Lability of RNA from the Large Cytoplasmic Ribosomal Subunit of the Protozoon *Crithidia oncopelti*

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(Received 13 May 1975)

SUMMARY

Cytoplasmic ribosomal RNA extracted from *Crithidia oncopelti* and analysed by gel electrophoresis at 4 °C consisted of two components, with molecular weights (relative to *E. coli* rRNA) of 1.30×10^6 and 0.83×10^6 daltons, present in equimolar amounts. On heating briefly at 51 °C followed by rapid cooling, the 1.30×10^6 RNA completely dissociated into two components of molecular weights 0.70×10^6 and 0.56×10^6 (present in equimolar amounts). Fifty per cent dissociation of the molecule occurred at 28 °C. That the integrity of the RNA molecule at low temperatures is maintained by its secondary structure was confirmed by electrophoresis under denaturing conditions (98 % v/v, formamide). To account for these phenomena, latent cleavage of the molecule *in vivo* is proposed.

INTRODUCTION

RNA from the large ribosomal subunit (L-RNA) from a variety of protozoa (Loening, 1968*a*; Stevens & Pachler, 1972; Bostock, Prescott & Lauth, 1971), insects (Applebaum, Ebstein & Wyatt, 1966; Greenberg, 1969; Ishikawa, 1973; Shine & Dalgarno, 1973), bacteria (Lessie, 1965; Borda, Green & Kamen, 1969) and chloroplasts (Ingle, 1968; Leaver, 1973) dissociates non-enzymically into two components of approximately the same size as the small subunit RNA (S-RNA). Dissociation of L-RNA occurs under conditions which cause disruption of non-covalent (hydrogen) bonds maintaining secondary structure (Herskovits, 1962; Cox & Littauer, 1962); the phenomenon is thought to reflect enzymic cleavage of the RNA *in vivo* in a single-stranded region of the molecule. In favourable conditions (high salt concentrations and low temperatures) the extracted molecules are held together by their secondary structure and the cleavage point is therefore only detectable under denaturing conditions.

This paper describes the presence of thermolabile L-RNA in the trypanosomatid flagellate *Crithidia oncopelti*, and it is suggested the cause of the break is enzymic cleavage of the molecule occurring *in vivo*.

METHODS

Organisms and growth conditions. *Crithidia oncopelti* was grown axenically in peptone-glucose medium (Newton, 1957). For experimental purposes, 500 ml batch cultures were harvested in mid-exponential phase of growth (2×10^7 cells/ml).

Escherichia coli (strain MRE600) was obtained as a frozen cake from the Microbiological Research Establishment, Porton Down, Wiltshire, and stored at -20 °C.

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RNA extraction. High molecular weight RNA was extracted from cells (Kirby, 1965; Parish & Kirby 1966), and stored at -20°C as an ethanolic suspension in 0.15 M-sodium acetate 0.5 % (w/v) sodium dodecyl sulphate (SDS) (2:1, v/v), pH 6.0. The RNA concentration was estimated from its extinction at 260 nm, assuming an $E_{1\%}^{1\text{cm}}$ of 250.

RNA was dissociated from *C. oncopelti* ribosomes (prepared by the method of Cross, 1970, and stored in small portions at -195°C in 50 mM-KCl, 10 mM-magnesium acetate, 50 mM-tris-HCl, pH 7.6) by dilution to the required concentration with gel running buffer containing either 0.05 % (w/v) SDS (for electrophoresis at 4°C) or 0.2 % (for electrophoresis at room temperature).

RNA analysis. RNA (5 to 40 μg in 5 to 60 μl) was analysed by polyacrylamide gel electrophoresis (Loening, 1968*b*) on 2.6 % (w/v) acrylamide gels at 4°C or at room temperature. The running buffer (acetate buffer) contained 20 mM-sodium acetate, 2 mM-EDTA, 40 mM-tris-HCl, pH 7.8 (plus 0.2 %, w/v, SDS at room temperature only). Phosphate buffer (30 mM- NaH_2PO_4 , 1 mM-EDTA, 36 mM-tris-HCl, pH 7.6, plus 0.2 %, w/v, SDS) was employed occasionally for electrophoresis at room temperature. Electrophoresis in denaturing conditions was performed at room temperature according to Staynov, Pinder & Gratzer (1972), using 4 % (w/v) gels in 98 % (v/v) formamide containing 20 mM-NaCl, pH 9.8.

