Discovery and characterization of *Acanthamoeba castellanii* mitochondrial 5S rRNA

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ABSTRACT

Although 5S rRNA is a highly conserved and universal component of eubacterial, archaeal, chloroplast, and eukaryotic cytoplasmic ribosomes, a mitochondrial DNA-encoded 5S rRNA has so far been identified only in land plants and certain protists. This raises the question of whether 5S rRNA is actually required for and used in mitochondrial translation. In the protist *Acanthamoeba castellanii*, BLAST searches fail to reveal a 5S rRNA gene in the complete mitochondrial genome sequence, nor is a 5S-sized RNA species detectable in ethidium bromide-stained gels of highly purified mitochondrial RNA preparations. Here we show that an alternative visualization technique, UV shadowing, readily detects a novel, mitochondrion-specific small RNA in *A. castellanii* mitochondrial RNA preparations, and that this RNA species is, in fact, a 5S rRNA encoded by the *A. castellanii* mitochondrial genome. These results emphasize the need for caution when interpreting negative results that suggest the absence of 5S rRNA and/or a mitochondrial DNA-encoded 5S rRNA sequence in other (particularly protist) mitochondrial systems.

Keywords: Protist; mtDNA; ribosome

INTRODUCTION

5S rRNA is a highly conserved and universal component of eubacterial, archaeal, plastid, and eukaryotic cytoplasmic ribosomes. This small (~120 nt) structured RNA interacts with ribosomal proteins (L5 in eukaryotes; L5, L18, and L25 in bacteria and organelles), and the resulting RNA–protein complex is found in the large ribosomal subunit (Moore 1996). Despite the fact that 5S rRNA was discovered some 40 years ago (Rosset and Monier 1963), its function is still not precisely defined; what is known is that the 5S ribonucleoprotein complex contributes importantly, albeit indirectly, to many of the functions of large ribosomal subunits that contain it (Moore 1996).

Surprisingly, in view of its otherwise ubiquitous distribution, 5S rRNA appears not to be universally present in mitochondrial systems. Plant mitochondrial ribosomes do contain a distinctive 5S rRNA species (Cunningham et al. 1976; Leaver and Harmey 1976; Spencer et al. 1981), encoded by the mitochondrial genome (Bonen and Gray 1980; Oda et al. 1992; Unseld et al. 1997; Kubo et al. 2000). A recognizable 5S rRNA gene is also present in some protist mitochondrial genomes, notably those of certain green, red, and brown algae (Wolff et al. 1994; Ohta et al. 1998; Burger et al. 1999; Turmel et al. 1999, 2002a, 2000b; Oudot-Le Secq et al. 2001, 2002) and jakobid flagellates (Lang et al. 1996, 1999). However, an obvious 5S rRNA gene has not been identified in other protist mtDNAs (Gray et al. 1998), or in any of the more than 100 animal mitochondrial genomes completely sequenced to date. Nor has a 5S rRNA species been detected in isolated animal mitochondrial ribosomes (O'Brien and Denslow 1996). By the same token, fungal mitochondrial systems evidently lack a 5S rRNA component (Lizardi and Luck 1971), although the possibility of preparative loss of 5S rRNA during isolation of fungal mitochondrial ribosomes has been debated (Datema et al. 1974; Michel et al. 1977).

A number of explanations could account for the absence of 5S rRNA in any given mitochondrial translation system. For example, the functional role of 5S rRNA may simply be dispensable in some cases. Other possibilities are that the functional role of 5S rRNA has been assumed by other ribosomal components (ribosomal proteins?) or that a 5S-equivalent sequence is covalently imbedded in the sequence of the large subunit rRNA (Nierlich 1982; Thurlow et al. 1984). There is no evidence to support the former suggestion, whereas the latter can be discounted by comparative analysis of rRNA secondary structure (Lang et al. 1987;

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Gutell et al. 1993). A fourth possibility is that the function of 5S rRNA has been assumed in mitochondria by an extramitochondrial 5S rRNA species imported into mitochondria. In this regard, intriguing recent evidence suggests that nucleus-encoded (cytoplasmic) 5S rRNA is a bona fide intramitochondrial component in animals (Yoshionari et al. 1994; Magalhães et al. 1998), and that human mitochondria are able to import 5S rRNA (Entelis et al. 2001).

