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Nucleotide sequences of Acanthamoeba castellanii 5S and 5.8S ribosomal ribonucleic acids: phylogenetic and comparative structural analyses

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ABSTRACT

Sequences of 5S and 5.8S rRNAs of the amoeboid protist *Acanthamoeba castellanii* have been determined by gel sequencing of terminally-labelled RNAs which were partially degraded with chemical reagents or ribonucleases. The sequence of the 5S rRNA is GGAUACGGCCAUACUGCGCAGAAAGCACCGCUUCCCAUCCGAACAGCG AAGUUAAGCUGCGCCAGGCGGUGUUAGUACUGGGGUGGGCGACCACCCGGGAAUCCACCGUGCCGUAUCCU$_{OH}$. This sequence is compared to eukaryotic 5S rRNA sequences previously published and fitted to a secondary structure model which incorporates features of several previously proposed models. All reported eukaryotic 5S rRNAs fit this model. The sequence of the 5.8S rRNA is AACΨCCUAACAACmGGAΨAUCUUGGUUCUCGCGAG GAUGAAGAmACGCAGCGAAAUGCGAUACGUAGUGUGAAUCGCAGGGAUCAGUGAAmUCAΨCGAAUCUΨUGAACGCA AGUUGCGCUCUCGUGGUUUAACCCCCCGGGAGCACGUUCGCUUGAGUGCCGCU(U)$_{OH}$. This sequence does not fit parts of existing secondary structure models for 5.8S rRNA, and we question the significance of such models.

INTRODUCTION

The large subunits of eukaryotic cytosol ribosomes contain 5S and 5.8S ribosomal ribonucleic acids (rRNAs) with lengths of approximately 120 and 160 nucleotides respectively. The ubiquity of these molecules within the eukaryotes makes them valuable for the analysis of phylogenetic relationships and for the comparative study of structure. Diverse eukaryotic 5S rRNAs have been sequenced, making possible the construction of phylogenies [1-3] and secondary structure models [2,4-6]. Reported nucleotide sequences for 5.8S rRNAs (or rRNA genes) have been limited to a variety of vertebrates, two insects, two ascomycetes, two flowering plants and a dinoflagellate protist [3,7-9]. The kingdom Protista (unicellular algae, slime molds and protozoa) is likely the most ancient and diverse of all eukaryotic kingdoms [10,11] and is therefore especially interesting for the study of 5S and 5.8S rRNAs. Indeed, recently published 5S rRNA sequences for a ciliated protozoan [12] and a

flagellated protozoan [13] indicate that, for this particular molecule, as much sequence diversity exists *within* the protists as exists *between* the protist, animal, plant and fungal kingdoms [13]. Sequencing of the 5S and 5.8S rRNA of the amoeboid protist *Acanthamoeba castellanii* should therefore provide valuable diversity for both phylogenetic and comparative structural analyses.

MATERIALS AND METHODS

Preparation of 5S and 5.8S rRNA. *Acanthamoeba castellanii* (ATCC 30010) was cultured for 10 days at 26°C with moderate agitation, in 2ℓ of the optimal growth medium of Neff *et al.* [14]. Cells harvested by centrifugation were stored at -70°C. Total RNA was extracted after suspending the cells in 20 volumes of 50 mM Tris-HCl pH 8.0, as described previously [15]. RNA soluble in 3M NaCl at 0°C (5S and tRNA) was, after ethanol precipitation, dried *in vacuo*, dissolved to 10 mg/ml in loading buffer [16] and resolved on 20 x 20 x 0.3 cm 10% polyacrylamide slab gels [17]. The 5S band was identified by UV-absorbance and eluted from gel slices by electrophoresis [13]. Eluted RNA was extracted once with buffer-saturated phenol [18], made 0.3 M in sodium acetate and precipitated with ethanol at -20°C. To purify 5.8S rRNA, total RNA insoluble in 3 M NaCl at 0°C was dissolved in 0.3 M sodium acetate, precipitated at -20°C with ethanol, dried *in vacuo*, dissolved to 4 mg/ml in loading buffer [16], heated to 60°C for 3 min to dissociate the 5.8S from the 26S rRNA and resolved on a composite polyacrylamide slab gel [9]. The 5.8S band was identified, eluted and precipitated as above for 5S rRNA.

