3′-TERMINAL NUCLEOTIDE SEQUENCE OF *CRITHIDIA FASCICULATA* SMALL RIBOSOMAL SUBUNIT RNA

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1. Introduction

The primary sequence at the 3′-end of SSU RNA is highly conserved in both prokaryotes and eukaryotes [1–9]. In all cases a stable 'hairpin' structure can be formed by intramolecular base pairing starting ~10–15 residues from the 3′-terminus [3,4,6,8]. The single-strand loop of this hairpin contains 2 adjacent N^6,N^6-dimethyladenosine (m_2^6A) residues, situated at an identical position in both prokaryotic and eukaryotic SSU RNA [1,3,8]. This remarkable preservation of primary sequence, secondary structure, and post-transcriptional modification argues that the 3′-end of SSU RNA has a crucial (and probably universal) role in protein biosynthesis.

It has been suggested that interaction between SSU RNA and mRNA may serve in positioning the latter correctly for initiation of translation [10], as well as in determining the intrinsic capacity of ribosomes to translate a particular cistron [11], while interaction between SSU RNA and 5 S rRNA may be involved in the association of large and small subunits during protein synthesis [12,13]. In fact, specific complexes between prokaryotic 16 S rRNA and mRNA [14,15], eukaryotic 18 S rRNA and mRNA [16], and eukaryotic 18 S rRNA and 5 S rRNA [12,17] have been observed, and possible sites of interaction in these complexes have been inferred from sequence data [2,9–11,13–16,18]. However, with the exception of the mRNA binding site in *Escherichia coli* 16 S rRNA [19,20] and the 18 S rRNA binding site in wheat 5 S rRNA [21], there is as yet little direct evidence supporting the particular sequences which are supposed to interact in the various proposed complexes between SSU RNA and other RNAs.

In the absence of such information, comparative sequence analysis of SSU RNA can be helpful in evaluating how generally applicable the proposed interactions between it and other RNAs may be (e.g., [9]). This approach can also provide valuable insights into the evolution of rRNA, although at the present time sequence information about eukaryotic 18 S RNA is limited to a few representatives of the animal [4,7,9], plant [6,9], and fungal [5] kingdoms. In order to gain a more comprehensive view of the function and evolution of the 3′-terminal region of SSU RNA, we have determined the sequence of the first 91 nucleotides at the 3′-end of SSU RNA from the protist *Crithidia fasciculata*, a trypanosomatid protozoan. We compare here the homology between this and other known SSU RNA sequences, and use this sequence information to evaluate proposed interactions between eukaryotic 18 S rRNA and other RNAs.

Abbreviation: SSU RNA, small ribosomal subunit RNA (16 S rRNA in prokaryotes, 18 S rRNA in eukaryotes)

2. Experimental

Ribosomal subunits were prepared by centrifuging freshly-isolated *C. fasciculata* ribosomes through sucrose density gradients containing 0.1 mM Mg^{2+} [22]. RNA extracted from the small subunit peak was homogeneous and intact by polyacrylamide gel electrophoresis [23]. We designate this rRNA species '18 S' to emphasize that it appears to have a higher molecular mass than typical eukaryotic 18 S rRNAs [24].

Crithidia '18 S' rRNA was radiolabeled at the 3′-terminus [25] using [5′-^{32}P]pCp synthesized [26] to a specific activity of 5000 Ci/mmol. The donor/acceptor/ATP molar ratio was 1:0.3:12 and RNA

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ligase (P-L Biochemicals) was 500 units/ml, in a total reaction volume of 20 μl. After 24 h at 4°C, the reaction mix was diluted with 80 μl 1 mM EDTA–10 mM Tris–HCl (pH 7.4) +10 μl 3 M $NaOOCH_3$ and the RNA was then precipitated by addition of 2 vol. 95% ethanol. The recovered RNA was solubilized, reprecipitated, and then washed once with 80% ethanol. After drying in vacuo, the precipitate was dissolved in 10 μl water and then 10 μl loading buffer [25] were added. The 3′-end-labeled RNA was resolved on a 2.5% polyacrylamide slab gel (20 × 20 × 0.15 cm) containing TBE buffer [50 mM Tris–50 mM boric acid–1 mM EDTA (final pH 8.3)] and 7 M urea; electrophoresis was carried out for 2 h at 500 V. Radioactivity was visualized by autoradiography and the band corresponding to intact 3′-end-labeled '18 S' rRNA was recovered by electrophoretic elution [27], as in [28].