On the other hand, a mitochondrial 5S rRNA species may be present but be too divergent in sequence and higher-order structure to be readily recognized as such. We present here one such case—in the amoeboid protist, *Acanthamoeba castellanii* —where previous approaches, both computational and experimental, were unable to detect either a 5S rRNA gene in the completely sequenced mitochondrial DNA or a mitochondrion-specific 5S rRNA.

RESULTS AND DISCUSSION

When *A. castellanii* RNA fractions were resolved by polyacrylamide gel electrophoresis, UV shadowing (Hassur and Whitlock 1974) revealed the presence of a novel, highly abundant, small RNA species (X) in purified mitochondrial (but not cytoplasmic) RNA (Fig. 1A). Species X was, however, invisible when gels were stained with ethidium bromide (Fig. 1B), an intercalating agent whose interaction with nucleic acids is strongly affected by the degree and stability of base pairing. Detection and isolation of species X

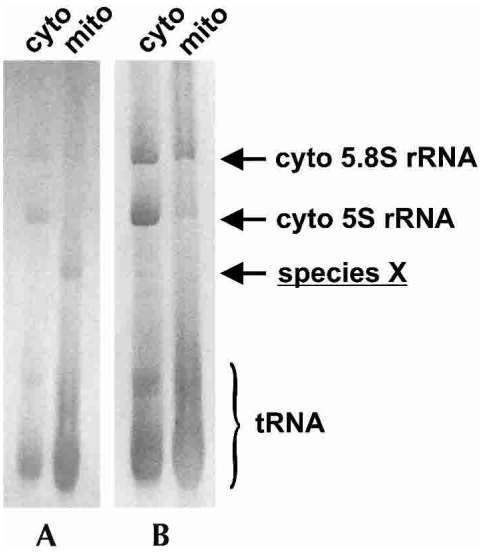

FIGURE 1. Visualization by (*A*) UV shadowing and (*B*) ethidium bromide staining of *A. castellanii* cytoplasmic (cyto) and mitochondrial (mito) RNAs separated on a 10% polyacrylamide gel. Note: The low-abundance RNA in the mitochondrial RNA preparation that comigrates with cytoplasmic 5.8S rRNA has a 3′-terminal sequence identical to that of cytoplasmic 5.8S rRNA (not shown). Our data indicate that this RNA has a single 3′-terminal U residue, whereas the number of U residues is ambiguous in the published sequence (MacKay and Doolittle 1981).

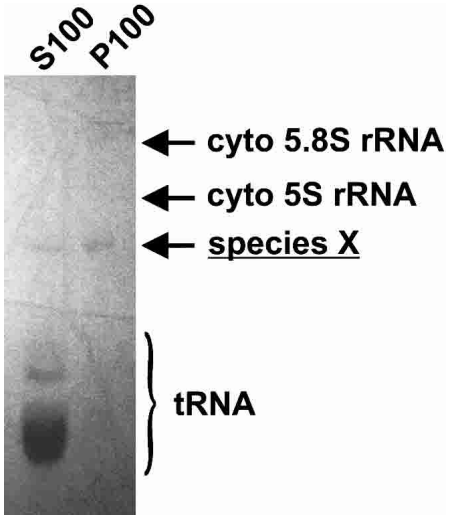

FIGURE 2. Visualization by UV shadowing of RNAs isolated from the supernatant (S100) and pellet (P100) of a 100,000*g* ultracentrifugation of a clarified Triton X-100 lysate of purified *A. castellanii* mitochondria. RNAs were separated on a 10% polyacrylamide gel. The positions of *A. castellanii* cytoplasmic (cyto) 5.8S and 5S rRNA markers are indicated.

was simplified by the unusually slow migration (relative to other 5S rRNAs) of *A. castellanii* cytoplasmic 5S rRNA (length 119 nt) under the gel electrophoresis conditions used.

The size and abundance of species X suggested that it could be a mitochondrial 5S rRNA. To test this hypothesis, we investigated the mitochondrial localization of this RNA by subjecting a clarified Triton X-100 lysate of purified *A. castellanii* mitochondria to centrifugation at 100,000*g* for 1.5 h. We expected that if species X is a bona fide 5S rRNA, it should remain associated with ribosomes and appear in the 100,000*g* pellet under the conditions used in this study. RNA was prepared from both the supernatant (S100) and pellet (P100) fractions, and, as expected, a significant portion of species X was found in the P100 (Fig. 2). The species X present in the S100 might be due to incomplete sedimentation of mitochondrial ribosomes; alternatively, a low-molecular-weight ribonucleoprotein complex containing species X (a putative 5S rRNA) may have dissociated from a fraction of the ribosomes (see Moore 1996). Localization of tRNAs exclusively in the S100 confirms that the high-speed centrifugation did not pellet small RNA species that are not associated with large complexes. Together, these observations support the hypothesis that species X is associated with mitochondrial ribosomes.