Gels were scanned at 265 nm in a Joyce-Loebl ultraviolet scanner. The apparent molecular weights of RNAs were calculated from their electrophoretic mobility relative to the two *E. coli* rRNAs (molecular weights taken as 1.07×10^6 and 0.56×10^6 daltons) present as internal markers, assuming the mobility of RNA is inversely related to the logarithm of its molecular weight (Loening, 1967; Bishop, Claybrook & Spiegelman, 1967). The molar concentration of each RNA species present was calculated from the area of its peak (determined using a planimeter) and the calculated molecular weight. In all Figures the molecular weights of RNA components are designated as daltons $\times 10^{-6}$.

RESULTS

rRNA from *C. oncopelti*, when extracted and analysed at 4°C , contained L-RNA of molecular weight 1.30×10^6 and S-RNA of molecular weight 0.83×10^6 in the molar ratio 1.0:1.07 (Fig. 1*a*). The additional minor components were coincidental with *E. coli* rRNA, and were derived from the bipolar body of the flagellate (Spencer & Cross, 1975). In contrast, rRNA from the same extract electrophoresed at 25°C contained a much lower amount of 1.30×10^6 L-RNA, but an increased amount of the 0.56×10^6 component together with an additional component of molecular weight 0.77×10^6 (Fig. 1*b*). Similarly, with RNA dissociated from purified ribosomes, the L-RNA present at 4°C was almost completely absent at room temperature (Fig. 1*c, d*).

Analysis at room temperature of RNA dissociated from the separated ribosomal subunits confirmed that the large subunit was the origin of the two smaller RNA components. As shown in Fig. 2, the large subunit contained two major components, in the molar ratio 1.0:1.05, of molecular weights 0.77×10^6 and 0.56×10^6 respectively, whereas the small subunit contained a single major peak of molecular weight 0.83×10^6 . The presence of a 1.07×10^6 component suggests some contamination of the cytoplasmic ribosomal subunits by bipolar body ribosomes (Spencer & Cross, 1975).

The additive molecular weight of the two smaller components was always approximately that of the L-RNA molecule at 4°C , and it was therefore proposed that the former were degradation products of the latter. Non-specific ribonuclease activity *in vitro* was eliminated as the cause of the degradation because the products were always the same two specific components, and no degradation of *E. coli* rRNA was observed when it was co-extracted

Fig. 1. *Crithidia oncopelti* rRNA analysed at 4 °C and at room temperature. rRNA was dissolved in acetate buffer containing 5 % (w/v) sucrose. Ribosomes were diluted with acetate buffer containing 5 % (w/v) sucrose and 0.05 % (w/v) SDS. Electrophoresis was performed for 2 h (c), 2.5 h (a) or 3 h (b, d). Marker RNA was *E. coli* (molecular weights 1.07×10^6 and 0.56×10^6). (a) rRNA extracted from cells and analysed at 4 °C (load: 6 μ g); (b) rRNA from the same extract as (a), analysed at room temperature (load: 18 μ g); (c) RNA dissociated from purified cytoplasmic ribosomes and analysed at 4 °C (load: 20 μ g ribonucleoprotein); (d) RNA dissociated from the same ribosome batch as (c), analysed at room temperature (load: 20 μ g ribonucleoprotein).

and analysed at 4 °C or at room temperature with *C. oncopelti* rRNA. Further degradation of extracted RNA from *C. oncopelti* was not demonstrable at room temperature even after storage for up to 3 months at -20 °C. It was therefore proposed that extracted L-RNA contained a single 'hidden' break; at 4 °C the molecule appeared to be intact, but the conditions of electrophoresis at room temperature were marginal for preservation of the secondary structure of the molecule, resulting in some dissociation and consequent exposure of the break.