To determine the 3′-terminal nucleoside residue, [5′-^{32}P]pCp-labeled '18 S' rRNA was hydrolyzed with alkali and the resulting 2′(3′)-nucleotides were resolved by two-dimensional thin-layer chromatography [28].

Nucleotide sequence analysis was carried out by the partial chemical degradation method [25], as detailed in [28]. End-labeled products were resolved on thin (33 × 40 × 0.05 cm) 20% or 10.5% polyacrylamide gels (19:1, w/w, acrylamide:*bis*-acrylamide) in the presence of 7 M urea.

3. Results and discussion

3.1. *Derivation of the 3′-terminal sequence*

The sequence of the first 91 residues at the 3′-end of *Crithidia* '18 S' rRNA can be read from the representative autoradiograms shown in fig.1A (20% sequencing gel) and 1B (10.5% gel). The identity of the 3′-terminal U residue was independently determined by alkaline hydrolysis of [5′-^{32}P]pCp-labeled '18 S' rRNA, which released most (96%) of the radioactivity as Up.

The band spacings in fig.1 provide evidence of modified residues at positions 3, 20, 21, 54 and 65. While these remain to be definitely characterized, some deductions about their identities can be made. Residue 3 appears to be a base-modified U probably ψ, as judged by the fact that it gives a very weak U-specific band in chemical sequencing gels but a strong band in enzyme (*Physarum, B. cereus*) gels. Pseudouridine, a known constituent of *Crithidia* rRNA [24], has been shown to be refractory to the U-specific chemical cleavage reaction [25]. Residues 20 and 21 did not give bands in the sequencing gels, suggesting, as in the case of residue 3, that the '18 S' rRNA is not efficiently cleaved at these positions. The analogous positions are occupied by 2 adjacent N^6,N^6-dimethyladenosine (m^6_2A) residues in other SSU RNAs [1,3,8,29] and by adjacent A residues in the rDNA sequences corresponding to the gene for SSU RNA [4,5,7,30]. N^6-Dimethylation is expected to block the A-specific chemical cleavage reaction, and blanks in chemical sequencing gels have been observed for the same residues in other SSU RNAs [6,9]. Since the dinucleotide m^6_2A-m^6_2Ap has in fact been isolated from *Crithidia* rRNA [24], we infer the presence of m^6_2A at positions 20 and 21 of the Crithidia '18 S' rRNA sequence. The absence of a band corresponding to position 20 in the alkaline ladder is consistent with the known alkaline resistance of the phosphodiester bond joining the two m^6_2A residues [31]. Residues 54 and 65 appear as G and C, respectively, in sequencing gels (fig.1B), but there are no corresponding bands in the alkaline ladder, suggesting that these are alkaline-stable residues, most likely O^2-methylnucleosides (Gm and Cm, respectively).

Specific, spontaneous breakdown of 3′-end-labeled *Crithidia* '18 S' rRNA occurred at positions 38 and 41 (see control lane, fig.1B), and we have observed the same phenomenon with wheat 18 S rRNA labeled and analyzed in the same way. The origin of this breakdown is unclear, but one possibility is that it is due to specific radiolytic cleavage at these positions, as a result of intermolecular complex formation during the electrophoretic elution and concentration step used to recover 3′-end-labeled 18 S rRNA from polyacrylamide gels. With both the *Crithidia* and wheat 18 S rRNAs, it is possible to base-pair the 3′-terminal sequences of 2 molecules so that the [5′-^{32}P]pCp terminus of one molecule is positioned close to (and can therefore irradiate) either C37(38) or U40(41) in the other. Evidence for the analogous intramolecular base-pairing in wheat 18 S rRNA appeared in [6].

As with other SSU RNA sequences, a stable (ΔG = –13.8 kcal [32]) m^6_2A stem and loop structure can be formed close to the 3′-terminus (residues 10–33) of *Crithidia* '18 S' rRNA (fig.2). The existence of such a hairpin structure in *Crithidia* is supported by the fact that its constituent residues are not susceptible to enzymatic hydrolysis [33] even in the presence of 7 M urea (not shown).

