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# Characterization of fragmented mitochondrial ribosomal RNAs of the colorless green alga *Polytomella parva*

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## ABSTRACT

**We have identified previously in mitochondrial DNA of the colorless, chlorophycean, green algal taxon, *Polytomella parva*, potential coding regions for four small subunit (SSU) and eight large subunit (LSU) rRNA fragments. In this study with *P.parva*, we isolated RNA from a mitochondrial-enriched preparation, characterized the 12 mitochondrial rRNA transcripts by either northern blot analysis or chemical sequencing and performed secondary structure modeling of the SSU and LSU rRNA sequences. The results show the following features about the mitochondrial SSU and LSU rRNAs of *P.parva*: (i) they are considerably shorter than their homologs from other green algae, although the main domains typical of conventional rRNAs are conserved; (ii) the rRNA fragmentation pattern is most similar to that of *Chlamydomonas reinhardtii* among green algae that have been characterized; (iii) three nucleotides are missing from the normally highly conserved GTPase center of the LSU rRNA; and (iv) post-transcriptional modification of the 3′-terminal region of the SSU rRNA is unusual in that it has the 'eubacterial' 3-methyluridine (corresponding to $m^3U$ at *Escherichia coli* 16S rRNA position 1498) but lacks the more highly conserved modifications at two adjacent A residues (corresponding to $N^6$,$N^6$-dimethyladenosine at *E.coli* 16S rRNA positions 1518 and 1519). This is the first report of the characterization by direct sequencing of fragmented mitochondrial rRNAs from a green alga.**

## INTRODUCTION

Complete mitochondrial genome sequences from four chlorophycean green algal taxa, i.e. *Chlamydomonas reinhardtii* (1,2), *Chlamydomonas moewusii* (= *Chlamydomonas eugametos*) (3), *Chlorogonium elongatum* (4) and *Scenedesmus obliquus* (5,6), revealed the existence of fragmented and scrambled small and large subunit (SSU and LSU, respectively) rRNA-coding regions and the lack of a 5S rRNA gene. The chlorophycean lineage is composed of two main sublineages as supported by both 18S rDNA sequence data and basal body configuration in flagellated cells (7); three of the four taxa discussed above associate with one of these sublineages while *S.obliquus* associates with the other. The fact that *S.obliquus* has fewer mitochondrial rRNA gene break points, all of which correspond to variable regions that are also interrupted in the other three taxa, implies that these break points were present in the ancestor of the four taxa. Moreover, the mitochondrial SSU and LSU rRNA break points of *C.moewusii* and *Chlorogonium* are in the same variable regions thus indicating a close phylogenetic relationship between these taxa.

The feature of fragmented mitochondrial rRNA-coding regions among chlorophycean algae is distinct compared with green algal taxa outside this group except for *Pedinomonas minor* whose phylogenetic position among the green algae is uncertain (5 and references therein, 8). However, knowledge of chlorophycean mitochondrial rRNAs is still rather limited. For example, rDNA sequencing, northern blot hybridization and S1 nuclease protection analysis were the main means previously employed to characterize these rRNAs; their direct sequencing has not been reported, principally because the species studied previously, all of which are photosynthetic, typically contain abundant chloroplast rRNA which hampers the isolation of relatively pure mitochondrial rRNA needed for such analysis. Moreover, potential secondary structures of mitochondrial SSU and LSU rRNAs in the Chlorophyceae have been reported only for *C.reinhardtii* and *C.moewusii* (1,9,10). Finally, information from additional chlorophycean species is required for understanding the evolutionary pathway of mitochondrial rRNA fragmentation in the chlorophycean lineage.

*Polytomella* (11) is a green algal genus consisting of colorless, wall-less unicells, which provides advantages in the isolation and characterization of mitochondrial components. For example, *Polytomella* strain SAG 198.80 has proven useful for the isolation of respiratory proteins (12–14). Similar studies have been difficult with *C.reinhardtii*, the green alga having the most well studied mitochondrial genetic system, because of contaminating thylakoid components (15,16). In addition, the fact that no plastid rRNAs could be detected in *Polytomella parva* (= *Polytomella agilis*), even by means of

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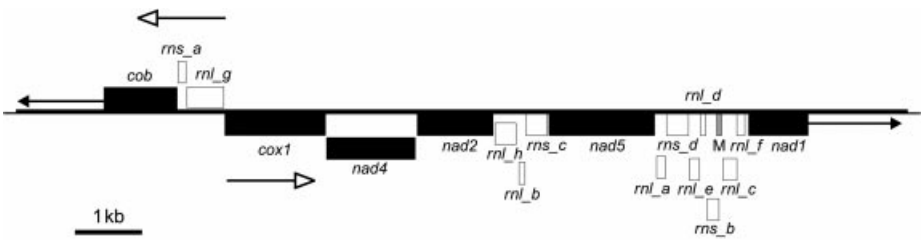



**Figure 1.** Map of the 13.5 kb mtDNA of *P.parva* showing the organization of mitochondrial rRNA genes (22). SSU and LSU rRNA genes are fragmented into four (*rns_a* through *rns_d*) and eight (*rnl_a* through *rnl_h*) potential modules (open rectangles), respectively. These gene modules are distributed on both strands and interspersed with each other and with protein (black rectangles)- and tRNA (hatched rectangle)-coding regions. Two open arrows indicate transcriptional orientations. Two solid arrows represent terminal inverted repeats. Thin lines at the ends indicate unsequenced parts. The partial sequence of this mtDNA is registered under GenBank accession no. AY062933.

northern blot analysis (17), implies either the absence or extremely low abundance of plastid ribosomes in this taxon, and thus makes it useful for the study of green algal mitochondrial ribosomes and rRNA. Information obtained about *Polytomella* mitochondria is particularly applicable to *C.reinhardtii*, because of the specific evolutionary connection between them among *Chlamydomonas*-like taxa (18,19).

