Evidence that the fragmented ribosomal RNAs of *Chlamydomonas* mitochondria are associated with ribosomes

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Abstract The discontinuous and scrambled organization of the small subunit and large subunit rRNA coding regions in *Chlamydomonas* mitochondrial DNA has been well documented. Our goals were to demonstrate that the small transcripts produced by these coding regions in *Chlamydomonas eugametos* are assembled into mitochondrial ribosomes and to characterize the sedimentation properties of these ribosomes and their subunits in sucrose gradients. Putative mitochondrial ribosomes (60–66S) and their large (44–50S) and small (35–39S) subunits were identified by slot blot hybridization which sedimented independently of the chloroplast and cytosolic ribosomes. A crude mitochondrial pellet prepared from *C. eugametos* was enriched for mitochondrial small subunit and large subunit rRNA subfragments thereby providing independent confirmation of the mitochondrial association of these rRNA molecules.

Key words: *Chlamydomonas*; Mitochondrion; Ribosome; Discontinuous gene; Green algae

1. Introduction

Distinctly different ribosomes function in the nuclear-cytosolic, mitochondrial and chloroplast protein synthesizing compartments of eukaryotic cells. Although both mitochondria and chloroplasts are generally agreed to have arisen from prokaryotic endosymbionts [1] mitochondrial ribosomes show fewer similarities to prokaryotic ribosomes than do chloroplast ribosomes and they are structurally and functionally more diverse than chloroplast ribosomes [2]. For example, in terms of the apparent sedimentation coefficent, the most common criterion for distinguishing different ribosome types, chloroplast ribosomes sediment like the 70S ribosomes of prokaryotes, while mitochondrial ribosomes are reported to be 55–60S in animals, 70–74S in fungi and *Euglena* and 77–80S in ciliate protozoans and land plants [2,3].

Extensive studies have been performed on the cytosolic and chloroplast ribosomes of *Chlamydomonas reinhardtii* [4]. The cytosolic ribosomes in these cells have an apparent sedimentation coefficient of approximately 83S and at Mg^{2+} concentrations below 2 mM, these ribosomes separate into subunits of 57–60S and 37–40S. Chloroplast ribosomes are typically described as having a sedimentation coefficient of 70S and at Mg^{2+} concentrations below 25 mM these ribosomes fractionate as a heterogenous population of molecules with lower sedimentation values and they begin to dissociate into subunits of 50–54S and 33–41S. At Mg^{2+} concentrations below 2 mM, chloro-

plast ribosomes become completely dissociated into individual subunits.

Although there is indirect evidence for the existence in *Chlamydomonas* of mitochondrial ribosomes and for a functioning mitochondrial protein synthesizing system, direct evidence for these conclusions is lacking [4]. Small subunit (SSU) and large subunit (LSU) rRNAs are encoded in the mtDNAs of *C. reinhardtii* and *Chlamydomonas eugametos*. The coding regions for these rRNAs, however, are distributed into separate gene pieces which are scrambled in order and are interspersed with each other and with protein-coding and tRNA genes [5–7]. This unusual gene organization raises the question as to whether these small transcripts function in mitochondrial ribosomes. The observation that these rRNA subfragments have the potential to form standard rRNA secondary structures through inter-molecular base pairing suggests that they could function in the *Chlamydomonas* mitochondrial ribosomes as a non-covalent network of small RNAs. In the case of *C. reinhardtii*, moreover, the mitochondrial rRNAs are more abundant than the protein-coding transcripts, as would be predicted for functional rRNA molecules [5]. Finally, the fact that *Chlamydomonas* mitochondrial DNA encodes several polypeptides which function in the mitochondrial electron transport chain [6, 8 and references therein] implies that these products are produced by a translational system resident within the *Chlamydomonas* mitochondria. In this paper, using probes specific to the mtDNA encoded SSU and LSU rRNA subfragments of *C. eugametos*, we have identified in sucrose gradients of total cellular ribosomes of *C. eugametos*, fractions which we argue correspond to mitochondrial ribosomes and their subunits. These results, therefore, support the hypothesis that the small transcripts produced by the dispersed and scrambled rRNA coding regions in the *Chlamydomonas* mitochondrial genome function in the assembly of mitochondrial ribosomes.