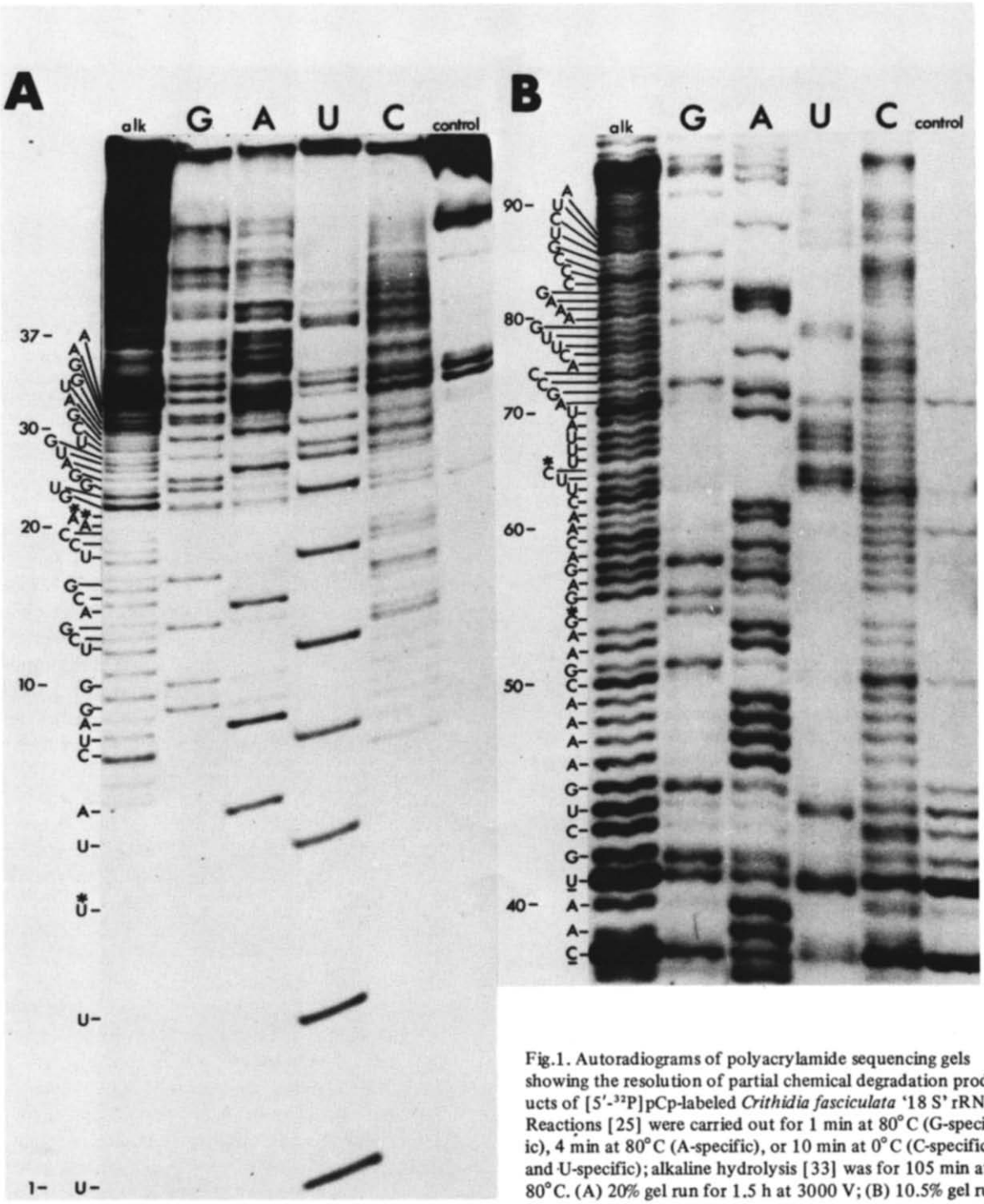

Fig.1. Autoradiograms of polyacrylamide sequencing gels showing the resolution of partial chemical degradation products of [5'-^{32}P]pCp-labeled *Crithidia fasciculata* '18 S' rRNA. Reactions [25] were carried out for 1 min at 80° C (G-specific), 4 min at 80° C (A-specific), or 10 min at 0° C (C-specific and U-specific); alkaline hydrolysis [33] was for 105 min at 80° C. (A) 20% gel run for 1.5 h at 3000 V; (B) 10.5% gel run for 2.5 h at 2000 V. Autoradiograms were prepared as in [28]. Numbering begins at the 3'-terminus. Asterisks mark probable modified nucleoside constituents, as described in the text, while underlined residues denote the positions of specific, spontaneous breakdown products (see control lane).