To date, sequences of the mitochondrial protein-coding genes, *cox1* and *cob*, from *Polytomella* strain SAG 198.80 have been reported (20,21), as has most of the mitochondrial DNA (mtDNA) of *P.parva*, which is in at least two linear pieces of 13.5 and 3.5 kb (22). The latter study (22) identified four SSU (*rns_a* through *rns_d*) and eight LSU (*rnl_a* through *rnl_h*) potential rRNA-coding modules, which are scattered over 8 kb of the *P.parva* 13.5 kb mtDNA component (Fig. 1). These coding modules are scrambled in order and interspersed with each other and with additional coding regions; *Polytomella*, therefore, shares these features with other chlorophycean green algae. The *P.parva* mitochondrial rRNA-coding modules, however, are distributed on both DNA strands, a feature not previously reported for chlorophycean mtDNA, although this has been reported for the fragmented rRNA genes in apicomplexan mtDNA (23).

In order to gain more information about the evolution of fragmented rRNA in green algae and to enhance the utility of *Polytomella* for studies of green algal mitochondrial biogenesis, we have characterized the mitochondrial rRNA transcript fragments of *P.parva* by either RNA sequencing or northern blot analysis, and performed secondary structure modeling of the SSU and LSU rRNA sequences as deduced from the rDNA regions.

## MATERIALS AND METHODS

### Strain and culture conditions

*Polytomella parva* (UTEX L 193) was obtained from the culture collection of the University of Texas at Austin. Cultures were grown at 25°C in the medium of Sheeler *et al.* (24) with shaking for small cultures (100–250 ml) or mild aeration for larger cultures (5–15 l). Cells were harvested in the logarithmic growth phase (~2.5 × $10^6$ cells/ml) by centrifugation (2000 *g*) at 4°C for 15 min.

### Preparation of a mitochondrial-enriched fraction

The mitochondrial fraction was prepared as described by Spencer *et al.* (25) with some modifications. A cell pellet from 6 l of culture was washed once in buffer A (50 mM Tris–HCl, pH 8.0, 300 mM mannitol, 0.1% bovine serum albumin, 1 mM β-mercaptoethanol, 3 mM EDTA). After resuspension in 30 ml of buffer A, the cells were disturbed manually in a 40 ml glass homogenizer (Kontes Glass Co.) until 90% of them were broken. Following centrifugation (1000 *g*) for 10 min, the supernatant was saved and subjected to another centrifugation (12 000 *g*) for 20 min to collect crude mitochondria. The resulting pellet was resuspended in 2 ml of buffer A and loaded onto a gradient consisting of 1.15 and 1.55 M sucrose (in buffer A). The gradient was centrifuged (25 000 r.p.m., Beckman SW 41 Ti rotor) for 1 h. The mitochondrial band at the interface of the 1.15 and 1.55 M sucrose layers was removed using an 18 gauge needle with a 90° bend. The fraction obtained was slowly diluted with two volumes of buffer B (50 mM Tris–HCl, pH 8.0, 20 mM EDTA), followed by centrifugation (12 000 *g*) for 15 min. All steps were carried out at 4°C.

### RNA isolation

For the isolation of total cellular RNA, cells were washed once in buffer C (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 50 mM EDTA) and lysed following resuspension in three volumes of buffer C containing 0.1% SDS. For the isolation of mitochondrial-enriched RNA, a mitochondrial-enriched fraction from 6 l of culture was resuspended in 9 ml of buffer D (10 mM Tris–HCl, pH 8.5, 50 mM KCl, 10 mM $MgCl_2$), followed by the addition of 1 ml of 20% Triton X-100 (in buffer D). Following centrifugation (10 000 *g*) for 10 min, SDS was added to the supernatant to a final concentration of 2%. RNA extraction from the cell and mitochondrial lysate followed the method of Rochaix and Malnoë (26). All steps were carried out at 4°C.

### RNA fractionation

RNA was fractionated in 8% polyacrylamide slab gels (20 × 20 × 0.15 cm) containing 7 M urea by electrophoresis at 350 V for 4 h (27) and was detected by staining with ethidium bromide.

### Determination of RNA sequences

RNA isolated from the mitochondrial-enriched fraction and semi-purified mitochondrial ribosomes (28) of *P.parva* was 3′-end-labeled with [$^{32}$P]pCp using RNA ligase (29). The end-labeled mitochondrial rRNAs were fractionated in 6% polyacrylamide gels (33 × 40 × 0.05 cm) at 1700 V and eluted according to Schnare *et al.* (30). The partial chemical

degradation method (29) was used to determine the 3′-terminal sequence of mitochondrial rRNAs.

### Northern blot hybridization

Following fractionation by polyacrylamide gel electrophoresis, RNA was blotted onto a nylon membrane (Hybond-N+; Amersham Pharmacia Biotech) by the method of capillary transfer (31) using 0.5 mM NaOH as the transfer solution. The Alkphos Direct Labeling and Detection System kit (Amersham Pharmacia Biotech) was employed for probe labeling and the subsequent hybridization, following the kit instructions. Oligonucleotide probes were labeled at 37°C for 2.5–3.5 h. Hybridization was carried out at 38°C overnight in a hybridization oven. The RNA blots were washed twice in the primary wash buffer at 38°C for 10 min and twice in the secondary wash buffer at room temperature for 5 min. Chemiluminescent detection was achieved by exposing the blots to autoradiography film. Oligonucleotide probes used in the northern blot hybridization are as follows: $S_1$, 5′ TTA TCT CAT AGT GAA AAG CTA GGC AAA GAC 3′; $S_2$, 5′ TGC GTA AAA CGA TAG TCC TTT GAG ACT ATT 3′; $L_1$, 5′ TTA TTC GTC TTT TTG TTC CAT CAC TGT ACT 3′; L2, 5′ ATA TTA AAT CGC TGG CCC ATG CTG CAA AAG 3′; $L_4$, 5′ ATC TCC TTT TGA ACC TTA ACC TAT CCG TTG 3′; $L_6$, 5′ CCT ATC GTC GCT TTT GTT ACT AAT GCC AGC 3′; $L_8$, 5′ AGG ATG CGA TGA TCC AAC ATC GAG GTG 3′.

## RESULTS

### Identification of mitochondrial rRNA fragments

Total cellular and mitochondrial-enriched RNA of *P.parva* were subjected to polyacrylamide gel electrophoresis (Fig. 2A). The total cellular RNA sample revealed prominent components typical of the nucleocytoplasm, including 25/28S, 18S, 5.8S and 5S rRNAs, as well as tRNAs. The RNA from the mitochondrial-enriched fraction contained, in addition, several potential mitochondrial rRNA species that had sizes significantly smaller than the 25/28S and 18S rRNAs.