Preparation of terminally-labelled RNA. 5S and 5.8S rRNAs (5-10 µg per reaction mix) were labelled at the 5'- and 3'-termini as described previously [13], using the protocols of Donis-Keller *et al.* [17] and Peattie [16] respectively. For 3'-labelling, the pCp : RNA : ATP molar ratio was 1 : 2 : 10. Labelled RNAs were resolved on 33 x 40 x 0.15 cm, 7M urea, 6% polyacrylamide gels (acrylamide : bis-acrylamide; 19 : 1), located by autoradiography and eluted as above.

Terminal nucleotide analyses. Not more than 2 µg 5'-labelled RNA was digested with 5 µg snake venom phosphodiesterase (prepared according to [19]) in 20 µl 125 mM ammonium formate pH 9.2, for 16-24 h at 37°C. A similar amount of 3'-labelled RNA was hydrolyzed for 90 h at room temperature in 10 µl 1 M NaOH and neutralized by the addition of 1 µl glacial acetic acid. 5'- and 3'-labelled nucleotides were identified by thin layer chromatography and quantitated by scintillation counting [13].

Sequencing of terminally-labelled RNAs. The partial chemical degradation method of Peattie [16] was used only on 3'-labelled RNA, while the partial ribonuclease digestion method of Donis-Keller *et al.* [17], supplemented by the use of ribonuclease PhyM [20], was used for both 3'- and 5'-labelled RNA. Ribonucleases T_1, U_2 and PhyM were obtained from P.L Biochemicals. The compositions of sequencing gels are given in the figure legends. Alkaline digests of terminally-labelled RNA were obtained by incubation of RNA in 20 µl of 0.15 M ammonium hydroxide for 1 min at 90°C followed by lyophilization and suspension of the digest in the buffer used for the ribonuclease digests [17].
Analysis of modified nucleotides in uniformly ^{32}P-labelled 5.8S rRNA. *A. castellanii* was cultured as described above in 50 ml of ATCC medium No. 354 (minus KH_2PO_4) with 7.5 mCi carrier-free [^{32}P]orthophosphate (New England Nuclear). 5.8S rRNA was purified as described above, hydrolyzed in 10 µl 1 M NaOH for 90 h at room temperature, and neutralized with 0.4 µl 90% formic acid. Separation of the hydrolysate into Np (nucleoside 2'(3')-monophosphate) and Nm-Np (alkali-stable dinucleotide) fractions was accomplished on a DEAE cellulose column [21]. The Np fraction (containing base-modified nucleotides) was chromatographed with 2'(3')-nucleotide marker (Ap, Cp, Gp, Up and Ψp) as described previously [9] for *in vivo* labelled mono- and dinucleotides. Labelled nucleotides were identified by autoradiography, scraped from the chromatography plate and quantitated by scintillation counting. The Nm-Np fraction was dissolved in 15 µl H_2O and divided in half. One half was made 100 mM in ammonium formate pH 9.2, and digested with calf intestine alkaline phosphatase (Sigma Chemical Co., 1 unit in 10 µl, 37°C, 30 min) to generate alkali-stable dinucleoside monophosphates (Nm-N); the other half remained Nm-Np. The Nm-Np fraction was chromatographed on glass-backed, cellulose thin layer plates (Merck) with fluorescent indicator, together with about 0.5 µmol Nm-Np marker isolated from wheat embryo 26S and 18S rRNA [22]. The solvent system used was that used for the Np fraction. The Nm-N fraction was chromatographed with wheat embryo Nm-N marker (generated from Nm-Np by phosphatase digestion) on cellulose plus indicator plates developed thrice in the first dimension with solvent system A of [23] and once in the second dimension with the solvent system of [24]. Comparison of autoradiograms with the position of the UV-absorbing markers allowed positive identification of most of the labelled Nm-Np and Nm-N derivatives; however, the compound subsequently identified as Cm-G required further characterization because of the coincidence of the Cm-G and Gm-C markers. The spot containing this dinucleotide was scraped from the glass plate, the labelled material was eluted with two 100 µl

volumes of 0.6 M ammonium hydroxide, lyophilized, and digested with snake venom phosphodiesterase to yield the nucleoside 5'-monophosphate (the 3'-constituent of the dinucleoside). This digest was then subjected to chromatography with pN markers as described above for 5'-terminal nucleotide analysis.