To further characterize this novel ribosomal component, RNA sequence data were obtained. Both 3′-end-labeling (Fig. 3A) and 5′-end-labeling (Fig. 3C) of isolated species X resulted in four labeled RNA species (X1–X4), differing in length by 1 nt. By chemical sequencing of the 3′-end-labeled RNAs (Fig. 3B), the four RNAs were shown to have exactly the same 3′ termini, indicating that they must be

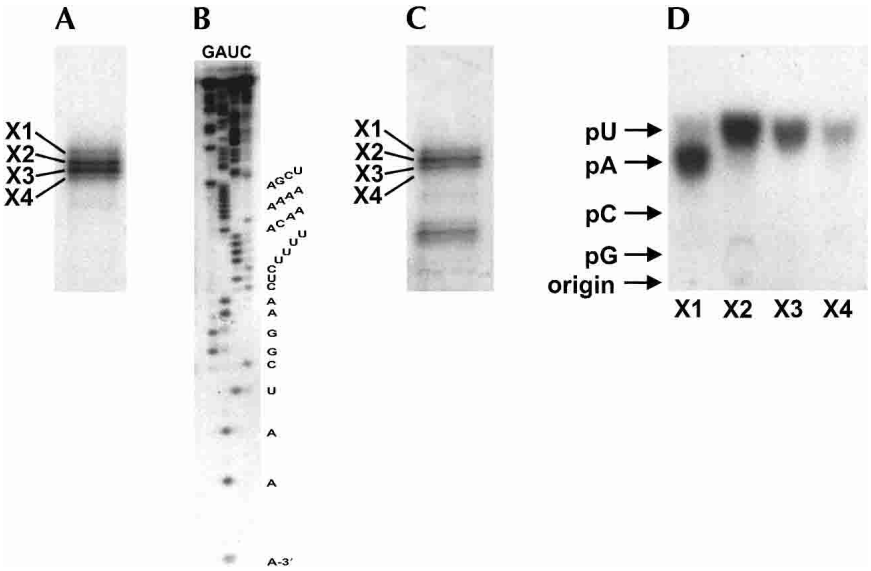

FIGURE 3. (*A*) Electrophoretic separation of 3′-end-labeled species X and (*B*) chemical sequencing of these labeled RNAs. A representative 20% polyacrylamide gel for RNA band X4 is displayed, with a portion of the deduced RNA sequence shown on the right. (*C*) Electrophoretic separation of 5′-end-labeled species X and (*D*) one-dimensional thin-layer chromatography of the products of P1 nuclease digestion of the four largest 5′-end-labeled RNAs (X1–X4). The positions of nucleoside 5′-monophosphate markers are indicated. Autoradiograms are shown in all cases.

heterogeneous at their 5′ends. Terminal analysis of 5′-end-labeled RNAs (Fig. 3D) revealed that the three shortest length variants (X2–X4, 116–118 nt) contained a U residue at their 5′ termini, whereas the longest variant (X1, 119 nt) had a 5′-terminal A. Because chemical sequencing gels of the 3′-end-labeled RNAs could be read to within a stretch of U residues near their 5′ ends, these combined analyses yielded RNA sequence data for every position in the molecule, demonstrating that species X is distinctly different in sequence from the nucleus-encoded cytoplasmic 5S rRNA of *A. castellanii* (MacKay and Doolittle 1981).

Alignment of the experimentally determined RNA sequence of species X with mitochondrial and eubacterial 5S rRNA sequences (Fig. 4A) revealed nucleotide similarity within a region highly conserved in other 5S rRNAs (outside of this stretch, very little primary structure conservation is evident among mitochondrial 5S rRNAs in general). In addition to displaying a diagnostic primary sequence motif, the RNA sequence of species X can be folded into a secondary structure (Fig. 4B) consistent with the consensus 5S rRNA secondary structure (Moore 1996). These data strongly suggest that species X is indeed a mitochondrial 5S rRNA. The high A + U content of this 5S rRNA (78%) with concomitant relatively weak base pairing in stem regions presumably accounts for the failure of ethidium bromide to bind efficiently to this RNA species in gels.