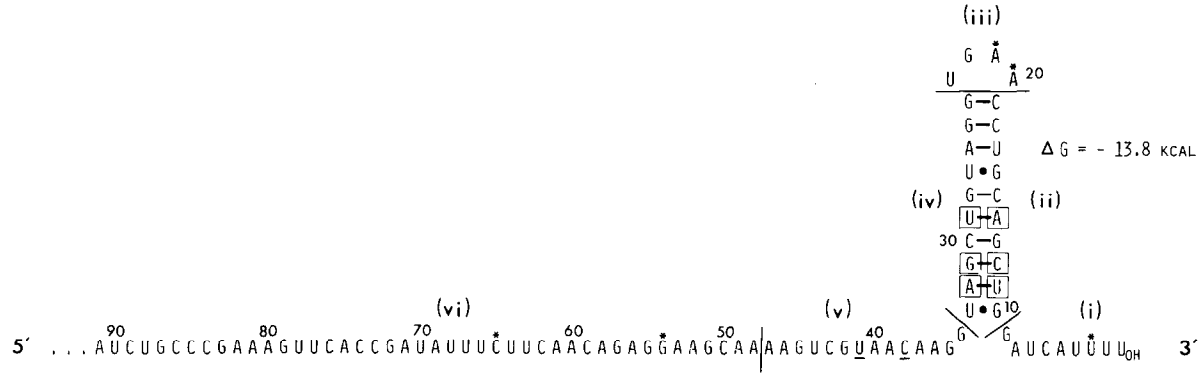

Fig.2. Sequence of the first 91 nucleotide residues at the 3′-end of *Crithidia fasciculata* '18 S' rRNA, indicating the m_2^6A hairpin structure encompassing residues 10–33. The sequence is divided into 6 sections, defined as follows: (i) the extreme 3′-terminal sequence (residues 1–9); (ii) the 3′-half of the m_2^6A stem (residues 10–19); (iii) the single-strand loop of the m_2^6A hairpin (residues 20–23); (iv) the 5′-half of the m_2^6A stem (residues 24–33); (v) homologous to a single-strand region which connects the m_2^6A helix to the next double-stranded region in the secondary structure model of *E. coli* 16 S rRNA [40] (residues 34–47); (vi) homologous to the 3′-half of the latter helix (residues 48–91). Asterisks and underlines denote the positions of probable modified residues and spontaneous breakdown products, respectively, as noted in the text.

3.2. *Evolutionary implications*

Fig.3 shows the extent of homology between the 3′-terminal sequence of *Crithidia* '18 S' rRNA and those of other eukaryotic 18 S rRNAs and *E. coli* 16 S rRNA. To facilitate this comparison, each SSU RNA sequence has been divided into 6 sections, as defined in fig.2. Like other eukaryotic SSU RNAs, *Crithidia* '18 S' rRNA lacks the prokaryotic 'Shine-Dalgarno' [10,11] sequence (CCUCC in *E. coli* 16 S rRNA: positions 4–8) and has a U instead of the prokaryotic G at position 23. However, *Crithidia* '18 S' rRNA differs from other eukaryotic 18 S rRNAs at 6 of the 20 positions (invariant in these other 18 S rRNAs) which constitute the m_2^6A stem. It is striking that the 3 substitutions in section (ii) of the *Crithidia* sequence are accompanied by reciprocal changes in section (iv), so that base-pairing (and therefore overall secondary structure) is strictly conserved throughout the m_2^6A stem (fig.2), in spite of the divergence in primary sequence. Overall, the *Crithidia* '18 S' rRNA sequence is not as closely related to the other 18 S rRNA sequences (64–74% homology in pairwise comparisons) as these are to each other (>84% homology between pairs), consistent with the view that the kingdom Protista is evolutionarily the most ancient of the eukaryotic kingdoms [34]. At the same time, *Crithidia* '18 S' rRNA appears no more or less divergent from *E. coli* 16 S rRNA than are other eukaryotic 18 S rRNAs. *Crithidia* '18 S' rRNA differs from most other eukaryotic 18 S rRNAs and from *E. coli* 16 S rRNA in its immediate 3′-terminal sequence, which is . . . $UUUU_{OH}$ rather than . . . UUA_{OH} or . . . UUG_{OH}. Of the eukaryotic 18 S rRNA sequences shown in fig.3, that of *Dictyostelium discoideum* bears the closest resemblance to *Crithidia* '18 S' rRNA: it appears to have an identical or very similar sequence at the immediate 3′-terminus and it probably differs from *Crithidia* '18 S' rRNA in only 2 rather than 3 of the constituent base-pairs of the m_2^6A stem. These data also indicate that additional sequences which might account for the apparent size difference between *Crithidia* '18 S' rRNA ($0.83–0.84 \times 10^6$ [24,35–37]) and other eukaryotic 18 S rRNAs (0.7×10^6 [38]) are not located within the first 100 nucleotides from the 3′-terminus of *Crithidia* '18 S' rRNA.