Two approaches were taken for mitochondrial rRNA identification. Northern blot analysis with individual probes derived from seven rRNA-coding modules each identified a single transcript (Fig. 2B). Five of these transcripts corresponded in size to an RNA component detected in the mitochondrial-enriched fraction (Fig. 2A). The other two of these seven transcripts, $L_2$ and $L_4$, were not detected by ethidium bromide staining; the former of these transcripts did not stain well because of its small size, and the latter fragment co-migrated with the tRNAs. For the remaining five RNA species, 3′-terminal chemical sequencing data (Fig. 3) established that each of these corresponds to a transcript of one of the rRNA-coding modules (Table 1). We note that the rRNA pairs $S_4$/$L_8$ and $S_2$/$L_3$ could not be well separated in standard 8% polyacrylamide gels due to their similar sizes within the pairs (Fig. 2A), but could be differentiated using 6% polyacrylamide sequencing gels (data not shown). Due to their similar sizes, rRNA fragments $L_1$, $L_6$ and $S_1$ also co-migrated (Fig. 2).

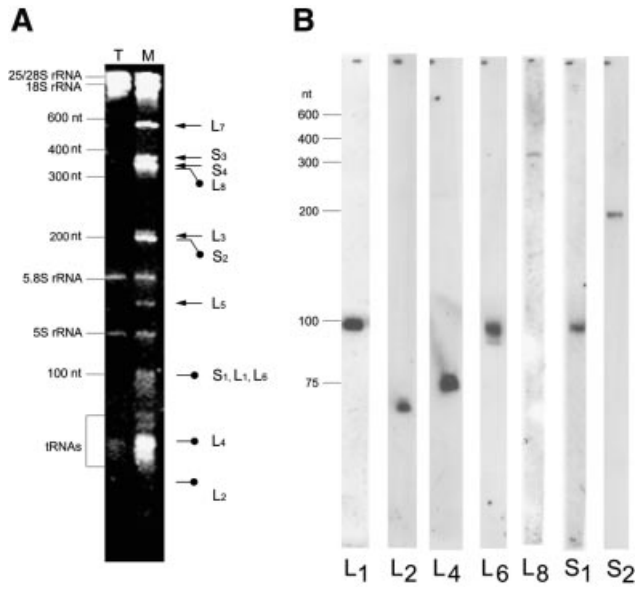



**Figure 2.** (**A**) Electrophoretic profile of *P.parva* total (T) and mitochondrial-enriched (M) RNA in an 8% polyacrylamide gel. Lines with a dot at one end indicate the positions of northern blot hybridization signals obtained with oligonucleotide probes derived from seven mitochondrial rDNA modules (B). Arrows point to the positions of the remaining five mitochondrial rRNA species whose 3′-termini were sequenced (Fig. 3). Positions of nucleocytoplasmic rRNAs including 25/28S, 18S, 5.8S, 5S rRNA and tRNAs, as well as RNA size markers (Sigma), are also shown. (**B**) Northern blot hybridization analysis to identify seven *P.parva* mitochondrial rRNA species. RNA species detected with oligonucleotide probes derived from rDNA modules *rnl_a*, *rnl_b*, *rnl_d*, *rnl_f*, *rnl_h*, *rns_a* and *rns_b* are designated $L_1$, $L_2$, $L_4$, $L_6$, $L_8$, $S_1$ and $S_2$, respectively. Locations of RNA size markers (Sigma) are indicated.

### Potential structures of *P.parva* mitochondrial SSU and LSU rRNAs

Figures 4 and 5 show the proposed mitochondrial SSU and LSU rRNA secondary structures of *P.parva* based on the sequences of the rDNA modules and modeled after the proposed secondary structures of their respective *Escherichia coli* homologs (10). The mitochondrial SSU and LSU rRNA fragments of *P.parva* have combined lengths of 979 and 1556 nt, respectively. In these structures, theoretically, the four SSU and eight LSU rRNA pieces could be brought together through intermolecular base-pairing to form structures containing three and six domains in the SSU and LSU rRNA, respectively, as in other fragmented chlorophycean counterparts (1,9).

The *P.parva* mitochondrial rRNAs appear structurally conventional throughout most of the evolutionarily conserved structural and functional cores [see Cannone *et al.* (10) for structure conservation diagrams], but deletions, relative to the corresponding regions of *C.reinhardtii* (1) and *C.moewusii* (9) mitochondrial and *E.coli* (10) rRNA, occur in three regions not reported previously to be variable. First, the 431–436 region of SSU rRNA lacks 7 nt (Fig. 4). Secondly, two sequences in the stem that results from the interaction of LSU rRNA fragments $L_6$ and $L_7$ are eliminated (Fig. 5A). Thirdly, 3 nt are missing from the GTPase center predicted for *P.parva* mitochondrial LSU rRNA (Fig. 5A); this center is otherwise rather conserved relative to the counterparts from *E.coli* as

