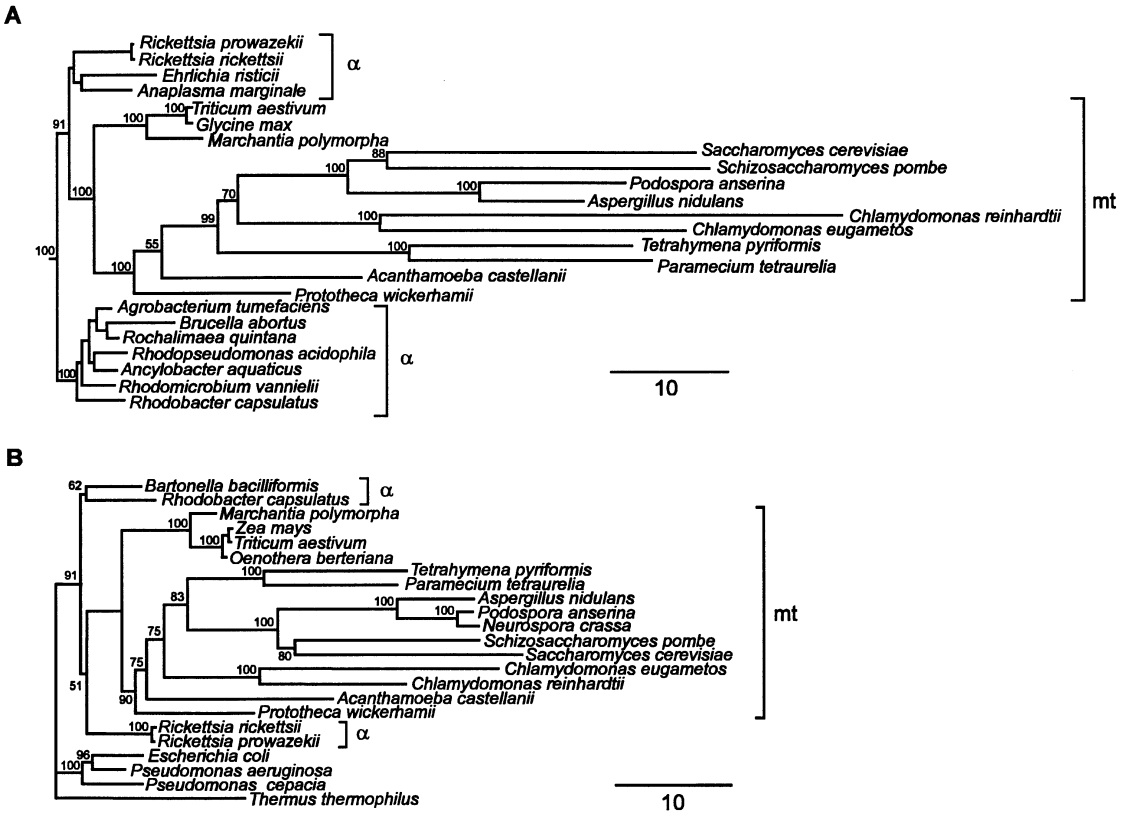

Fig. 1. Neighbor-joining phylogenetic analyses of ribosomal RNA gene sequences. **A** SSU rRNA gene tree representing the mitochondrial and α-proteobacterial section of a larger tree containing 108 taxa including eubacterial, chloroplast, and mitochondrial sequences. **B** LSU rRNA gene tree of mitochondrial and eubacterial sequences. The mitochondrial (*mt*) and α-proteobacterial (α) sequences are *bracketed. Numbers* at the nodes connecting the branches are bootstrap values. The *bars* represent 10 substitutions per 100 nucleotides.

mitochondrial SSU and LSU rRNA sequence regions used in this data analysis, 49.7 and 50.5%, respectively, are similar to the mitochondrial counterparts of plants (52–56%), while those of *C. eugametos* at 39.9 and 43.5%, respectively, are closer to the values of the mitochondrial counterparts of fungi, yeast, and ciliates (32–44%).