A search of the complete mtDNA sequence of *A. castellanii* (Burger et al. 1995; GenBank accession number NC_001637) located the mitochondrial 5S rRNA gene (*rrn5*) within a previously unassigned 240-nt spacer between the *cox1/2* and *rps4* genes. The 5′ ends of this molecule map 109–112 nt downstream from the 3′ end of the *cox1/2* reading frame, whereas the 3′ end maps 22 nt upstream of the 5′ end of the *rps4* coding region. The *rrn5* gene is in the same transcriptional orientation as all other genes in this mitochondrial genome. When the *A. castellanii* mitochondrial 5S rRNA sequence was used as a query in BLAST searches of public domain databases, no other 5S rRNA sequences (including any of the known mitochondrial ones) were detected.

CONCLUSIONS

Even though the *A. castellanii* mitochondrial 5S rRNA displays a degree of primary and secondary structure conservation clearly sufficient to mark it as a homolog of other 5S rRNAs, extensive analysis of the complete mtDNA sequence (Burger et al. 1995) had previously failed to identify the corresponding gene. The 5S RNA species itself had also escaped detection over a number of years in experiments where ethidium bromide was routinely used to visualize gel-purified *A. castellanii* mitochondrial RNA species. The results reported here suggest that a 5S rRNA species may be encoded in more mitochondrial genomes than is currently appreciated. Clearly, direct characterization of mitochondrial RNAs by several methods remains the most reliable approach to identifying mitochondrial 5S rRNAs and their genes.

In this regard, it is noteworthy that even with the *A. castellanii* mitochondrial 5S rRNA sequence in hand, we are not able to identify a homologous sequence in the mitochondrial genome of *Dictyostelium discoideum* (Ogawa et al. 2000), a member of the same protist phylum (Amoebozoa) to which *A. castellanii* belongs (Cavalier-Smith 1998). The *A. castellanii* and *D. discoideum* mitochondrial genomes share a number of features in common, including an almost identical gene content, a single open reading frame (*cox1/cox2*) encoding subunits 1 and 2 of cytochrome oxidase, and a similar set of tRNA genes whose transcripts require the same type of 5′ editing (Burger et al. 1995; Ogawa et al. 2000). *D. discoideum* mtDNA does encode a 129-nt RNA species (Pi et al. 1998) that, however, lacks the diagnostic primary and secondary structural features of a conventional 5S rRNA, and which displays no convincing sequence similarity to the *A. castellanii* mitochondrial 5S rRNA sequence described here. The fact that this novel RNA component does not appear to be associated with *D. discoideum* mito-