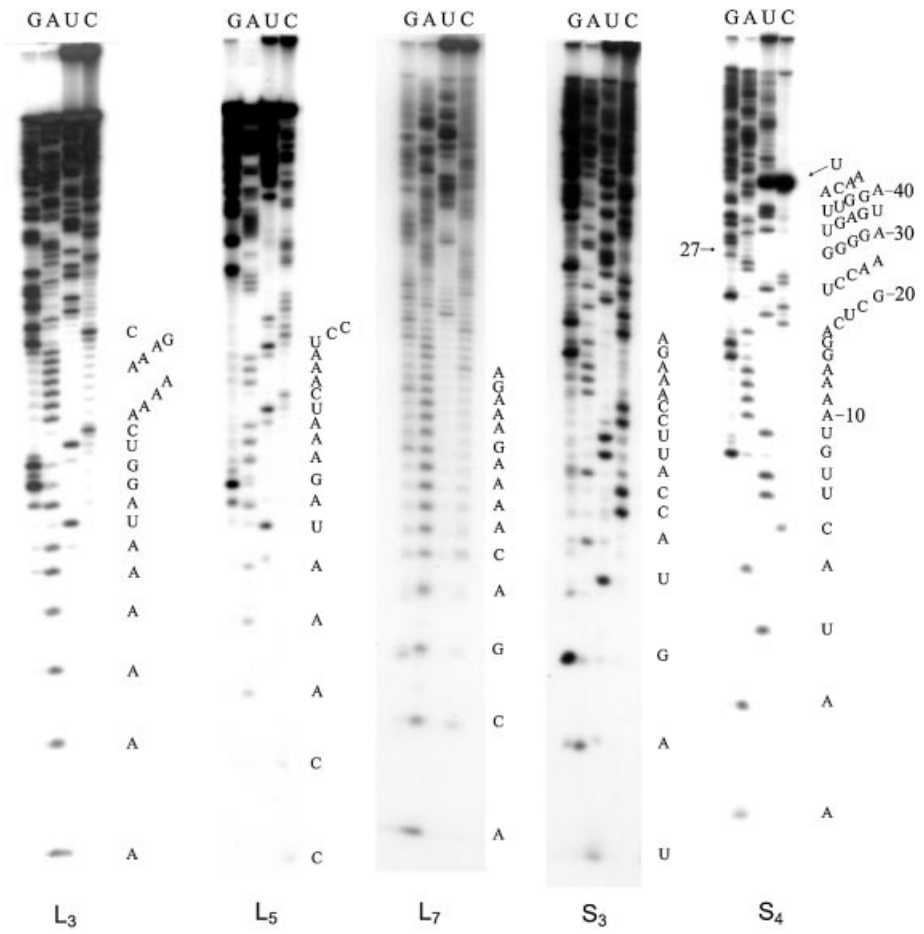



**Figure 3.** Autoradiograms of 20% sequencing gels showing the resolution of limited chemical digests of five 3′-end-labeled *P.parva* mitochondrial rRNA species. These RNAs were identified as transcripts of rDNA modules *rnl_c*, *rnl_e*, *rnl_g*, *rns_c* and *rns_d*, and are designated $L_3$, $L_5$, $L_7$, $S_3$ and $S_4$, respectively. In the gel for $S_4$, the arrows indicate the positions of reduced G-lane and enhanced C-lane cleavage; this sequence is numbered from the 3′-terminus.

**Table 1.** Summary of features of *P.parva* mitochondrial rRNA-coding modules and their transcripts[a]

| Coding module | rRNA | Minimum length[b] | Maximum length[c] | Actual 3′-terminus[d] | Length determined by gel electrophoresis[e] | Length and location in structure diagrams[f] |
|---|---|---|---|---|---|---|
| SSU | | | | | | |
| *rns_a* | $S_1$ | 95 (2485–2391) | 116 (2502–2387)[g] | | 99 | 99, 1–99 (2485–2387) |
| *rns_b* | $S_2$ | 167 (10 188–10 354) | 188 (10 168–10 355) | | 198 | 179, 100–278 (10 177–10 355) |
| *rns_c* | $S_3$ | 362 (7493–7854) | 387 (7482–7868) | 7854 | 362 | 362, 279–640 (7493–7854) |
| *rns_d* | $S_4$ | 339 (9599–9937) | 408 (9533–9949) | 9937 | 339 | 339, 641–979 (9599–9937) |
| LSU | | | | | | |
| *rnl_a* | $L_1$ | 67 (9466–9532) | 102 (9459–9560) | | 99 | 100, 1–100 (9461–9560) |
| *rnl_b* | $L_2$ | 48 (7434–7481) | 85 (7408–7492) | | 60 | 60, 101–160 (7433–7492) |
| *rnl_c* | $L_3$ | 176 (10 429–10 604) | 196 (10 429–10 624) | 10 624 | 200 | 196, 161–356 (10 429–10 624) |
| *rnl_d* | $L_4$ | 71 (10 097–10 167) | 120 (10 068–10 187) | | 73 | 73, 357–429 (10 095–10 167) |
| *rnl_e* | $L_5$ | 127 (9941–10 067) | 159 (9938–10 096) | 10 078 | 141 | 141, 430–570 (9938–10 078) |
| *rnl_f* | $L_6$ | 92 (10 625–10 716) | 185 (10 605–10 789) | | 99 | 96, 571–666 (10 625–10 720) |
| *rnl_g* | $L_7$ | 552 (3054–2503) | 613 (3098–2486)[g] | 2500 | 558 | 558, 667–1224 (3057–2500) |
| *rnl_h* | $L_8$ | 321 (7087–7407) | 364 (7070–7433) | | 332 | 332, 1225–1556 (7085–7416) |

[a]Lengths are indicated in nucleotides; numbers in parentheses refer to coordinates in the GenBank entry (AY062933).
[b]Based on secondary structure modeling.
[c]Based on the positions of flanking genes, assuming no overlap; in cases where the gene of interest is flanked by another rRNA gene the coordinates of the neighboring gene were taken from the previous column.
[d]Genomic position determined by RNA sequencing.
[e]Detected by ethidium bromide staining or northern hybridization.
[f]Estimates based on values in previous columns.
[g]Transcribed from the opposite strand relative to most of the other rRNA-coding modules.