The addition of *C. eugametos* and several other sequences to the database did not alter the general topology of mitochondrial phylogenetic trees that have been observed previously (Gray et al. 1989). In agreement with these earlier reports, the mitochondrial rRNA-based phylogenetic trees consistently showed a fundamental split. The more conservative (less divergent) land plant sequences affiliate into one group while the sequences of all other taxa (including the chlorophytes) form a second group characterized by long individual branch lengths with relatively short internodal distances. Neighbor-joining (distance) (Fig. 1A)- and parsimony (data not shown)-based phylogenetic trees constructed using the more comprehensive SSU rRNA gene database support the hypothesis (Yang et al. 1985; Gray et al. 1989; Wolff and Kück 1990; Lonergan and Gray 1994) that all mitochondrial SSU rRNA genes descended from a common ancestor originating within the α-proteobacteria. As can be seen in the phylogenetic analysis presented in Fig. 1A, the corrected number of substitutions (as inferred from the branch lengths) for taxa in the second, rapidly evolving group, including ciliates, fungi, yeast and *Chlamydomonas*, is severalfold higher than the accumulated substitutions in the mitochondrial SSU rRNA gene sequences of land plants. Analysis of LSU rRNA gene sequences yielded similar results (Fig. 1B).

The group with rapidly evolving mitochondrial rRNA genes includes *Prototheca wickerhamii*, the only other complete green algal mitochondrial rRNA gene sequence available. However, there is no specific affiliation between the *Chlamydomonas* sequences and those of *P. wickerhamii* in SSU (Fig. 1A) and LSU (Fig. 1B) rRNA gene-distance-based trees. Closer apparent affiliations were observed between the *Chlamydomonas* pair and the yeast, fungal, and ciliate groups, although the specific affiliations with these groups do not appear significant because SSU and LSU distance trees are not consistent, nor was there consistency between treeing methods. This conclusion is further supported by the low bootstrap values associated with the node connecting the *Chlamydomonas* rRNA gene sequences to those of other taxa within the yeast/fungal/ciliate group in SSU and LSU rRNA gene neighbor-joining trees (Fig. 1).

The long branches observed for most mitochondrial taxa are an indication that the number of transitional

substitutions has saturated, a condition that can lead to branching artifacts. To test whether the fungal, yeast, ciliate, and *Chlamydomonas* mitochondrial rRNA genes were clustering because of such treeing artifacts, neighbor-joining trees were constructed using simple transversional dissimilarity matrices. However, the resulting SSU and LSU distance trees (not shown) were nearly identical in branching pattern to those based on nucleotide substitutions (Fig. 1) with the exception that the relative position of *A. castellanii* and *P. wickerhamii* changed between the two SSU distance-based trees. Moreover, 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 thus the separation of *C. reinhardtii* from the metaphytes) were not due to the ''long-branch length attracts'' artifacts (Gray et al. 1989) to which parsimony analyses are sensitive.

If mitochondria and chloroplasts each arose by single endosymbiotic events, the phylogenetic trees based on nucleus-, plastid-, and mitochondrial-encoded rRNA sequences should be parallel (Gray and Doolittle 1982; Gray 1989, 1992). This clearly is not observed. The affiliation of green algae (chlorophytes) and land plants, to the exclusion of other eukaryotes, is supported by nucleus (Sogin et al. 1986; Gunderson et al. 1987; Hendricks et al. 1991) and plastid (Cedergren et al. 1988) encoded rRNA sequence data and other biochemical, cytological, and molecular information (Chapman and Ragan 1980; Mattox and Stewart 1984). It continues to be perplexing, therefore, that the mitochondrial rRNA sequences of *Chlamydomonas* do not affiliate in phylogenetic trees with those of land plant mitochondria (Gray et al. 1989). Additional mitochondrial rRNA gene sequences from the other members of the Chlorophyta must be added to the databases before the apparent discrepancy between the mitochondrial rRNA gene trees and those of the nucleus- and plastid-encoded counterparts is likely to be resolved.