It is evident from fig.3 that the primary sequence of sections (i)–(v) at the 3′-end of SSU RNA is very highly conserved, not only among eukaryotes but between eukaryotes and prokaryotes as well. Particularly notable is the apparent absolute conservation of the primary sequence of section (v), which in secondary structure models of *E. coli* 16 S rRNA is largely [39] or entirely [40] a single-strand region connecting two base-paired helices. This suggests that it is primary structure per se in section (v) that participates in and indeed is crucial for the same specific function(s) in both eukaryotic and prokaryotic SSU RNAs. Homology between prokaryotic and eukaryotic SSU RNAs is abruptly and greatly reduced in section (vi), which constitutes the 3′-half of a long double-stranded region

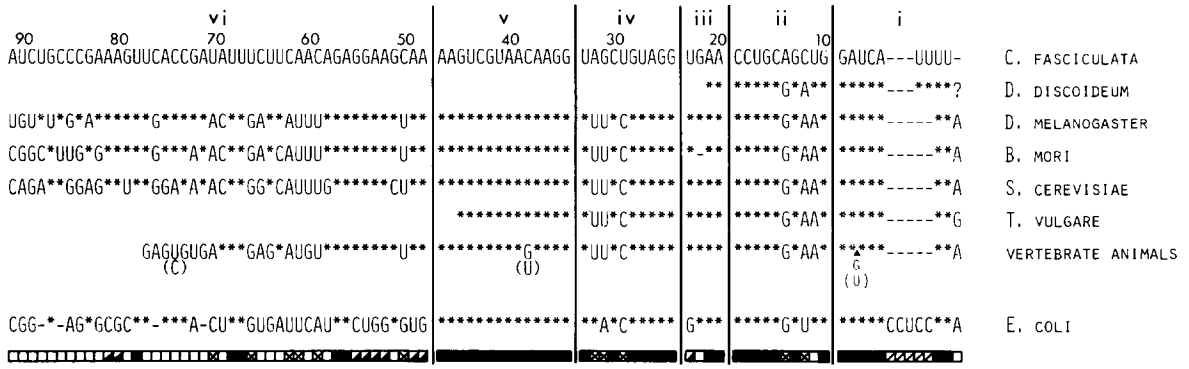

Fig.3. Homology between the 3′-terminal nucleotide sequence of *Crithidia fasciculata* '18 S' rRNA and those of: *Dictyostelium discoideum* [2]; *Drosophila melanogaster* [7]; *Bombyx mori* [4]; *Saccharomyces cerevisiae* [5]; *Triticum vulgare* [6]; and vertebrate animal [3,9] 18 S rRNAs and *E. coli* 16 S rRNA [29,30]. Positions occupied by the same residue as in *Crithidia* '18 S' rRNA are marked by *. Alignment between *E. coli* 16 S rRNA and the 18 S rRNAs is based on [4]. In [9] a G(U) substitution was noted at position 37 of vertebrate animal and barley embryo 18 S rRNA but wheat embryo 18 S rRNA has the expected C at this position [6]. Position 73 is U in hen reticulocyte and rat liver 18 S rRNA, but C in mouse sarcoma 18 S rRNA [9]. To facilitate comparison, the sequences have been divided into 6 sections [(i)–(vi)] as defined in fig.2. Degree of homology (bottom line) is indicated as follows: (■) residue the same in all SSU RNAs, both prokaryotic and eukaryotic; (◪)* residue the same in all eukaryotic 18 S rRNAs but different in *E. coli* 16 S rRNA; (▣)* residue the same in all eukaryotic 18 S rRNAs except that of *Crithidia*; (□) residue variable among 18 S rRNAs. The position of the prokaryotic 'Shine-Dalgarno' [10,11] sequence in *E. coli* 16 S rRNA is denoted by ◪*.