The extent to which L-RNA dissociation was temperature-dependent is shown in Fig. 3. Extracted rRNA was stable to brief heating up to 19 °C; beyond this temperature dissociation of the L-RNA (1.30×10^6) occurred, and by 51 °C the molecule was completely dissociated (via an intermediate 0.77×10^6 molecule) into two components of molecular weights 0.70×10^6 and 0.56×10^6 . The dissociation products were present in approximately equimolar amounts to each other and to the 0.83×10^6 RNA (S-RNA), which remained unaffected by the treatments. From the data replotted in Fig. 4, it was estimated that 50 % dissociation of the L-RNA occurred at 28 °C, in agreement with other systems where the effect has been observed in solutions of ionic strength about 0.2 (Bostock *et al.*, 1971; Leaver, 1973). Whether raising the salt concentration had any effect on the melting temperature of the L-RNA was not investigated.

In the absence of degradative enzymes, RNA molecules containing an intact phosphodiester backbone can withstand heating to 80 °C for a few minutes (Stanley & Bock, 1965), but any ribonuclease activity in the sample would be accelerated at this temperature (Atchison, Bourque & Wildman, 1973). That complete dissociation of the L-RNA from *C. oncopelti* at 51 °C is due to a latent break in the molecule, and is not a consequence of RNAase

Fig. 2

Fig. 3

Fig. 2. RNA content of separated ribosomal subunits. Subunits were prepared by sucrose gradient centrifugation. Up to 20 mg ribonucleoprotein were layered on to 50 ml linear gradients of 10 to 25 % sucrose in 50 mM-KCl, 1 mM-magnesium acetate, 50 mM-tris-HCl, pH 7.6, and centrifuged at 62 300 *g* for 10 h at 4 °C in the SW 25.2 rotor of the Beckman L2-65B preparative ultracentrifuge. After centrifugation the gradients were displaced at 4 °C through a Uvicord 1 mm flow cell, and the extinction monitored at 254 nm. Fractions containing 60s and 40s subunits (Cross, 1970) were pooled separately, diluted with 50 mM-KCl, 50 mM-tris-HCl, pH 7.6, and the Mg^{2+} adjusted to 3 mM. The subunits were collected by centrifugation at 165 000 *g* for 3 h at 4 °C, and immediately diluted with phosphate buffer + 0.2 % (w/v) SDS. Electrophoresis was for 3 h at room temperature. (a) RNA dissociated from the large subunit (35 μ g ribonucleoprotein); (b) RNA dissociated from the small subunit (17 μ g ribonucleoprotein).

Fig. 3. The effect of brief incubation of rRNA at various temperatures before analysis. Samples (0.2 ml) of an rRNA extract (0.5 mg/ml in acetate buffer without SDS) were heated in a water bath at various temperatures for 5 min and then plunged on to ice. After the last sample had been cooled at 0 °C for 30 mins, 20 μ l portions of each sample were electrophoresed at 4 °C for 2.5 h. (a) Control sample, kept on ice before analysis; (b) to (f), RNA heated for 5 min at 19, 25, 31, 40 and 51 °C, respectively.

activity *in vitro*, was confirmed by analysis of the RNA in formamide gels. In completely denaturing conditions, without prior heat treatment, *C. oncopelti* rRNA consisted of three major components of average molecular weights 0.83×10^6 , 0.70×10^6 and 0.56×10^6 (Fig. 5). The molecular weights of *C. oncopelti* rRNA determined in aqueous and denaturing conditions are summarized in Table 1.

Fig. 4

Fig. 5

Fig. 4. Effect of temperature on the stability of L-RNA from *C. oncopelti*. The percentage of intact L-RNA is expressed relative to S-RNA after the different temperature treatments shown in Fig. 3.

Fig. 5. Electrophoresis of *C. oncopelti* rRNA under fully denaturing conditions. rRNA was dissolved in 98 % (v/v) formamide containing 20 mM-NaCl and 5 % (w/v) sucrose. Electrophoresis was performed at 20 °C for 4 h. (a) *Crithidia oncopelti* rRNA (30 µg); (b) *C. oncopelti* rRNA (30 µg) + *E. coli* rRNA (7 µg).