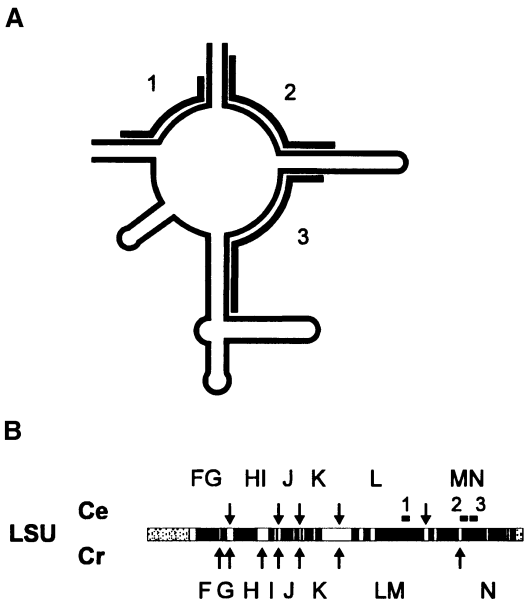

Fig. 2. Relative positions of the peptidyl-transferase-center-specific oligonucleotide probes used to detect *Chlamydomonas* mitochondrial LSU rRNAs. **A** Skeleton drawing of the peptidyl transferase center within the 3′ half of LSU rRNA secondary structure showing the relative positions of probes 1, 2, and 3. **B** Relationship between the discontinuities in the mitochondrial LSU rRNAs of *C. eugametos* (*Ce*) and *C. reinhardtii* (*Cr*) relative to the conserved (*solid*) and variable (*open*) rRNA regions described by Gray and Schnare (1995), drawn to the scale of the *Escherichia coli* 23S (*LSU*) rRNA; the relative positions of the oligonucleotide probes within a linear representation of the *E. coli* LSU rRNA are indicated. The positions of the points of discontinuity observed in the *C. eugametos* and *E. reinhardtii* LSU rRNA are indicated by *arrows.* Homologous coding regions are indicated by the *letters F–N. Stippled regions* represent the terminal nucleotides missing from the *Chlamydomonas* mitochondrial LSU rRNAs relative to the *E. coli* LSU rRNA.

Calculation of the Minimal Transpositional Distance Between Mitochondrial rRNA Gene Piece Orders in C. eugametos *and* C. reinhardtii

The mitochondrial rRNA genes in the last common ancestor of *C. eugametos* and *C. reinhardtii* likely shared breakpoints currently observed in the mitochondrial rRNA of these taxa (Denovan-Wright and Lee 1994). These ancestral genes could have had a scrambled structure or one that was co-linear with conventional rRNA genes. The DERANGE program of Sankoff et al. (1992) was employed to measure the minimal number of transpositions required to convert the mitochondrial rRNA gene organization of *C. eugametos* to that of *C. reinhardtii* and to compute the minimal transpositional distance of the *C. eugametos* and *C. reinhardtii* patterns from an rRNA coding structure that is co-linear with eubacterial genes. The discontinuities in the mitochondrial rRNA coding regions of *C. eugametos* and *C. reinhardtii* are found exclusively in variable rRNA domains; some of these sites are in corresponding variable regions in the two taxa while several variable rRNA regions are interrupted in one taxon but not the other (Denovan-Wright and Lee 1994). To compare the arrangement of mitochondrial rRNA coding information, homologous sections of these regions reflecting the smallest uninterrupted coding regions in either or both taxa were designated by the same letter; A–E and F–N represent the SSU and LSU rRNA homologous coding regions, respectively, and are designated in alphabetical order according to the 5′ to 3′ order in which their transcripts appear in secondary-structure models (see Table 1 and Fig. 2). Based on the observations that the *C. reinhardtii* and *C. eugametos* rRNAs are all encoded from the same DNA strand, it is assumed that inversions of mitochondrial rRNA gene pieces are incompatible with function. Moreover, *C. eugametos* and *C. reinhardtii* each encodes only one copy per unit genome of the same conserved mitochondrial rRNA core and therefore addi-