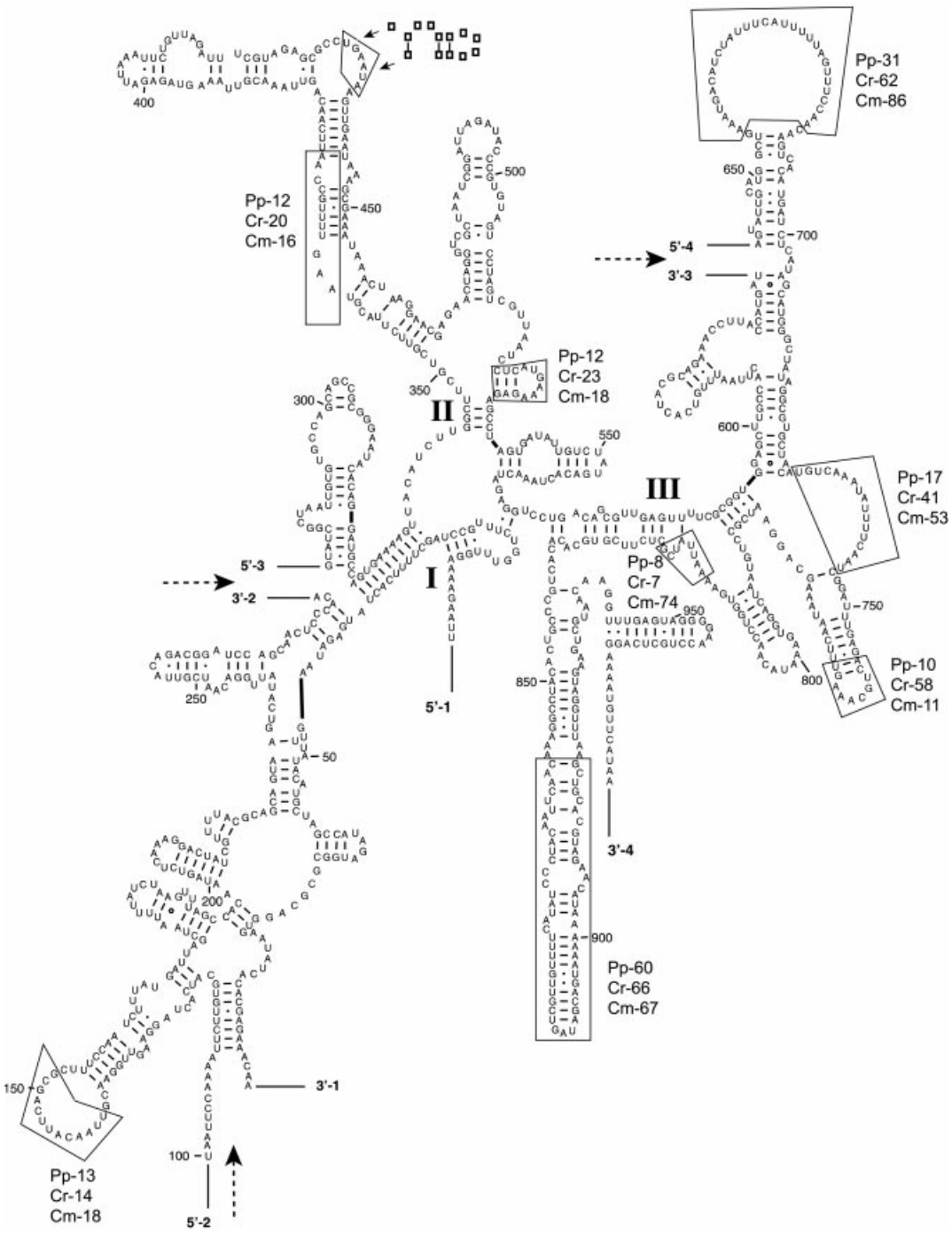
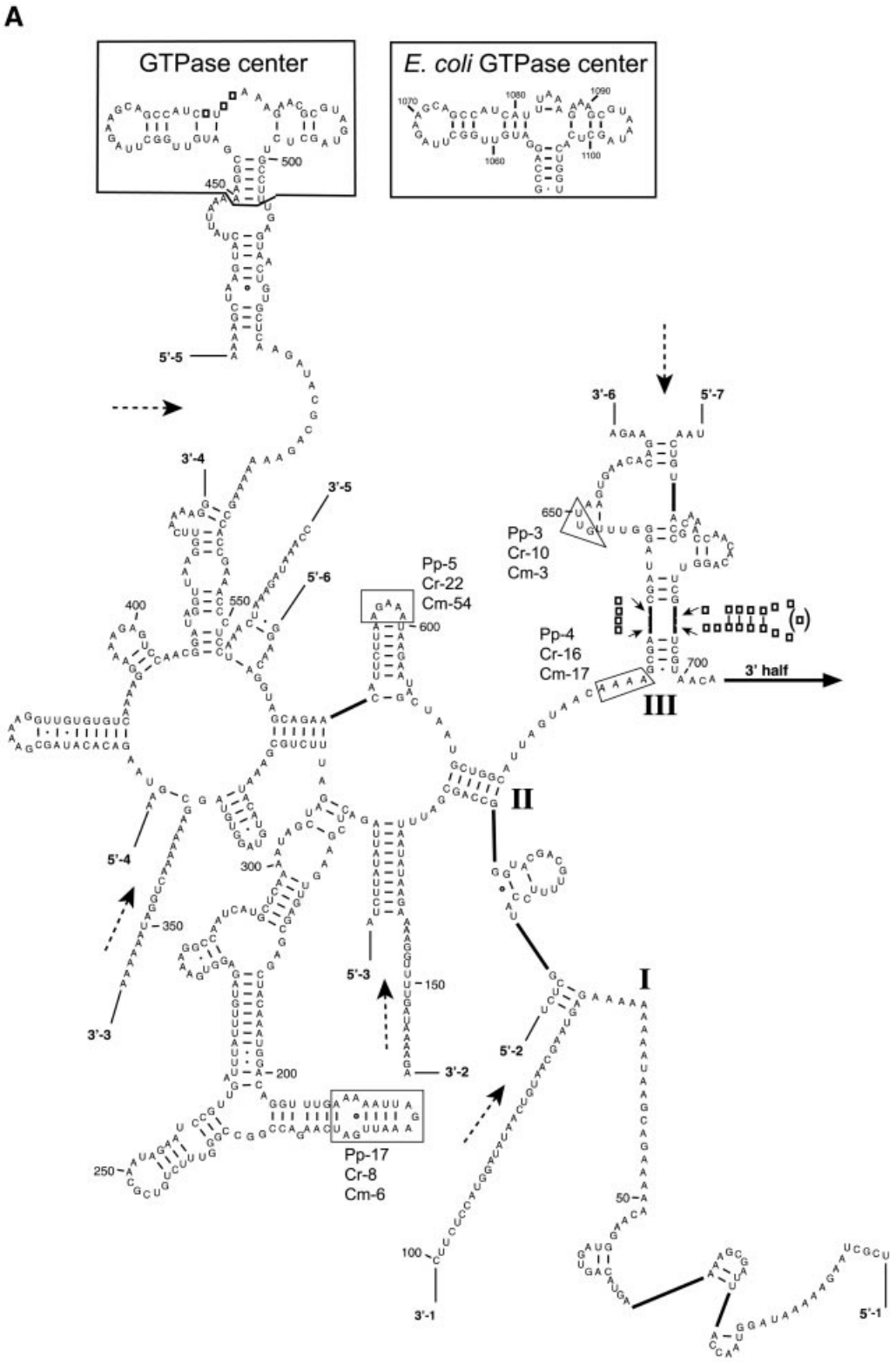