2. Materials and methods

2.1. Preparation and sucrose gradient fractionation of total cellular ribosomes

Synchronous, phototrophic cultures of wild-type *C. eugametos* (UTEX 9) and *C. reinhardtii* (137c, mt^+) were grown to a cell density of 3–4 × 10⁶ cells/ml as previously described [9]. Six hours after the onset of the light period (L-6), between 1 × 10¹⁰ and 1.6 × 10¹⁰ cells were subjected to centrifugation at 5000 × g for 5 min at 4°C and resuspended to a final concentration of 2 × 10⁹ cells/ml in TKM buffer (25 mM Tris-HCl, pH 7.8, 25 mM KCl, 25 mM MgOAc; 5 mM glutathione). The cells were lysed by two passages through a French Pressure cell (Aminco) at 5000 psi. The cell lysate was subjected to centrifugation at 40,000 × g for 30 min at 4°C; the resulting supernatant was designated as the S40 fraction. Sixty A_{260} units of the S40 fraction or of the pellet resulting from centrifugation of the S40 fraction through a 1.85 M sucrose cushion (134,000 × g for 4 h at 4°C) were layered on top of sucrose step gradients composed of 9, 14, 19, 24, 29 and 34% sucrose

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in either TKM buffer or TK buffer (25 mM Tris-HCl, pH 7.8, 25 mM KCl; 5 mM glutathione) containing either 0 or 5 mM MgOAc. After centrifugation at 22,500 rpm at 2°C in a Beckman SW28 rotor for the times indicated, 0.5 ml fractions of the sucrose gradients were collected using a syringe pump to upwardly displace the gradient, and aliquots of each fraction were analyzed spectrophotometrically.

Another 5 μl aliquot of each fraction was denatured by incubation at 50°C for 60 min in the presence of glyoxal [10], diluted with 20 × SSPE (3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.4) to a final concentration of 5 × SSPE, and applied to a Hybond N (Amersham) membrane in a slot blot manifold. Each slot blot was rinsed twice with 20 × SSPE, allowed to air dry and baked at 80°C under vacuum for 2 h. All solutions and glassware were treated to inhibit the activity of RNases [10]. The apparent S values of sedimenting material in the *C. eugametos* gradients were calculated [11] using as a reference the migration of *C. reinhardtii* cytosolic ribosomes run in parallel gradients containing TKM buffer; these reference ribosomes were assumed to be 83S.

2.2. Hybridization

C. eugametos mitochondrial ribosomes were identified using hybridization probes derived from clones of the *C. eugametos* mtDNA and synthetic oligonucleotide probes. The 2.1 kb *Hind*III (probe L_6/S_2) and 3.2 kb *Sma*I–*Hind*III (probe L_5/S_3) DNA fragments of *C. eugametos* were previously designated probes III and VI, respectively [7]. The sequences of the *C. eugametos* mitochondrial rRNA-specific oligonucleotide probes and their corresponding co-ordinates relative to the numbering of the *E. coli* rRNAs are as follows:

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

Filter-bound RNA isolated from the sucrose gradients was hybridized with ^{32}P-labelled DNA restriction fragments or oligonucleotide probes as described previously [7] except that the final low-salt washes were omitted in hybridizations using oligonucleotide probes.

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

A crude mitochondrial fraction was isolated from synchronous, phototrophically grown wild-type *C. eugametos* cells. The cells were collected at the beginning of the light period (L-0) when the culture density was 2.7 × 10⁶ cells/ml. The cell pellet was washed twice by resuspending the cells in buffer A (300 mM mannitol, 3 mM EDTA, 0.1% bovine serum albumin, 1 mM β-mercaptoethanol, 50 mM Tris, pH 8.0) and subjecting the suspension to centrifugation at 6000 × g. The cells were resuspended to a final density of 1.4 × 10⁸ cells/ml in buffer A and lysed at 3000 psi in the French pressure cell into an equal volume of cold buffer A. The minimum cell pressure required to lyse 95% of the cells, leaving 5% of the cells undamaged or cracked as judged by light microscopy, was chosen to minimize the damage to mitochondria. The cell suspension was subjected to two rounds of centrifugation at 3000 × g for 15 min and the resulting supernatant was subjected to centrifugation at 10,000 × g for 20 min at 4°C. The crude mitochondrial pellet was resuspended in 2.5 ml of buffer A and 10 ml of buffer B (150 mM NaCl, 100 mM EDTA, 10 mM Tris, pH 8.0) and centrifuged twice at 10,000 × g for 20 min at 4°C. The mitochondrial pellet was incubated on ice for 15 min after the addition of 0.2% SDS, 100 mM Tris, pH 9.0. The RNA was then purified by phenol extraction and subjected to denaturing polyacrylamide gel electrophoresis and ethidium bromide staining as described previously [7].