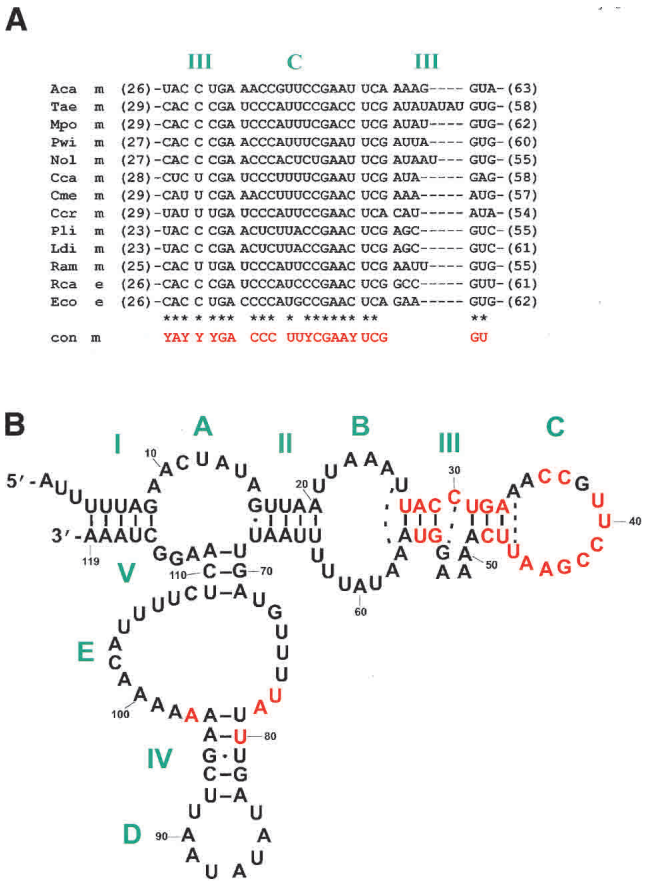

FIGURE 4. (*A*) Alignment of a conserved portion of mitochondrial (m) and eubacterial (e) 5S rRNA sequences. Sequences used (GenBank accession number in parentheses): *Acanthamoeba castellanii* (Aca; this work), *Triticum aestivicum* (Tae; M10361), *Marchantia polymorpha* (Mpo; M68929), *Prototheca wickerhamii* (Pwi; U02970), *Nephroselmis olivacea* (Nol; AF110138), *Cyanidium caldarium* (Cca; Z48930), *Cyanidioschyzon merolae* (Cme; D89861), *Chondrus crispus* (Ccr; Z47547), *Pylaiella littoralis* (Pli; AJ277126), *Laminaria digitata* (Ldi; AJ344328), *Reclinomonas americana* (Ram; U59762), *Rhodobacter capsulata* (Rca; X04585), and *Escherichia coli* (Eco; X00414). The spacing in the alignment represents the alternation of single- and double-stranded regions. Dashes (-) indicate alignment gaps. Numbers in parentheses indicate nucleotides not shown. The consensus (con m) indicates positions that are either identical in at least 9 of the 11 mitochondrial sequences or of the same type (Y = pyrimidine, R = purine) in all 11 mitochondrial sequences. Asterisks indicate positions in the mitochondrial consensus that are also conserved in the eubacterial sequences. (*B*) Potential RNA secondary structure of the longest sequence variant of the *A. castellanii* mitochondrial 5S rRNA. Red indicates nucleotides identical to the mitochondrial consensus. Additional potential base-pairing is indicated by broken lines. Helices I to V and loops A to E are denoted as in Burger et al. (1999).

chondrial ribosomes (Pi et al. 1998) argues against the possibility that it is a nonhomologous but functional equivalent of a conventional 5S rRNA. If *D. discoideum* mitochondrial ribosomes do in fact lack the equivalent of a 5S rRNA component (mtDNA-encoded or otherwise), this disparity between two otherwise very similar amoebozoan mitochondrial systems would strengthen the view that the mitochondrial translation system is unusually flexible in its requirement for a 5S rRNA component.

MATERIALS AND METHODS

Isolation of mitochondrial and cytoplasmic RNA

A. castellanii strain Neff (ATCC 30010) was grown at 30°C with moderate shaking to an $O.D._{550}$ of ~1.0. Mitochondria were purified (Price and Gray 1999) and cytoplasmic and mitochondrial RNAs were isolated as described (Spencer et al. 1992). RNAs were separated on a 1.5-mm-thick 10% polyacrylamide gel (all polyacrylamide gels used in this study contained 7 M urea; Spencer et al. 1992) and eluted from a homogenized gel slice by shaking overnight at 4°C in a 1:1 mixture of phenol-cresol:buffer [0.5 M NH_4OAc, 10 mM $Mg(OAc)_2$, 1.0 mM EDTA]. RNAs were precipitated twice with ethanol, redissolved in water, and stored at −20°C in 50% ethanol.

Fractionation of mitochondria

Purified mitochondria were gently lysed in a solution containing 2% Triton X-100, 10 mM Tris-HCl (pH 8.5), 50 mM KCl, and 10 mM $MgCl_2$ (Spencer et al. 1992) and centrifuged at 9,000*g* in a fixed-angle rotor for 10 min. The clarified supernatant was further fractionated by ultracentrifugation at 100,000*g* for 1.5 h in a fixed-angle rotor. RNAs were separated on a 1.5-mm-thick 10% polyacrylamide gel and visualized by UV shadowing (Hassur and Whitlock 1974).

Chemical sequencing of RNA

RNAs were 3′-end-labeled with [5′-^{32}P]pCp and RNA ligase (Peattie 1979) and purified on a 6% polyacrylamide sequencing gel. Chemical sequencing reactions were performed as described (Peattie 1979), and products were resolved in 6% and 20% polyacrylamide gels.

5′-End analysis of RNA

RNAs were 5′-end labeled with [γ-^{32}P]ATP and polynucleotide kinase (Schnare et al. 1985) and purified as for the products of 3′-end-labeling. Labeled products were excised and extracted from the gel and treated with P1 nuclease, which generates nucleoside 5′ monophosphates (pN). The products of P1 digestion were separated by one-dimensional thin-layer chromatography using cellulose plates (predipped in a 10% dilution of a saturated solution of NH_4SO_4) and a 4:1 mixture of 95% ethanol:water as the solvent (Lane 1963).

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