**Figure 4.** Potential secondary structure of *P.parva* mitochondrial SSU rRNA. The structure is constituted by four RNA species, whose 5′- and 3′-termini are indicated. Roman numerals denote the three domains. Thin lines define regions variable among and continuous in rRNAs of *P.parva*, *C.reinhardtii* and *C.moewusii* mitochondria; number of nucleotides in these regions is indicated for *P.parva* (Pp), *C.reinhardtii* (Cr) and *C.moewusii* (Cm). The broken arrows indicate the break points. Small squares, each of which represents a nucleotide, describe structures that are conserved in *C.reinhardtii* and *C.moewusii* mitochondria and in *E.coli*, but altered or absent in the mitochondria of *P.parva*.

well as *C.reinhardtii* and *C.moewusii* mitochondria. Although the DNA used for sequencing the region encoding the GTPase center was obtained by PCR (22), the possibility of amplification errors was minimized by a parallel PCR with *C.reinhardtii* DNA which produced a product having the same sequence found for cloned DNA that had not been PCR amplified (1). In addition, a cDNA of the *P.parva* mitochondrial GTPase center was obtained by reverse transcriptase–PCR, and its sequence exactly matches the DNA prepared by PCR, thus supporting the absence of the 3 nt in the GTPase center of *P.parva* LSU rRNA. All DNA sequences were determined for both strands.




Comparisons involving 775 and 1174 nt of mitochondrial SSU and LSU rDNA, respectively, that could be aligned among *P.parva*, *C.reinhardtii* and *C.moewusii*, revealed a nucleotide identity of 61 and 69%, respectively, between *P.parva* and *C.reinhardtii*, and an identity of 58 and 68%, respectively, between *P.parva* and *C.moewusii*. These results

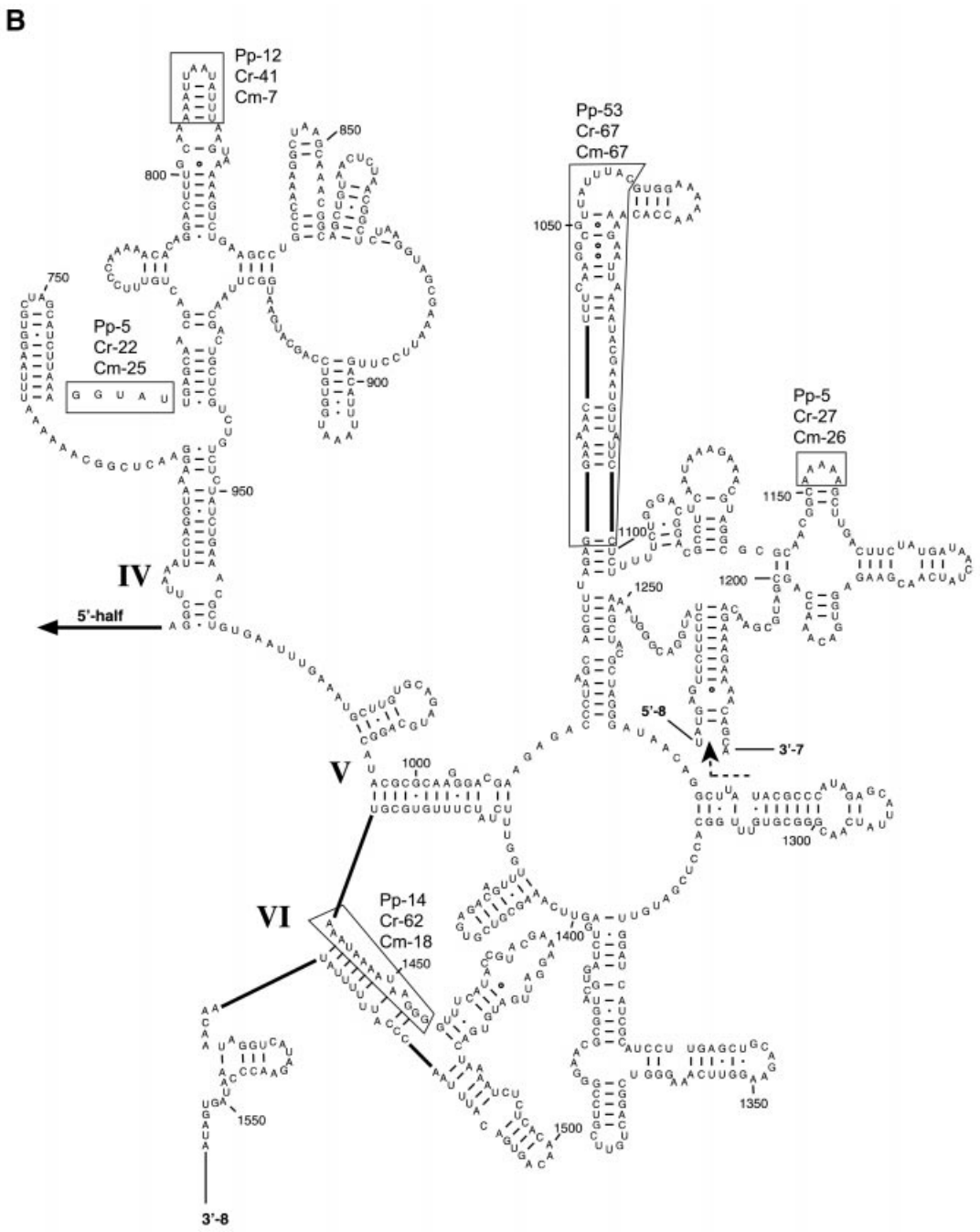



**Figure 5.** (Opposite and above) Potential secondary structure of *P.parva* mitochondrial LSU rRNA. (**A**) 5′ half; (**B**) 3′ half. The structure is constituted by eight RNA species. Roman numerals denote the six domains. The GTPase center of the LSU rRNA is enclosed by thick lines as is the potential structure of the *E.coli* GTPase center included as an inset for reference. The square in brackets indicates an extra nucleotide in *E.coli*. Other explanations are as described in Figure 4.

underscore the considerable evolutionary divergence, at the primary sequence level, between the *P.parva* mitochondrial rDNA sequences and those of *C.reinhardtii* and *C.moewusii*.