3. Results

3.1. Identification of mitochondrial ribosomes within the population of total C. eugametos cellular ribosomes

Total cellular *C. eugametos* ribosomes, partially purified by centrifugation through a 1.85 M sucrose cushion, were fractionated by sucrose gradient centrifugation and aliquots of the
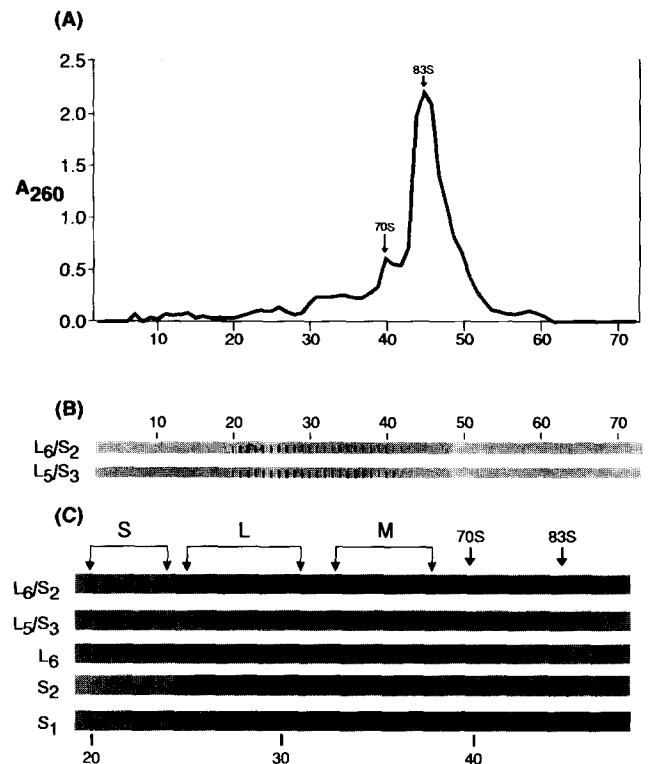

Fig. 1. (A–C) Sucrose gradient fractionation of *C. eugametos* total cellular ribosomes in buffers containing 25 mM Mg^{2+} and identification of fractions with probes specific to mitochondrial rRNA. Total cellular ribosomes were layered on top of the sucrose gradient and subjected to centrifugation at 22,500 rpm in a Beckman SW28 rotor at 2°C for 8 h; the fractions (indicated below A and C and above B) are numbered in ascending order from the top of the gradient. (A) Absorbance of gradient fractions at 260 nm; the positions of the chloroplast (70S) and cytosolic (83S) ribosome UV-absorbing peaks are indicated. (B) Slot blot hybridization analysis with radio-labelled *C. eugametos* mitochondrial DNA restriction fragments containing the majority of the coding regions of rRNA gene pieces L_6 and S_2 (L_6/S_2) or L_5 and S_3 (L_5/S_3). (C) Slot blot hybridization analysis of fractions 20–48. The results with probes L_6/S_2 and L_5/S_3 are repeated from above. The same hybridization membranes were stripped and reprobed with oligonucleotides specific to the mitochondrial rRNA subfragments L_6, S_2 and S_1. Fractions containing putative mitochondrial ribosome small subunit (S), large subunit (L) and monosome fractions (M) are indicated as are the positions of chloroplast (70S) and cytosolic (83S) ribosomes.