in the secondary structure models of *E. coli* 16 S rRNA [39,40]. This region may also be largely double-stranded in eukaryotic 18 S rRNA [41]. Our data further illustrate a phenomenon noted by others [40] with regard to rRNA sequence evolution, namely, that there has been a very high degree of primary sequence conservation in regions that are single-stranded, whereas double-stranded regions have diverged in sequence to a greater event. Nevertheless, the latter regions show a pronounced tendency toward reciprocal base changes which maintain overall secondary structure.

3.3. *Functional implications*

Knowledge of the 3′-terminal sequence of *Crithidia* '18 S' rRNA allows us to ask whether this molecule could participate in specific base-paired interactions which have been postulated to involve the 3′-end of other eukaryotic 18 S rRNAs. One proposal [2] is that a conserved purine-rich sequence (GCGGAAGG) near the 3′-terminus of eukaryotic 18 S rRNA interacts with a complementary sequence within the 5′-leader region of eukaryotic mRNA during the initiation of protein synthesis. In the position of this putative mRNA binding site, *Crithidia* '18 S' rRNA has the sequence GCAGCUGG (residues 9–16), a difference of 3 out of 8 residues. Divergence to this extent would not be expected if this sequence had a universal function in binding eukaryotic mRNAs. In fact, we find that the base changes in the *Crithidia* sequence either entirely preclude pairing between it and postulated 18 S rRNA binding sites in eukaryotic mRNAs [2,9,13,16,18] or render such paring much less likely, since the hybrid structures which can be written have substantially reduced thermodynamic stability. Thus, the mRNA–18 S rRNA binding model [2], which has been criticized [42,43], does not appear to be applicable to *Crithidia*. For eukaryotes, an alternative 'scanning' model has been proposed [44,45] in which the 5′-terminus per se, rather than a specific sequence of nucleotides in the 5′-leader region, is the primary determinant for binding eukaryotic mRNAs to small ribosomal subunits.

It has also been postulated that interaction between 5 S rRNA and the 3′-end of eukaryotic 18 S rRNA may play a role in the association of large and small subunits during initiation of protein synthesis [12,13]. Formation of a specific complex in vitro has been demonstrated between wheat 18 S and 5 S rRNAs [12,17], and the 18 S rRNA binding site in wheat 5 S rRNA has been identified and sequenced [21]. The complementary 5 S rRNA binding site in 18 S rRNA has not been directly determined, but it may encom-