RESULTS

Sequence of Acanthamoeba castellanii 5S rRNA. Fig. 1 shows overlapping sections of autoradiograms used to determine the nucleotide sequence of this 5S rRNA (Fig. 3). Terminally-labelled RNA was digested with ribonucleases (Fig. 1A to 1D) or degraded with chemicals (Fig. 1E). The 5'-terminal nucleotide was found to be entirely pG. Positions 4 and 38 were each confirmed as U and position 51 as G by chemical sequencing of 3'-labelled RNA. The nucleotide sequence of positions 77-92 was determined with 5'-labelled RNA digested with ribonucleases (Fig. 1D) and with chemically degraded 3'-labelled RNA which confirmed each of positions 78 and 88 as C, position 84 as U, and position 90 as A. It is interesting to note that 3'-labelled RNA was unsuitable for sequencing positions 78 to 86 by either ribonuclease digestion (see bottom of Fig. 1C) or chemical degradation methods. The problem in this region is the very close spacing of bands, believed to be due to base-pairing between positions 78 to 86 and nucleotide positions 3' to this region. This view is consistent with the observation that ribonucleases U_2 and PhyM cleave poorly in this region (see Fig. 1D). Nucleotide positions 91 to 119 can be read from Fig. 1E, an autoradiogram of 3'-labelled RNA degraded with chemicals. The identification of each of positions 100 and 115 as A and positions 103 and 112 as C was confirmed by ribonuclease sequencing of 3'-labelled RNA. The U residue at position 119 was consistent with the terminal nucleotide analysis which yielded 91% Up.

Sequence of Acanthamoeba castellanii 5.8S rRNA. Fig. 2 shows overlapping sections of autoradiograms of terminally labelled RNA digested with ribonucleases which were used to determine most of the nucleotide sequence of this RNA. The 5'-terminal nucleotide (not shown) was blurred in Fig. 2A, but was identified by the terminal nucleotide analysis as 95% pA. The presence of weak PhyM and OH^- (alkaline digest) bands at positions 4 and 17 suggested that these residues may be pseudouridines (Ψ). These assignments were strengthened when 3'-labelled RNA degraded with chemicals was found to be resistant at these positions to the U-specific reaction (for previous examples of this resistance see [9] and [16]. Quantitation of the Np fraction of an alkaline digest of 5.8S labelled *in vivo* yielded 3.8 molar copies of Ψp, thus allowing these two