gradient were subjected to spectrophotometric analysis. Two UV-absorbing peaks were observed with apparent sedimentation coefficients of 70S and 83S (Fig. 1A) which presumably represent the chloroplast and cytosolic ribosome fractions (based on comparisons with *C. reinhardtii* ribosomes). No other UV absorbance peaks significantly above background absorbance were observed within the sucrose gradients. Slot blot hybridization analysis was employed to identify mitochondrial rRNA-containing fractions of this gradient with radio-labelled restriction fragments of *C. eugametos* mtDNA containing the coding regions for the LSU rRNA species L_6 and the SSU rRNA species S_2 (probe L_6/S_2) and the coding regions for LSU rRNA species L_5 and SSU rRNA species S_3 (probe L_5/S_3) and with radio-labelled oligonucleotides complementary to *C. eugametos* mitochondrial rRNA species S_1, S_2, L_4, L_5 and L_6 [7]. The hybridization conditions employed here were the same as those employed previously [7] which showed that these
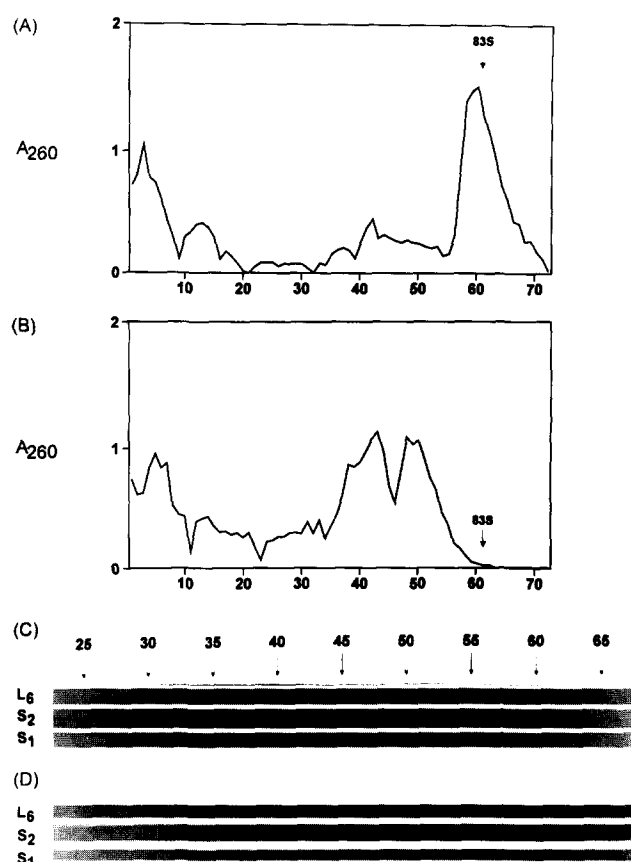
Fig. 2. (A–D) Sucrose gradient fractionation of *C. eugametos* total cellular ribosomes in buffers containing 5 and 0 mM Mg^{2+} and identification of fractions with probes specific to mitochondrial rRNA. Total cellular ribosomes were subjected to sucrose gradient centrifugation as before (Fig. 1) except that the centrifugation time was increased to 14 h; fractions (indicated below A and B and above C) are numbered in ascending order from the top of the gradient. Absorbance at 260 mM of fractions from (A) 5 mM and (B) 0 mM Mg^{2+} gradients. Slot blot hybridization analysis of fractions from the (C) 5 mM and (D) 0 mM Mg^{2+} gradients with radio-labelled oligonucleotides specific to the mitochondrial rRNA subfragments L_6, S_2 and S_1. The fractions corresponding to the UV absorbance of cytosolic (83S) ribosomes from a parallel gradient containing 25 mM Mg^{2+} are indicated as a reference.

probes hybridized specifically to the corresponding mitochondrial SSU and LSU rRNA subfragments. As shown in Fig. 1B, the L_6/S_2 and L_5/S_3 probes both hybridized with the same sucrose fractions and these hybridizing fractions showed no correspondence with the distribution of chloroplast or cytosolic ribosomes. On close inspection, the hybridizing fractions in Fig. 1B resolve into three overlapping sets of fractions (Fig. 1C) which we propose correspond to the small subunit (S), large subunit (L) and monosome (M) mitochondrial ribosome components of *C. eugametos*. In further experiments, the radiolabelled probes were removed from the membranes and end-labelled oligonucleotides complementary to L_6, S_2 and S_1 were individually allowed to anneal to the same denatured RNA fractions of the gradient. The oligonucleotide probes specific to S_1 and S_2 both hybridized exclusively with the proposed small subunit and monosome mitochondrial ribosome fractions while the oligonucleotide probe specific to L_6 (and those specific to L_4 and L_5, data not shown) hybridized exclusively to the pro-

posed large subunit and monosome mitochondrial ribosome fractions. Based on the three or four most intensely hybridizing fractions associated with the proposed small unit (S), large subunit (L) or monosome (M) mitochondrial ribosome fractions in Fig. 1C, we calculate the apparent sedimentation coefficient of these particles to be 35–39S, 44–50S and 60–66S, respectively. An accurate determination of these values, however, would require purification of the mitochondrial ribosome components and analytical ultracentrifugation. Judged by the relative hybridization signals, approximately 50% of the mitochondrial ribosomes are dissociated into ribosomal subunits in the TKM buffer employed (25 mM Mg^{2+}).