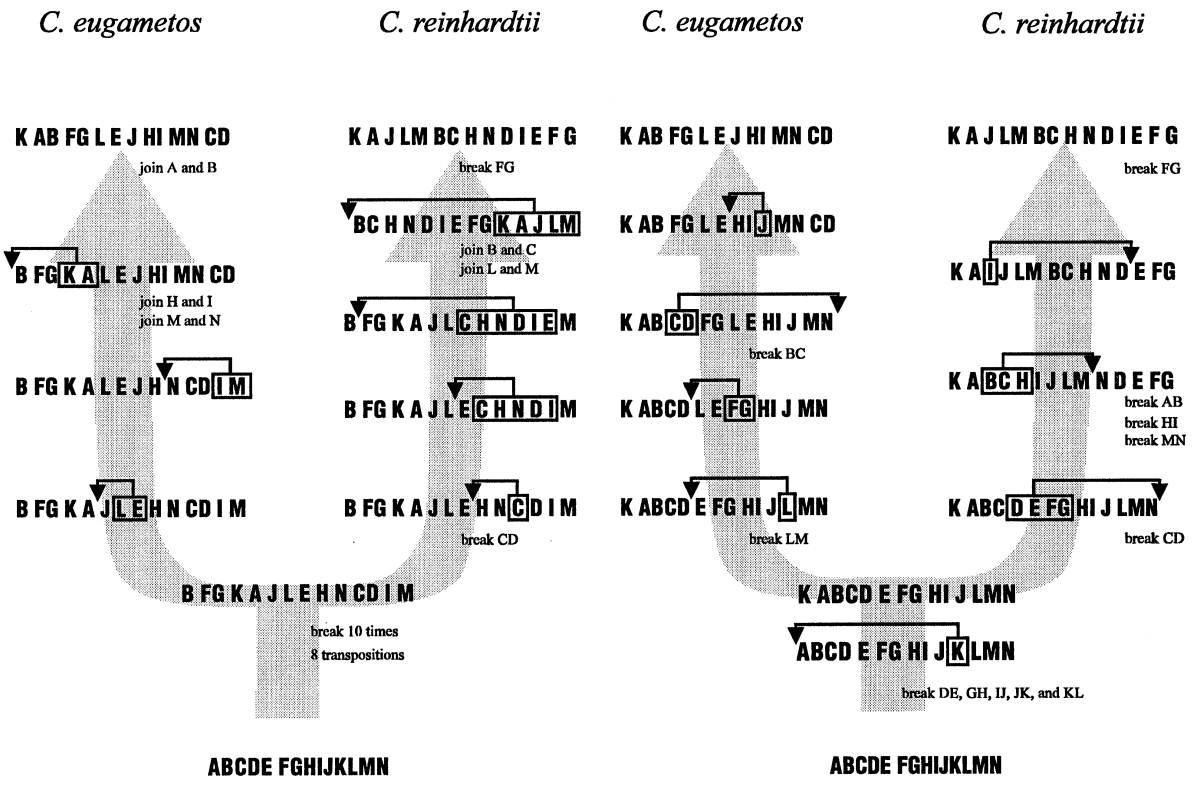

Fig. 3. Models for the origin of the *C. eugametos* and *C. reinhardtii* mitochondrial rRNA gene piece organization from a last common ancestor with **A** a highly scrambled gene piece structure or **B** a structure that was nearly co-linear with *E. coli* rRNA genes and that contained the SSU rRNA gene encoded upstream of the LSU rRNA gene. Homologous sections of the *C. eugametos* and *C. reinhardtii* mitochondrial SSU (*A–E*) and LSU (*F–N*) rRNA coding regions are designated by the same letter (see Table 1 and Fig. 2). Individual letters or groups of letters represent gene pieces that encode single transcripts. 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 encode 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 minimal transpositional distances between the observed gene orders; alternate pathways involving a different order of the same transpositional events are possible.

tion/deletion differences were not considered. The rearrangement of mitochondrial rRNA gene pieces in these computations was therefore limited to transpositional events. In the evolutionary pathways generated, rRNA coding domains that were altered from the conventional 5′ to 3′ order were assumed to encode separate rRNA transcripts because there are no reported examples where conserved rRNA sequences within one continuous rRNA molecule are not in the usual 5′ to 3′ order. It is further assumed that coding regions were altered to produce individual transcripts prior to the rearrangement of the gene pieces from their conventional order. The introduction of new break points within SSU or LSU rRNA coding regions is assumed to be compatible with function because the resulting individual transcripts could function in composite rRNA structures. Following these rules, we liberally introduced or removed breakpoints without penalty in this analysis. Finally, this analysis was simplified by linearizing the circular *C. eugametos* mitochondrial gene map at a point which minimized the number of transpositions needed to interconvert the two rRNA gene orders.