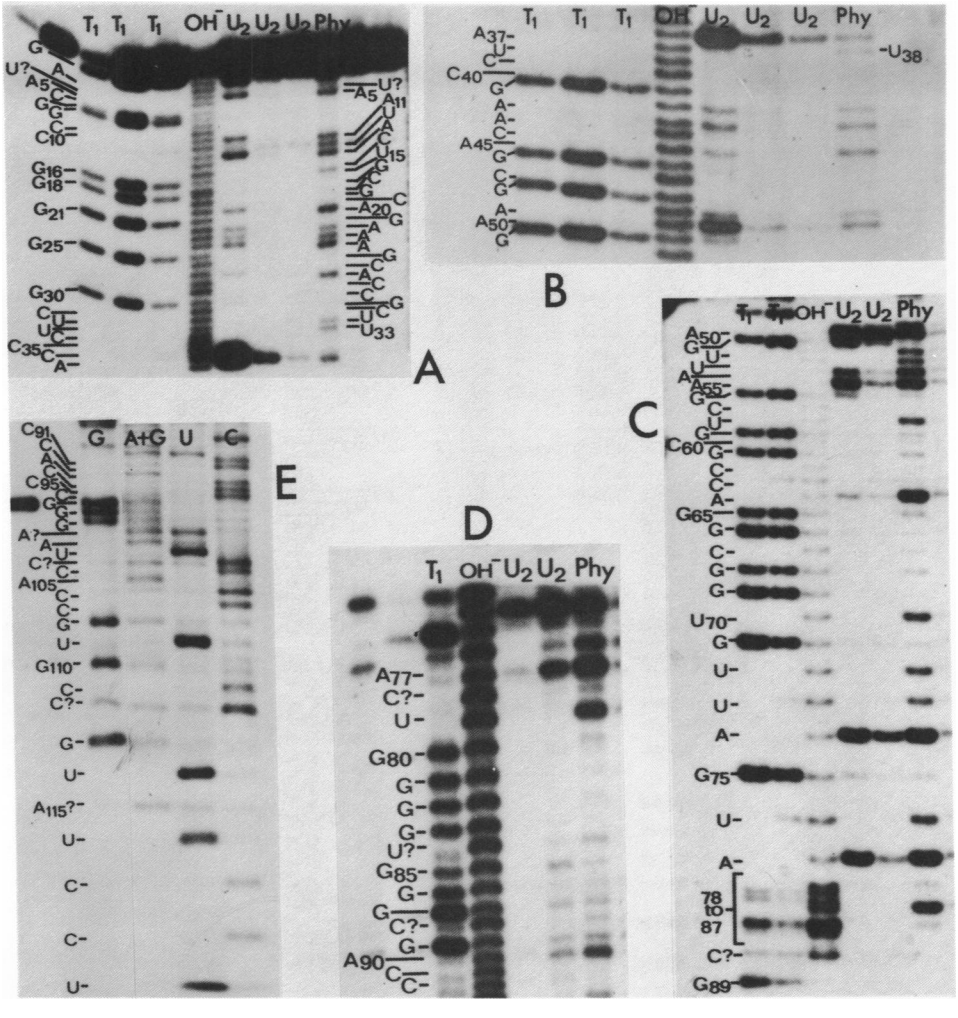
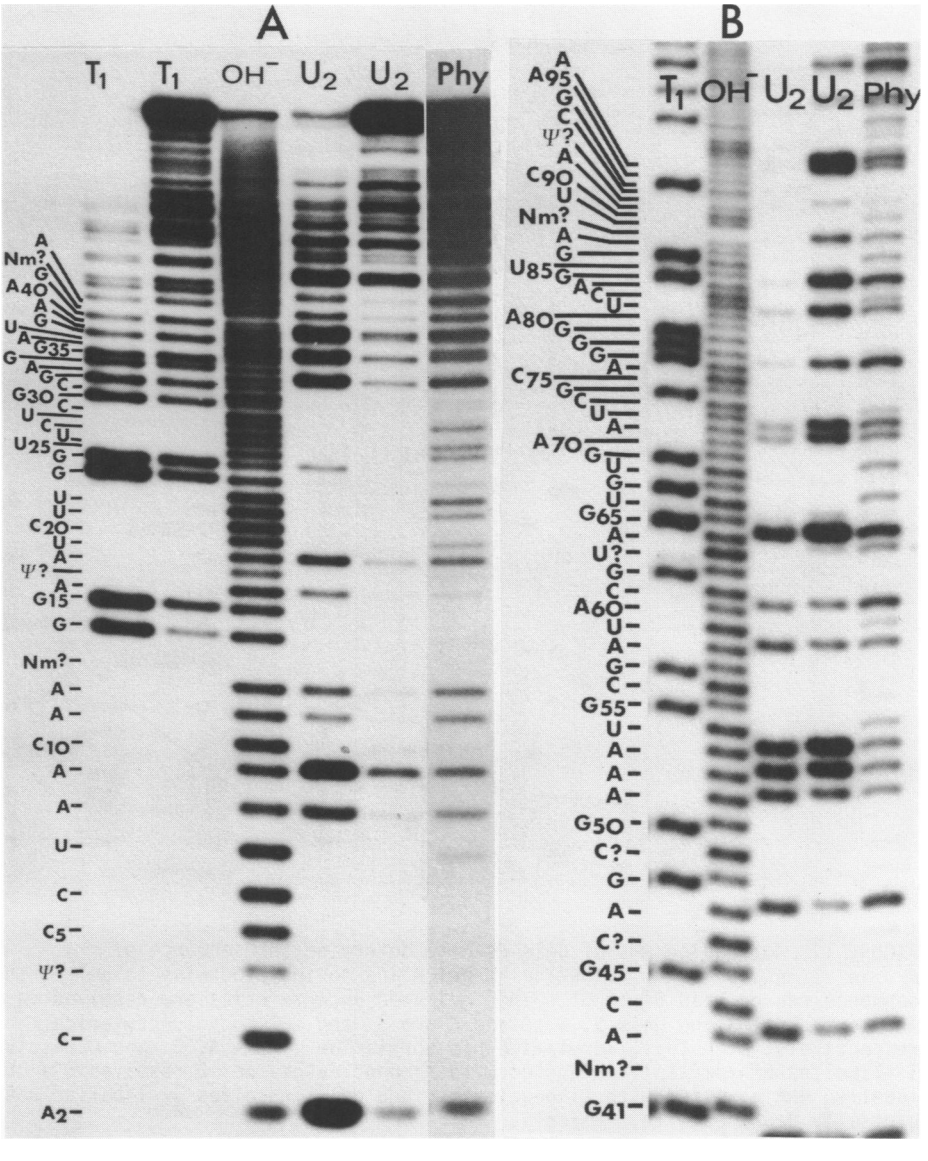


FIGURE 1. Autoradiograms of gels used to determine the sequence of *A. castellanii* 5S rRNA. Starting at gel A and moving clockwise to gel E, the sequence can be read 5' to 3'. The indicated uncertainties are resolved in the text. Gel A and gels B, C, D and E are 5% and 10% in polyacrylamide respectively; with 19:1, acrylamide:bis-acrylamide. Gels A, B, and C resolve 3'-labelled RNA partially digested with ribonucleases; gel D resolves 5'-labelled RNA digested with ribonucleases; and gel E resolves 3'-labelled RNA partially degraded with chemicals.