Features of the 12 *P.parva* mitochondrial rRNA-coding modules and their transcripts are summarized in Table 1. Approximate locations of the 5′ and 3′ ends of these mitochondrial rRNAs, judged to be within 5–10 nt of the true termini, were estimated on the basis of their sizes as determined by secondary structure modeling, gel electrophoresis and the proximity of neighboring coding regions as determined by DNA sequencing. The 3′ ends of rRNA species $L_3$, $L_5$, $L_7$, $S_3$ and $S_4$ were precisely located by direct chemical sequencing. The estimated size of each rRNA fragment (Table 1) is consistent with the size of the corresponding rDNA module identified previously by DNA sequencing, therefore arguing against the presence of additional points of

**Table 2.** Length[a] of green algal mitochondrial rRNAs in nucleotides

| Species | SSU rRNA | LSU rRNA | Accession nos |
|---|---|---|---|
| *Mesostigma viride* | 1558 | 2843 | AF353999 |
| *Nephroselmis olivacea* | 1509 | 2760 | AF110138 |
| *Prototheca wickerhamii* | 1674 | 3009 | U02970 |
| *Pedinomonas minor* | 1178 | 2110 | AF116775 |
| *Scenedesmus obliquus* | 1747 | 3028 | AF204057 |
| *Chlorogonium elongatum* | 1449 | 1921 | Y07814, Y13644 |
| *Chlamydomonas moewusii* | 1240 | 1916 | AF008237 |
| *Chlamydomonas reinhardtii* | 1200 | 2085[b] | U03843 |
| *Polytomella parva* | 979 | 1556 | AY062933 |

[a]In most cases precise termini have not been determined.
[b]Length of LSU rRNA does not include $L_{2b}$ and $L_{3a}$ (1).

discontinuity within any of the coding modules. The size of $S_2$ was estimated at 179 nt (Table 1) and could be only 9 nt larger when the positions of flanking genes are considered; it therefore seems likely that its electrophoretic mobility is artifactually slow since it migrates near the 200 nt RNA marker in polyacrylamide gels (Fig. 2). A similar phenomenon has been observed for *Euglena gracilis* LSU rRNA species 4 (27).

### Chemical cleavages of rRNA fragment $S_4$

Sequencing of the 3′-terminus of the *P.parva* mitochondrial rRNA fragment $S_4$, which corresponds to the 3′ end region of SSU rRNA from other sources, revealed two sites where chemical cleavage was unusual compared with other sites in this rRNA (Fig. 3). The residue located 45 nt from the 3′ end, where the DNA sequence predicts a U, exhibited an extraordinarily strong C-specific chemical cleavage. This enhanced cleavage is diagnostic for 3-methyluridine ($m^3U$) in the RNA, as was observed, for example, at the homologous position in SSU rRNA from *E.coli*, wheat mitochondria and *E.gracilis* chloroplasts (32,33). In addition, the residue located 27 nt from the 3′-terminus, where the DNA sequence predicts a G, was resistant to G-specific chemical cleavage. It is not certain whether this lack of cleavage indicates post-transcriptional modification because there are examples where some unmodified G residues give blanks in the G lane of chemical sequencing gels (29; M.N.Schnare, unpublished data).

Normal chemical cleavage was observed at the two A residues of *P.parva* (24 and 25 nt from the 3′-terminus of S4 rRNA; Fig. 3) corresponding to *E.coli* 16S rRNA positions 1518 and 1519. In *E.coli* and many other eubacterial and eukaryotic SSU rRNAs (32), as discussed later, these sites contain two adjacent $N^6$,$N^6$-dimethyladenosine residues that are resistant to A-specific chemical cleavage.

## DISCUSSION

### General structure of *P.parva* mitochondrial rRNAs

One of the most distinguishing features of the fragmented mitochondrial rRNAs of *P.parva* is their combined SSU rRNA and LSU rRNA lengths, which are considerably shorter than the fragmented and continuous rRNA counterparts so far characterized from other green algae (Table 2). The observed small size of the *P.parva* mitochondrial rRNA-coding regions seems to be the result of a trend for smaller SSU and LSU rRNA-coding regions in the lineage leading to *P.parva*. The existence of such a trend is supported by the lengths of homologous SSU and LSU rRNA variable regions that are unbroken in *P.parva*, *C.reinhardtii* and *C.moewusii*; most of these regions are shorter, sometimes drastically so, in *P.parva* relative to the other two taxa (Figs 4 and 5).

In addition, deletions have been detected in three regions of the *P.parva* mitochondrial rRNA that are usually conserved in other systems. First, the GTPase center, which corresponds to positions 1051–1108 of *E.coli* LSU rRNA, is very highly conserved in terms of its 58 nt length and proposed secondary structure. Due to the reduced length of the *P.parva* GTPase center (55 nt), however, two base pairs, corresponding to pair 1058:1080 and pair 1082:1086 in the secondary structure model for the *E.coli* GTPase center (Fig. 5A), are not possible. Comparative (34), thermodynamic (35) and crystal structural (36) studies emphasize the fundamental importance of the 1082:1086 base pair. We note, however, that disruption of this base pair also occurs in the mitochondrial rRNA of trypanosomatid protozoa and metazoan animals (10), all of which have highly reduced LSU rRNA sequence lengths. Secondly, length reduction in SSU rRNA fragment $S_3$ near coordinates 431–436, which corresponds to the 722–733 region of the *E.coli* homolog, alters the potential secondary structure in this region. Different approaches reveal that this region plays an important role in *E.coli* protein synthesis [see Zimmermann (37) for a review]. Thirdly, two sequences in the LSU region of interaction between $L_6$ and $L_7$, which correspond to positions 1299–1302 and 1626–1641 of *E.coli* LSU rRNA, are deleted. It has been shown that parts of the missing sequences are in a region involved in ribosomal protein binding (38). Deletions in these three regions may be the result of relaxed functional constraints on the mitochondrial ribosomes of *P.parva*; it is also possible that proteins may have replaced certain functions of the rRNAs.