3.2. Effect of lowered Mg^{2+} concentrations on the dissociation of mitochondrial ribosomes

Total cellular ribosomes were prepared for fractionation in 5 and 0 mM Mg^{2+} sucrose gradients as described above except that the partial purification by centrifugation through a 1.85 M sucrose cushion was omitted during these ribosome isolations to ensure that small mitochondrial rRNAs or ribonucleoprotein particles were not excluded from hybridization analysis. In addition, to better resolve such slower sedimenting material, sucrose density gradients were centrifuged longer than in the previous experiments. The UV-absorbance profiles of these fractionations is shown in Fig. 2A and 2B. At 5 mM Mg^{2+} the 70S chloroplast peak was absent and a broad peak of 50–60S appeared in the UV profile of the ribosome population. When the Mg^{2+} concentration was lowered to 0 mM the 83S cytosolic ribosome class disappeared and the majority of the UV-absorbing material was 30–60S as reported for *C. reinhardtii* [12]. Slot blots of denatured *C. eugametos* RNA from aliquots of the gradients containing 5 and 0 mM Mg^{2+} were prepared and allowed to anneal to oligonucleotides specific for the *C. eugametos* rRNAs L_6, S_2 and S_1. As shown in Fig. 2C and 2D, the S_2 and S_1 specific probes hybridized most intensely to fractions 35–38 (40–44S) and 34–37 (38.5–42.5S) in the 5 and 0 mM Mg^{2+} gradients, respectively, while the L_6 specific probe (and those specific to L_4 and L_5, data not shown) hybridized most intensely to fractions 40–43 (47–51S) and 39–41 (45.5–48.5S) in the 5 and 0 mM Mg^{2+} gradients, respectively. No hybridization of the probes was detected to any other fractions including those from fractions 49–52 which correspond to 60–66S. These results indicate that the *C. eugametos* mitochondrial ribosomes were completely dissociated into subunits when the Mg^{2+} concentration was reduced to 5 and 0 mM. Because small ribonucleoprotein (RNP) particles and free rRNA were not removed by centrifugation through a sucrose cushion prior to sucrose gradient fractionation using the 5 and 0 mM Mg^{2+} concentrations, and because no hybridization was observed to the fractions isolated from the top (left) of the sucrose gradient, it appears that very little if any of the mitochondrial rRNAs screened are present as free rRNA. Moreover, it appears that once the monosomes have dissociated into ribosomal subunits, the subunits do not further dissociate into smaller RNP particles.

3.3. Crude mitochondrial fractions are enriched for mitochondrial rRNA subfragments

The six LSU and three SSU mitochondrial rRNA transcripts corresponding to gene pieces of *C. eugametos* mtDNA were identified by slot blot hybridization analysis of total cellular RNA with mtDNA restriction fragments and oligonucleotide
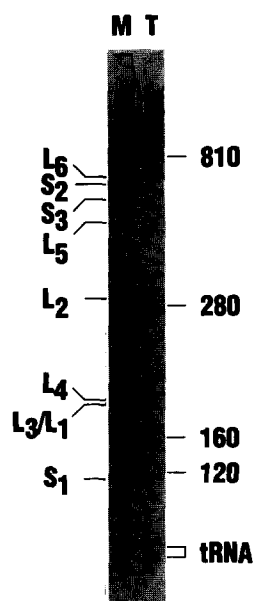
Fig. 3. Gel electrophoresis and ethidium bromide staining of RNA isolated from a mitochondrial sub-cellular fraction of *C. eugametos*. Total cellular RNA (T) and mitochondrial-enriched (M) RNA (2 μg of each) was fractionated in denaturing polyacrylamide gels and stained with ethidium bromide. The positions of the cytosolic and chloroplast 5S rRNA (120 nt), cytosolic 5.8S rRNA (160 nt), chloroplast LSU rRNA α fragment (280 nt), chloroplast LSU rRNA γ fragment (810 nt) and tRNAs (60–80 nt) and the *C. eugametos* mitochondrial RNAs (S₁–S₃ and L₁–L₆) identified by size are indicated [4,7,17].