A
T1
T1
OH⁻
U2
U2
Phy
B
T1
OH⁻
U2
U2
Phy

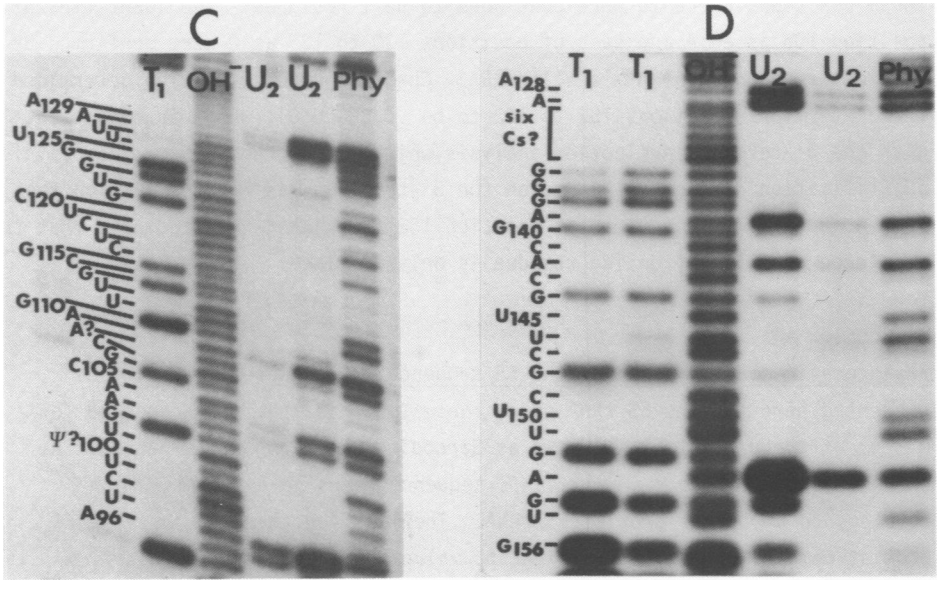


FIGURE 2. Autoradiograms of gels used to determine the sequence of *A. castellanii* 5.8S rRNA, which can be read 5' to 3' from gels A to D. Uncertainties are resolved in the text. Gel A is 20% and gels B, C and D are 10% in polyacrylamide; with 29:1 and 19:1, acrylamide:bis-acrylamide, respectively. Gels A, B and C resolve 5'-labelled RNA, and gel D resolves 3'-labelled RNA; all RNA has been partially digested with ribonucleases.

Ψ residues and requiring one or two more (see below). The blanks observable in Fig. 2A at positions 13 and 42 indicated the presence of sugar-modified nucleotides, and the parent residues at these positions were determined to be C and A respectively by chemical degradation of 3'-labelled RNA. The presence of Cm-Gp and Am-Ap (where m indicates $O^{2'}$-methylation of the parent nucleotide) in RNA labelled *in vivo* was demonstrated chromatographically. The identities of each of positions 46 and 49 as C and position 63 as U were confirmed by chemical sequencing of 3'-labelled RNA. Position 88 yields a blank in Fig. 2B, suggesting another $O^{2'}$-methylated residue; indeed, chemical sequencing identified the parent nucleotide as A and Am-Up was present in RNA labelled *in vivo*. Positions 92 and 100 in Fig. 2B and 2C respectively, yield weak PhyM and OH^- bands, indicating that these might be the location of the one or two Ψ residues required by the quantitation of the Np fraction (see above). Chemical sequencing of 3'-labelled RNA showed no degradation at

these positions and both were concluded to be Ψ residues. The identities of position 108 as an A and each of positions 130 to 135 as C were confirmed by chemical sequencing of 3'-labelled RNA. Chemical sequencing also determined the 3'-terminal positions 157 to 162 to be ...CCGCU(U), which is consistent with the 3'-terminal nucleotide analysis which yielded Up > 90%. The positive identification of two U residues on the 3'-terminus was not possible on the sequencing gels; hence the U at position 162 is bracketed to indicate that the existence of this nucleotide residue is only probable.