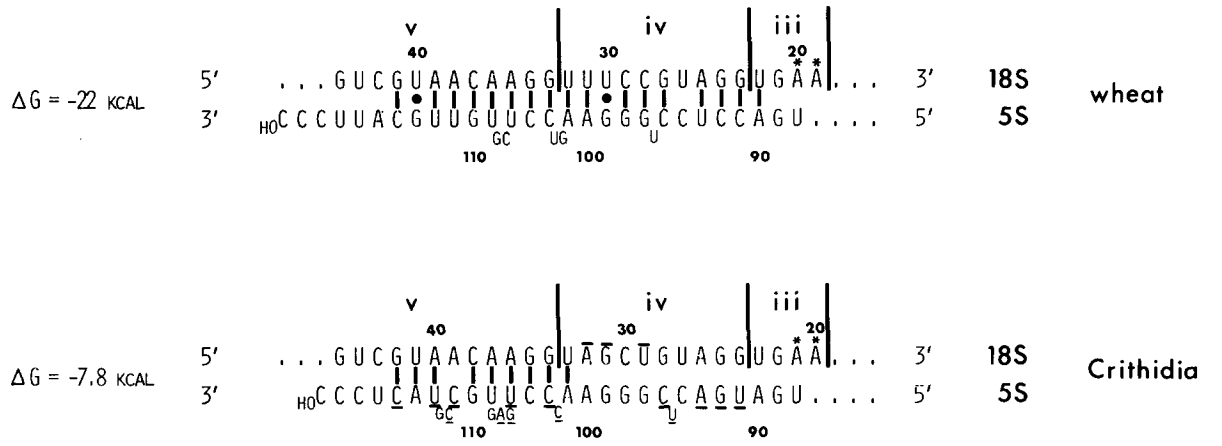

Fig.4. Potential base-paired complexes between 18 S and 5 S rRNAs from wheat and *Crithidia*. The 18 S rRNA binding site in wheat 5 S rRNA has been identified by sequence analysis [21], whereas the 5 S rRNA binding site in wheat 18 S rRNA is inferred [9,13]. The wheat 5 S rRNA sequence shown in fig.4 is the corrected version determined in our laboratory [28], so that the wheat 5 S–18 S complex as written is slightly, but not significantly, different from that postulated in [9,13]. The *Crithidia* 5 S–'18 S' complex has been constructed using the strictly-homologous sections of *Crithidia* 5 S rRNA [46] and *Crithidia* '18 S' rRNA (fig.3). Residues which differ between *Crithidia* and wheat are overlined (18 S rRNA) and underlined (5 S rRNA) in the *Crithidia* complex.

passe all of section (iv) and much of section (v) [13], as shown in fig.4. The base-paired structure which can be written between the known binding site in 5 S rRNA and the postulated one in 18 S rRNA is thermodynamically very stable in the case of wheat, in agreement with the observed thermal stability of the complex formed in vitro [17]. The proposed 5 S rRNA binding site is totally conserved in the 18 S rRNA of various vertebrate animals and of barley embryos [9], as would be expected if this sequence is generally important for interaction with a conserved sequence in 5 S rRNA.

Since the sequence of *Crithidia* 5 S rRNA has recently been determined [46], we examined the relevant regions of *Crithidia* 5 S and '18 S' rRNAs to ascertain whether a stable complex, analogous to that between wheat 18 S and 5 S rRNAs, could be formed. As shown in fig.4, base changes occur in both the '18 S' rRNA and 5 S rRNA of *Crithidia* in the regions homologous to the proposed sites of interaction in wheat 18 S and 5 S rRNAs. These sequence changes are nonreciprocal in the sense that a change in the '18 S' sequence is not accompanied by a corresponding change in the 5 S sequence which would allow retention of base-pairing, and the same is true for changes in the 5 S sequence. As a result, the possibilities for base-pairing between *Crithidia* '18 S' and 5 S rRNAs are greatly reduced in this region, and the complex which can be formed is much less stable thermodynamically than the wheat complex. In addition, base-pairing cannot extend into region (iv) of *Crithidia* '18 S' rRNA, so that disruption of the m_2^6A stem, an important feature of the model in [9,13], is not possible. In examining the sequence of *Crithidia* 5 S rRNA, we have noted another region (residues 57–74) which could potentially form a stable duplex ($\Delta G = -17.6$ kcal [32]) with the 3′-end of *Crithidia* '18 S' rRNA (residues 22–41), effectively disrupting the m_2^6A stem in the process. However, since we have not been able to observe a complex between *Crithidia* 5 S and '18 S' rRNAs in vitro, under conditions [17] where a control complex between wheat 5 S and 18 S rRNAs readily forms, the significance of this alternative potential interaction is unclear.

The sequence we have obtained for the 3′-end of *Crithidia* '18 S' rRNA does not lend support to functional models which involve complementary base-pairing between eukaryotic 18 S rRNA and either mRNA [2] or 5 S rRNA [13]. However, the ribosome of *Crithidia* has some unusual structural characteristics, not the least of which is the presence of 3 or 4 low M_r rRNAs (in addition to 5 S and 5.8 S rRNAs) in the large ribosomal subunit [23]. This opens the possibility that functional interactions between *Crithidia* '18 S' rRNA and other RNAs might differ in certain respects from those postulated for other eukaryotes.

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