In calculating the minimum number of transpositional events that could directly convert the mitochondrial rRNA gene piece order of *C. eugametos* to that of *C. reinhardtii*, the program revealed a pathway involving seven transpositions (Fig. 3A, model A). Assuming that the midpoint region in this pathway approximates the rRNA gene piece order of their last common ancestor, the hypothetical ancestral mitochondrial rRNA genes would have been equally or more fragmented and scrambled than is the present case for either taxon. According to this model, the ancestral gene would have been altered by subdivision of some rRNA gene pieces and the rearrangement and reassembly of other dispersed gene pieces into larger rRNA gene pieces during the evolution of both the *C. eugametos* and *C. reinhardtii* mitochondrial rRNA patterns.

Calculations performed to find a minimal transpositional distance from rRNA coding structures that are dis-

continuous but co-linear with typical eubacterial rRNA gene arrangements (Gray and Schnare 1995) revealed a minimum pathway requiring five transpositions to convert this hypothetical ancestral pattern to that of *C. eugametos* and four to that of *C. reinhardtii* (Fig. 3B, model B). 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 last common ancestor of these two taxa 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 orders from this ancestor would be seven. Using this approach, therefore, the DERANGE program revealed a second pathway that could interconvert the mitochondrial rRNA gene piece organization of *C. eugametos* and *C. reinhardtii* in seven steps.

Although the transpositional pathways which generate the two extant *Chlamydomonas* mitochondrial rRNA gene patterns from the hypothetical last common ancestors depicted in models A and B require the same number of transpositions, we favor model B because any ancestral *Chlamydomonas* rRNA gene structure was ultimately derived from the protogenote-type continuous rRNA genes (Gray and Schnare 1995) and, in total, far fewer transpositional events are necessary with this model to generate the *C. eugametos* or *C. reinhardtii* mitochondrial rRNA gene organization from such an ancestor. Moreover, in this evolutionary pathway, ancestral rRNA gene pieces have been subdivided or remained the same; there is no rejoining of rRNA coding regions as depicted in model A. The latter would require the precise realignment of appropriate gene pieces and the loss of processing sites leading to the division of the RNA.

Identification of Mitochondrial LSU rRNAs in Other Chlamydomonas *Taxa*

Information about the position of mitochondrial rRNA discontinuities in other members of the genus *Chlamydomonas* may provide information relevant to the models considered above. As shown in Fig. 2B, both *C. eugametos* and *C. reinhardtii* have an rRNA discontinuity in a common variable rRNA region, between coding regions K and L, which separates the 5′ and 3′ halves of their mitochondrial LSU rRNA. The 3′ side of this interruption, corresponding to regions L, M, and N, is composed of two separate rRNAs in both *C. eugametos* and *C. reinhardtii,* which together make up the mitochondrial rRNA peptidyl transferase center (Noller 1984). However, the breakpoint within the 3′ half of the LSU rRNA is between L and M in *C. eugametos* and between M and N in *C. reinhardtii* (Fig. 2B). Using northern blot analysis of electrophoretically separated total RNA, we undertook a survey of the size of molecules encoding the 3′ half of the mitochondrial LSU rRNA in other evolutionarily diverse *Chlamydomonas* species. One goal of this work was to determine whether the shared point of discontinuity separating the 5′ and 3′ halves of the mitochondrial LSU rRNA in *C. eugametos* and *C. reinhardtii* is also shared by the other taxa and is therefore likely to be an ancestral feature of the genus. We also tried to test whether algae within the *C. eugametos* or *C. reinhardtii* clades share fragmentation sites within the 3′ half of the mitochondrial LSU rRNA which are unique to *C. eugametos* or *C. reinhardtii,* respectively. The probes employed were complementary to different portions of the peptidyl transferase center of the mitochondrial LSU rRNAs of *C. reinhardtii* and *C. eugametos* (probes 1 and 3) and *Scenedesmus obliquus* (probe 2) as shown in Fig. 2A and B; probes 2 and 3 should detect the same fragment in *C. eugametos* and *C. reinhardtii.* In addition to *C. eugametos* and *C. reinhardtii,* which act as controls, the taxa surveyed include *Chlamydomonas pitschmanii* and *Chlamydomonas humicola,* which appear to represent middle and early diverging branches of the *C. eugametos* lineage, and *Chlamydomonas iyengarii* and *Chlamydomonas frankii,* which appear to be middle and early diverging representatives of the *C. reinhardtii* lineage (Turmel et al. 1995).