DISCUSSION

Phylogenetic consideration of the 5S sequence. The nucleotide sequence presented here for the 5S rRNA of *A. castellanii* is the first reported for a member of the protist class known as Sarcodina or Rhizopoda (amoebae). As shown in Table 1, the *A. castellanii* sequence bears no especially high homology to any other protist 5S rRNA. The mean for these *A. castellanii* : protist comparisons is 67%, similar to values obtained when *A. castellanii* is compared to representatives of the other eukaryotic kingdoms. Plant (wheat [9]), animal (human [26]) and fungal (*Saccharomyces carlsbergensis* [27]) 5S rRNAs all bear 65% homology to *A. castellanii* 5S. On the basis of overall homology between 5S rRNAs, *A. castellanii* is just as divergent from fellow protists as it is from members of other kingdoms.

The values obtained for the protist : protist comparisons in Table 1 (mean = 64%) are well below values obtained within the animal kingdom. (The 5S rRNAs of the insect *Drosophila melanogaster* [28] and the echinoderm *Lytechinus variegatus* [29] show 76% and 82% homology, respectively, with human 5S, and 85% homology with each other). They are in the same range as values

Table 1 Percent Homologies Between 5S rRNAs of Members of the Kingdom Protista

		A.c	T.t	C.f	D.d	C.p	C.c
amoeboid protozoan	*Acanthamoeba castellanii*	///	68	66	66	64	69
ciliate protozoan	*Tetrahymena thermophila* [12]		///	66	61	62	70
flagellate protozoan	*Crithidia fasciculata* [13]			///	66	62	57
cellular slime mold	*Dictyostelium discoideum* [25]				///	57	57
green alga	*Chlorella pyrenoidosa* [6]					///	63
dinoflagellate alga	*Crypthecodinium cohnii* [3]						///

5S nucleotide sequences have been aligned for maximum homology in all pairwise comparisons.

for interkingdom comparisons (plant : animal, 67%; animal : fungal, 60% fungal : plant, 61%; protist : plant, 65%, protist : animal, 65%; protist : fungal, 59%). The four eukaryotic kingdoms are about equally remote from each other, and the protist kingdom may be the most internally diverse.

A secondary structure model for *A. castellanii* 5S rRNA and comparative analysis of this model. The secondary structure model of *A. castellanii* 5S rRNA shown in Fig. 3A closely resembles the extensively base-paired model of Nishikawa and Takemura [5]. We propose that this model is generally acceptable for eukaryotic 5S rRNAs on the grounds that it meets the criteria suggested by Luehrsen and Fox [6]. That is, (1) proposed helices can be formed in *all* available sequences (see list in Table 2) at *homologous* locations, and (2) the nucleotide sequences of these regions exhibit phylogenetic diversity. Our model differs from the revision of the Fox and Woese [4] model recently suggested by Luehrsen and Fox [6] in that the helices designated II and IV in the latter model (and so designated in Fig. 3A) have been extended by two and three base-pairs respectively. These extensions require a single unpaired ("looped-out") nucleotide in each helix (homologous to positions 63 and 83 or 84 in the *A. castellanii* 5S).

We believe the proposed helix II extension is justified by sequence diver-

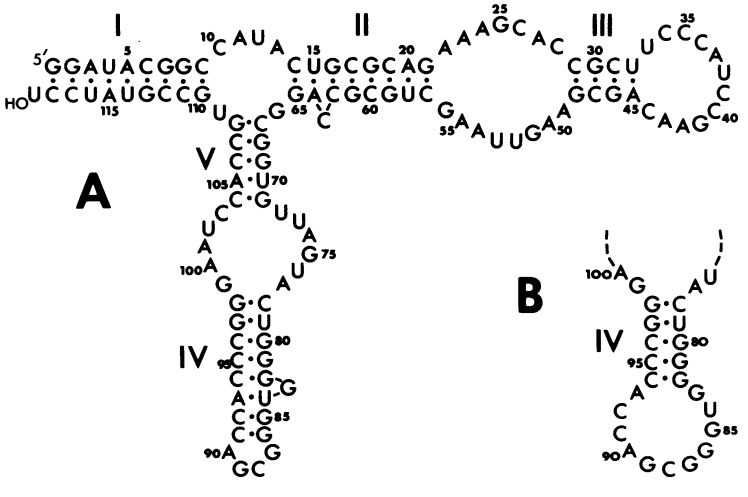


FIGURE 3. (A) Nucleotide sequence of *A. castellanii* 5S rRNA arranged in a secondary structure model which closely resembles that of Nishikawa and Takemura [5] and which we believe to be universally acceptable for eukaryotic 5S rRNAs. (B) An alternative structure after Fox and Woese [4] and Luehrsen and Fox [6] for helix IV.

sity in this region. *A. castellanii, T. thermophila, D. discoideum* and *C. fasciculata* base-pair 5'-CU-3' to 3'-GA-5'; *C. pyrenoidosa*, wheat, *C. cohnii* and the animals base-pair 5'-CC-3' to 3'-GG-5'; and the yeasts base-pair 5'-UC-3' to 3'-AG-5'. The 5S rRNAs of three non-yeast fungi can also form this extension and the primary sequences involved differ from each other and from all the above (W.F. Walker, in preparation).