probes specific to these rRNAs [7]. In order to confirm the mitochondrial association of these rRNA subfragments, we compared their abundance in RNA prepared from crude mitochondrial pellets and whole cells. Following polyacrylamide gel electrophoresis and ethidium bromide staining (Fig. 3), none of the mitochondrial rRNA subfragments are visible in the total cellular RNA preparation. For the mitochondrial enriched RNA sample, however, molecules of the sizes reported [7] for subfragments L_2, L_5, L_6, S_2 and S_3 are clearly visible; rRNA subfragments smaller than 280 nucleotides and corresponding to L_1, L_3, L_4 and S_1 are only barely visible or not visible, presumably because of their small size. The yield of mitochondrial-enriched *C. eugametos* RNA was approximately 0.1% of the amount of total cellular RNA isolated from a similar number of cells.

4. Discussion

Using probes specific to the mtDNA encoded rRNA subfragments of *C. eugametos* we have identified what appear to be mitochondrial ribosomes and their subunits in sucrose density gradients of *C. eugametos* total cellular ribosomes. Confirmation of the mitochondrial association of these subfragments was provided by an enrichment of these RNAs in crude mitochondrial pellets. Under conditions of 25 mM Mg^{2+}, the apparent sedimentation coefficient of the mitochondrial ribosomes at approximately 60–66S is distinct from that of the chloroplast (70S) and cytosolic (83S) ribosomes of *C. eugametos*, as characterized here, and of *C. reinhardtii* as reported in the literature [4]. In terms of their sedimentation value, the *C.*

eugametos mitochondrial ribosomes contrast with the 77–78S ribosomes of land plant mitochondria [13, 14] and appear intermediate between the 55–60S ribosomes of animal mitochondria and the 70–74S mitochondrial ribosomes of *Euglena* and fungi [3]. At 25 mM Mg^{2+}, the chloroplast and cytosolic ribosomes of *C. reinhardtii* [12], and apparently those of *C. eugametos*, are not appreciably dissociated. At this Mg^{2+} concentration approximately half of the *C. eugametos* mitochondrial ribosomes were dissociated into small and large subunits. It remains to be determined whether the high proportion of dissociated mitochondrial ribosomes at 25 mM Mg^{2+} reflects the *in vivo* state of these ribosomes or is an artifact resulting from the conditions employed. At 5 and 0 mM Mg^{2+} the mitochondrial ribosomes are fully dissociated into subunits and these subunits do not appear to dissociate into smaller RNP particles, suggesting that under these conditions there is not a reversible association between rRNA subfragments or small RNP particles within the mitochondrial ribosomal subunits. Similarly, *C. reinhardtii* chloroplast large ribosomal subunits are stable in the absence of Mg^{2+} [12] despite the demonstration that the chloroplast LSU rRNA is composed of four individual species [15–17]. The coding regions for these chloroplast LSU RNA subfragments are organized in the conventional 5′ to 3′ order as continuous rRNA genes and the individual mature transcripts they produce are liberated by the post-transcriptional removal of internal spacer sequences from large precursor RNA.

Because the mitochondrial ribosomal subunits and monosomes are such a minor component of total steady-state cellular ribosomes in *C. eugametos* it is not surprising that we were unable to detect precursors of mitochondrial ribosomes or their subunits. As a result, we cannot distinguish between the hypotheses that mitochondrial ribosome biogenesis in *Chlamydomonas* proceeds either by the association of small RNP particles composed of individual mitochondrial rRNAs and ribosomal-proteins [5] or that a transcript(s) containing interspersed rRNA domains form(s) the ribosomal subunit cores through base-pairing in larger RNP particles that are later processed to generate the mature mitochondrial rRNA termini.

Boer and Gray [5], in considering whether the various LSU and SSU rRNA gene pieces identified in *Chlamydomonas* mtDNA are functional, suggested that rigorous proof for such a conclusion would require a demonstration that the RNAs transcribed from these coding regions are indeed present in subunits of *Chlamydomonas* mitochondrial ribosomes and that such ribosomes are active in mitochondrial protein synthesis. Based on the results presented here, we propose that the first of these steps has been accomplished. A positive answer to the second of these steps would be technically difficult because of the relatively low number of mitochondrial ribosomes in *Chlamydomonas* cells and because of the lack of any in vitro translational system involving land plant or algal mitochondrial ribosomes.

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