Total cellular RNA isolated from all taxa of the study group and fractionated by 1.2% agarose gel electrophoresis revealed very similar ethidium bromide staining patterns (Fig. 4). The cytosolic LSU (3.5 kb) and chloroplast SSU (1.5 kb) as well as comigrating chloroplast LSU (1.7 kb) and cytosolic SSU (1.8 kb) rRNAs were identified in the RNA samples, based on abundance and mobility relative to RNA molecular size standards (Fig. 4).

Results of the northern blot hybridization experiments of electrophoretically separated total cellular RNA are presented in Fig. 4. Depending on the probe, strong or moderate to weak hybridization signals corresponding to the position of the cytosolic LSU rRNA (3.5 kb) and the chloroplast LSU rRNA (1.7 kb) fragments can be seen in most RNA preparations. Other large RNAs of unknown function such as a 2.5-kb RNA of *C. frankii* and a 1.5-kb RNA of *C. pitschmanii* were detected with probe 1 and 2, respectively. The hybridization signals corresponding to molecules below 700 nt in size that are likely to be fragments of the 3′ half of the mitochondrial LSU rRNA varied considerably in intensity or were absent depending on the probe and the source of RNA. The size of the small RNAs detected by the three probes is summarized in Table 2. These sizes were confirmed by hybridization analysis of the RNA preparations fractionated by denaturing polyacrylamide gel electrophoresis (data not shown). For probe 1, an arrowhead (Fig. 4) marks the position of a weak signal in the *C. humicola* lane which is visible on the original photograph; in addition, the

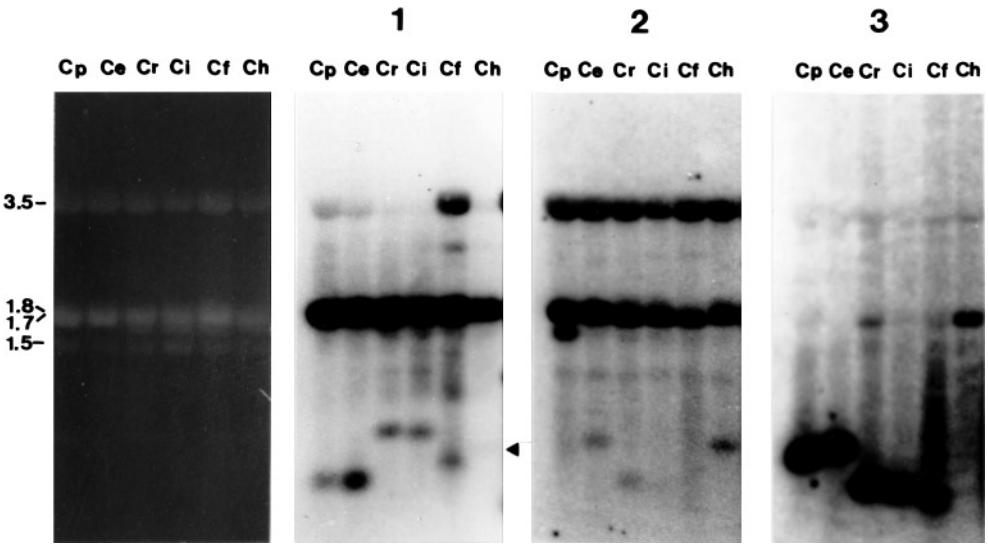

Fig. 4. Ethidium bromide staining pattern and northern hybridization analysis of RNA isolated from six species of *Chlamydomonas* and fractionated by 1.2% denaturing agarose gel electrophoresis using peptidyl-transferase-center-specific oligonucleotide probes 1, 2, and 3. The *lettering* above each lane 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.* The ethidium-bromide-stained gel is shown on the left and the oligonucleotide probes used in northern blot analysis are indicated above each blot. The *arrowhead* marks a weak hybridization signal in the *C. humicola* lane. The sizes of the cytosolic and chloroplast rRNAs are indicated as follows: 3.5 kb, cytosolic LSU rRNA (Hoober and Blobel 1969); 1.8 kb, cytosolic SSU rRNA (Bourque et al. 1971); 1.7 kb, chloroplast LSU δ fragment (Rochaix and Darlix 1982; Lemieux et al. 1989; Turmel et al. 1993); 1.5 kb, chloroplast SSU rRNA (Dron et al. 1982; Durocher et al. 1989).