The extension of helix IV is also supported by comparative analysis. Most vertebrates, *D. melanogaster, Bombyx mori, L. variegatus, Lingula anatina, T. thermophila* and *C. cohnii* base-pair 5'-UGG-3' to 3'-GCC-5'. The yeasts and *A. castellanii* base-pair 5'-UGG-3' to 3'-ACC-5'. *D. discoideum* base-pairs 5'-UUG-3' to 3'-AAC-5'. *C. fasciculata* base-pairs 5'-UCA-3' to 3'-AGU-5'. *C. pyrenoidosa* base pairs 5'-UGA-3' to 3'-AUU-5'. Each of these base-pair triplets is formed by looping out the nucleotide homologous to *A. castellanii* position 83. Three 5S rRNAs form the extension by looping-out the nucleotide homologous to *A. castellanii* position 84. These are the 5S rRNAs of wheat, turtle and chicken, which base-pair 5'-ANGG-3' to 3'-UCC-5' (where N designates the looped-out nucleotide).

Table 2 demonstrates that calculations of free energies for the extended (Fig. 3A) and Luehrsen and Fox (Fig. 3B) versions of helix IV generally favour the former. In some cases the more favourable configuration shows a positive, zero, or marginally negative ΔG, and some interaction with other ribosomal components must be invoked for helix stabilization. However, the fact that *all* available eukaryotic 5S rRNA sequences will form structures homologous to helix IV of Fig. 3A and will do so in spite of divergence in primary sequence throughout the helix indicates evolutionary constraints upon and thus the functional significance of this structure.

For *A. castellanii*, a third configuration for helix IV can be drawn. If the 5'-side of helix IV above U84 were shifted upwards one nucleotide position, base-pairing could occur between positions 78 to 83 and 94 to 99, with a single internal mis-match. It may also be noted that positions 24 to 26 could base-pair with positions 51 to 53, as suggested in the original Nishikawa and Takemura model. We reject both of these potential alternative structures because they fail to meet criterion (1) above.

Recently reported sequences for the 5S rRNAs of *Chlamydomonas reinhardii* [33] and the ascomycetes *Aspergillus nidulans* and *Neurospora crassa* [34] support our model by providing further phylogenetic diversity of sequence for the proposed extensions to helices II and IV and confirm the unacceptability of the alternative structures mentioned in the preceding paragraph.

Table 2 Free Energies of Two Alternative Structures for Helix IV in Eukaryotic 5S rRNAs

	A	B
A. castellanii	-11.4	-4.0
T. thermophila	- 0.4	+2.6
C. fasciculata	- 1.0	+1.6
D. discoideum	- 2.0	+1.6
C. cohnii	- 7.0	-4.0
C. pyrenoidosa	+ 2.8	-0.2
wheat [9]	- 8.6	-2.4
yeast species [7]	- 5.0	+4.0
chicken and turtle [7]	- 4.4	+3.0
human, *Xenopus* species iguana and trout [7]; brachiopod *L. anatina* [30], *L. variegatus* [29] and silkworm *B. mori* [31].	0.0	+3.0
D. melanogaster [28]	+ 2.8	+5.8

Free energies (ΔG in kcalories at 25°C) were calculated according to the rules of Tinoco *et al.* [32]. The alternative structures A and B are depicted in Fig. 3 for *A. castellanii* 5S rRNA. The first six 5S rRNAs are referenced in Table 1. All the vertebrate and fungal sequences listed in [7] have been included, except those of *X. laevis* oocytes, the *S. carlsbergensis* sequence designated (a) and *Pichia membranaefaciens*, which are only tentative in the regions of helix II or IV. The *C. pyrenoidosa* and flowering plant 5S rRNA sequences presented in [7] are incorrect; revised versions [6,9] are used.