Finally, we could not identify any RNAs corresponding in sequence to the two RNA species, $L_{2b}$ and $L_{3a}$, from *C.reinhardtii* mitochondria; these abundant RNAs were initially presumed to correspond to non-core LSU rRNA regions based on their cotranscription with and precise cleavage from LSU rRNA fragments $L_{2a}$ and $L_{3b}$ (1). However, as sequences homologous to $L_{2b}$ and $L_{3a}$ have also not been identified in other LSU rRNAs, including *C.moewusii* mitochondrial LSU rRNA (9), it now appears likely that these are not components of *C.reinhardtii* mitochondrial ribosomes.

### Mitochondrial rRNA break points

All the interrupted points, three and seven in *P.parva* mitochondrial SSU and LSU rRNAs, respectively, are located in regions previously identified as variable in sequence and secondary structure in other rRNAs (39). Figure 6 shows the comparison of mitochondrial SSU and LSU rRNA discontinuity patterns among the *Chlamydomonas*-like chlorophycean algae. The number and position of broken variable regions in LSU rRNA is identical between *P.parva* and *C.reinhardtii* (1), while these two organelles share two out of three break points in their SSU rRNAs. The other break point in *P.parva* SSU rRNA (separating $S_1$ and $S_2$ in the structure models) is shared with *C.moewusii*/*Chlorogonium* (4,9). Overall, the fragmentation patterns of *P.parva* mitochondrial

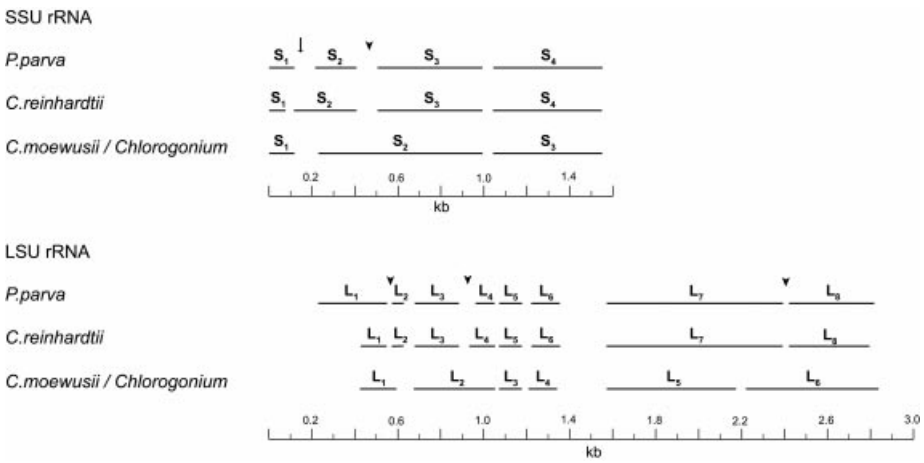



**Figure 6.** Comparison of mitochondrial rRNA fragmentation patterns among *Chlamydomonas*-like algae. The rRNAs are drawn to the scale of the *E.coli* homologs. The arrow and arrowheads indicate the break points unique to *P.parva*/*C.moewusii*/*Chlorogonium* and *P.parva*/*C.reinhardtii*, respectively.

rRNAs are more similar to those of *C.reinhardtii* than to their counterparts from *C.moewusii* (Fig. 6), consistent with phylogenetic analysis of nuclear 18S rDNA sequences (18,19).

### Post-transcriptional modification near the 3′-terminus of *P.parva* mitochondrial SSU rRNA

Information about the post-transcriptional modification pattern of rRNA is limited because of technical challenges involved in identifying and localizing modifications in large RNAs (40). However, direct chemical sequencing of 3′-end-labeled SSU rRNA has proven useful for evaluating the presence or absence of the 'eubacteria-specific' $m^3U$ (corresponding to $m^3U$ at *E.coli* 16S rRNA position 1498) and the two adjacent $N^6$,$N^6$-dimethyladenosine residues (corresponding to *E.coli* 16S rRNA positions 1518 and 1519) that are found in both eubacterial and eukaryotic cytoplasmic SSU rRNA (32). The $m^3U$ and the two $m^6_2A$ modifications are present in wheat (32) and *Acanthamoeba castellanii* (41) mitochondrial rRNAs, two systems in which the rRNA sequences/structures have retained striking similarity to their eubacterial homologs (41,42). The more divergent mitochondrial rRNAs from *Tetrahymena pyriformis* (43) and fungi (44,45) are missing all three of these modifications, while the small-sized SSU rRNAs from animal mitochondria have the two $m^6_2A$ modifications but do not have the $m^3U$ (46–48). The data presented here for *P.parva* mitochondria provide the first example of any SSU rRNA that has the 'eubacterial' $m^3U$ without also having at least one of the adjacent $m^6_2A$ modifications. Note that the *E.gracilis* chloroplast 16S rRNA has the $m^3U$ but only one of the $m^6_2A$ residues (33).

## CONCLUSION

The highly divergent and fragmented nature of chlorophycean mitochondrial rRNAs raises the question of whether they can actually function in mitochondrial protein synthesis. Indirect evidence that these rRNAs are functional comes from the observations that they are associated with ribosome-sized particles in *C.moewusii* (28), and that a mutation in the GTPase center of the *C.reinhardtii* LSU rRNA gene is linked to the suppression of a frame shift mutation in the mitochondrial *cox1* gene (49). The work presented here and in Fan and Lee (22) provides the necessary background for more direct functional studies of fragmented mitochondrial rRNAs, using *P.parva*. In addition, novel structural and post-transcriptional modification features of *P.parva* mitochondrial rRNAs revealed in this study present interesting questions regarding their potential effect on ribosomal function.

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