Table 2. Sizes of RNAs that hybridized with mitochondrial peptidyl transferase center-specific probes in northern blots of total cellular RNA isolated from *Chlamydmonas* algae

Taxon	Morphological group[a]		Sizes of LSU RNA fragments detected[b]	
			1	2/3
C. eugametos	Chlamydella	(1,120)	480	640
C. pitschmanii	Chlorogoniella	(1,100)	480	620
C. humicola	Chlorogoniella	(1,210)	580	630
C. reinhardtii	Euchlamydomonas	(1,170)	680	490
C. iyengarii	Euchlamydomonas	(1,140)	680	460
C. frankii	Euchlamydomonas	(970)	540	430

[a] Ettl (1976).

[b] Sizes of the RNAs (distinct from the chloroplast and cytosolic LSU rRNAs) that hybridized with probe 1 (1) and with probe 2 and/or 3 (2/3) in northern blot analysis of total cellular RNA. For each taxon, the sum of the sizes of the two RNAs detected is shown in parentheses

same fragment was detected on blots of RNA separated by polyacrylamide gel electrophoresis (data not shown). Moreover, in the *C. humicola* lanes, probe 2 hybridized moderately to a small RNA but probe 3 was uninformative. In each case, probe 1 detected a different small RNA than probes 2 and 3. In the two cases (*C. eugametos* and *C. reinhardtii*) where probes 2 and 3 both hybridized to a small RNA, they appeared to hybridize to the same RNA molecule as expected because these probes correspond to the same conserved rRNA domain. The sizes of the small RNAs detected in both the *C. eugametos* and *C. reinhardtii* RNA preparations are consistent with the size of the gene pieces from which they are transcribed and with previous northern blot studies employing DNA clones as probes (Boer and Gray 1988; Denovan-Wright and Lee 1994); these rRNA fragments account for a 3′ half LSU rRNA of the *C. eugametos* and *C. reinhardtii* LSU rRNA consisting of 1,120 and 1,170 nt, respectively. For the remaining taxa, the combined size of the two small RNAs detected with probe 1 and with probe 2 or 3 was within 150 nt of this size range (Table 2). This suggests that an interrupted variable region separating the 5′ and 3′ halves of the mitochondrial LSU rRNA is a common feature of the study group and thus likely to be an ancient feature of *Chlamydomonas* algae as assumed in our models. This possibility is not unreasonable considering the sequence length differences (up to 60 nt) between corresponding mitochondrial LSU rRNA variable regions in *C. eugametos* and *C. reinhardtii* (Boer and Gray 1988; Denovan-Wright and Lee 1994).

The similarity in size between the mitochondrial rRNA fragments detected with probe 2/3 in *C. eugametos, C. pitschmanii,* and *C. humicola,* within one clade, and in *C. reinhardtii, C. iyengarii,* and *C. frankii,* within the other clade, supports the possibility that species within each clade share a common interrupted variable region within the 3′ half of their mitochondrial LSU rRNA. This interpretation is further supported by the similarity in size of fragments detected with probe 1 for two out of three taxa within each clade. The fragments detected by probe 1 from the earliest-diverging taxa of each clade, *C. humicola* and *C. frankii,* are intermediate

in size between those of the other clade members examined. This observation could be explained by changes in the size of variable regions in this portion of the LSU rRNA. Our interpretation of the results in Table 2, although preliminary for the early diverging taxa within each clade, is more easily understood in terms of model B, where early after the division of the two major lineages, the ancestral gene piece LMN sustained a break (processing signal) between L and M in one lineage and between M and N in the other lineage. Model A states that L, M, and N are in widely separate gene pieces in the last common ancestor, so precise rearrangement and joining events (loss of processing signals) would have been required to generate the MN and LM gene pieces. It seems less likely that these complex steps would have occurred in both of the two major lineages before the divergence of the deeply branching sublineages. Model B is also favored because it requires the fewest number of rearrangements from the protogenote-type continuous rRNA gene.

Further progress in the testing and refinement of the models presented here requires detailed knowledge about the mitochondrial rRNA coding regions and their transcripts in additional *Chlamydomonas* taxa. Considering the extreme differences between the structure and organization of these coding regions in *C. eugametos* and *C. reinhardtii*, it would be prudent to examine these features in taxa which show simpler arrangement differences relative to *C. eugametos* or *C. reinhardtii.*

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