A. castellanii 5.8S rRNA. Nazar *et al.* [35] and Luoma and Marshall [36] have proposed different secondary structure models for 5.8S rRNA. Both models derive much of their credibility from the fact that yeast and vertebrate 5.8S rRNAs could be made to conform to them. Plant 5.8S does not fit either of these models as well as do vertebrate or yeast [9]. A diversity of 5.8S rRNA nucleotide sequences should allow the acceptance of one, or the rejection of both, of these models.

Fig. 4 shows the nucleotide sequence of *A. castellanii* 5.8S rRNA arranged to fit, as well as possible, the secondary structure model of Nazar *et al.* [35]. Helix e (the "G-C rich stem" common to both the Nazar *et al.* and Luoma and Marshall models) is particularly convincing because of a favourable ΔG [32] of -11.6 kcalories and, more importantly, because all the 5.8S rRNAs

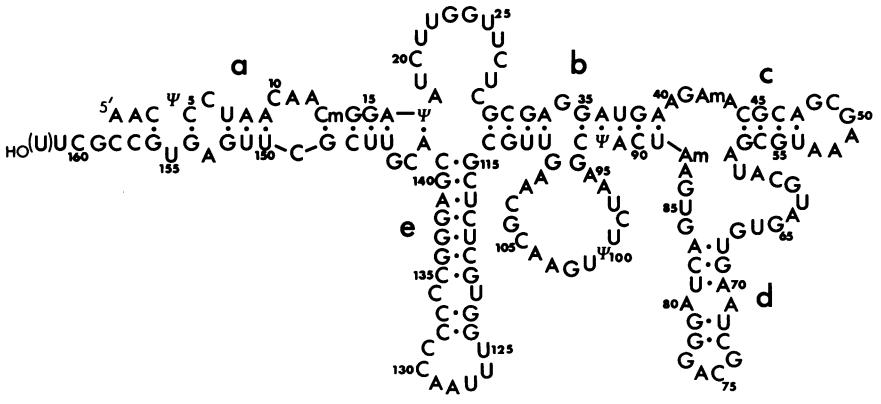


FIGURE 4. An attempt to fit *A. castellanii* 5.8S rRNA to the model of Nazar *et al.* [35]. The letter m at positions 13, 42 and 83 indicates $0^{2'}$-methylation of the respective residue.

referred to above can form this helix with homologous regions, while exhibiting phylogenetic diversity of sequence in these regions. Although helix b can be formed with the *A. castellanii* 5.8S, this potential structure is disturbing in that not all 5.8S rRNAs can form it with homologous regions, and those which can exhibit limited sequence diversity in this region. Helix c can be formed in all 5.8S rRNAs with homologous regions; however, the nucleotide sequences of these regions are almost invariant. *A. castellanii* 5.8 does not form helices a and d as well as do yeast or vertebrates [35]. Helices a and d also exhibit the short-coming that, in those 5.8S rRNAs which can potentially form these helices, use is often made of non-homologous regions for base-pairing. It is conceivable that a, b, c and d all represent helices present in some 5.8S rRNAs *in vivo* and that 5.8S has been evolving in a manner which does not result in the satisfaction of criteria (1) and (2), yet these criteria *do* apply to a part of this molecule, the G-C rich stem (helix e).

Helix a can also be criticized on the grounds that the 5'- and 3'-terminal regions of 5.8S rRNA are considered to be involved in specific base-pairing with sequences in 26S rRNA [37-40]. The extensive mis-matching and looping-out of nucleotides required to form helix a in *A. castellanii* and other 5.8S rRNAs may indicate that this helix is formed only in free molecules in solution [41,42] and has little significance in the ribosome.

Attempts to fit the *A. castellanii* 5.8S sequence to the model of Luoma and Marshall were equally unsatisfactory and we conclude that a universally

acceptable model for 5.8S rRNA secondary structure has yet to be proposed and that perhaps only limited regions of the molecule are involved in intramolecular interactions *in vivo*. The diversity of the protist kingdom indicates that further sequencing of protist 5.8S rRNAs should aid in the construction of a 5.8S secondary structure model as satisfactory as that arrived at with 5S rRNA, and which may involve extensive interactions with other rRNAs.

NOTE ADDED

The extended helices II and IV (we have claimed to be valid on the basis of comparative analysis) were originally proposed by Nishikawa and Takemura [5]. Recent publications of comparative work on eukaryotic 5S rRNAs have considered these extensions [6] and concluded that they are valid [43]. Recent models [43,44] propose that helix II of prokaryotic 5S rRNA is extended (by two base-pairs past a looped-out nucleotide) in a manner precisely homologous to that of the extended helix II of eukaryotic 5S rRNA.

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