Table 1. Average molecular weights of cytoplasmic rRNA extracted from *C. oncopelti* and analysed under various conditions

Conditions of electrophoresis	No. of experiments	$10^{-6} \times$ Molecular weights of major components present ($\pm$ S.D.)*
Aqueous, 4 °C	14	1.30 ($\pm$ 0.03), 0.83 ($\pm$ 0.02)
Aqueous, room temperature (19–25 °C)	15	[1.28 ($\pm$ 0.04)], 0.86 ($\pm$ 0.03), 0.77 ($\pm$ 0.02), 0.56†
98 % (v/v) formamide	3	0.83 ($\pm$ 0.03), 0.70 ($\pm$ 0.01), 0.56†

* Values in square brackets indicate variable amounts present.

† RNA was coincident with *E. coli* marker RNA.

DISCUSSION

Evidence is presented which suggests RNA from the large ribosomal subunit of *C. oncopelti* (L-RNA, 1.30×10^6) contains a 'hidden' break at a unique position. At normal salt concentrations and low temperatures the extracted molecule is held together by its secondary structure. Procedures which disrupt non-covalent bonds (raised temperatures, formamide treatment) cause unfolding of the molecule, resulting in its conversion to fragments of molecular weights 0.70×10^6 and 0.56×10^6 .

Dissociation was independent of whether the RNA had been extracted directly from cells or dissociated from purified ribosomes, suggesting the break is caused *in vivo* by a ribonuclease associated with the ribosomes. Intact rRNA is not essential for ribosome functioning *in vitro* (Szer, 1969, Hübös, Gaál & Vereczkey, 1972; Grove & Johnson, 1973), and if this is also true for ribosomes functioning *in vivo* then lability of the molecule could reflect its random digestion within the ribosome. Non-specific association of ribonuclease with ribosomes has been demonstrated in many organisms (see Barnard, 1969, for review) and

the specificity of degradation could reflect the accessibility of RNA within the ribosome to non-specific enzyme action (Dessev & Grancharov, 1973). There is, however, some evidence from 3'-terminal sequence analyses that the latent scissions in insect rRNA reflect specific nuclease activity (Shine, Hunt & Dalgarno, 1974). Furthermore, in organisms where precursor molecules to labile L-RNA have been studied by pulse-chase experiments, newly synthesized L-RNA was stable (Applebaum *et al.*, 1966; Greenberg, 1969). Thus lability of the L-RNA of *C. oncopelti* may be a result of specific cleavage occurring during RNA maturation.

Whether the change in mobility of the larger dissociation product from 0.77×10^6 to 0.70×10^6 (see Fig. 3*d*) at intermediate temperatures reflects a conformational change within the molecule, or the further dissociation of a small fragment as has been found in other eukaryotes (Pene, Knight & Darnell, 1968; Stevens & Pachler, 1972) is uncertain, since the occurrence of low molecular weight RNAs after denaturation of L-RNA was not investigated.

Reijnders *et al.* (1973) briefly reported that *C. luciliae* L-RNA contains a hidden break at a unique position. Their estimates for the molecular weights of rRNA from this organism were 1.38×10^6 and 0.83×10^6 (at 4 °C) and 0.83×10^6 , 0.74×10^6 and 0.56×10^6 (in 8 M-urea at 60 °C), in agreement with those reported here for *C. oncopelti*. They found that in denaturing conditions the L-RNA from *C. luciliae*, isolated by sucrose gradient centrifugation from total cell sap RNA, consisted of the 0.83×10^6 and 0.56×10^6 components. However, the present work, in which the RNA species were obtained from separated ribosomal subunits in addition to whole cells, suggest that the 0.83×10^6 RNA component was incorrectly assigned to the large subunit by Reijnders *et al.* (1973).

In the course of this work, similar dissociation products were noted for the L-RNA from *C. fasciculata* and *Trypanosoma brucei*. Thus labile L-RNA may be a feature common to all trypanosomatid flagellates.

Loening (1968*a*) noted a constant molecular weight of about 0.7×10^6 for S-RNA from eukaryotes, and distinguished two classes of L-RNA: plants and protozoa contain L-RNA of constant molecular weight 1.3×10^6 whereas L-RNA from higher animals has evolved in size from 1.4×10^6 to 1.75×10^6 . However, two exceptions to this general classification were rRNAs from *A. castellanii* (1.53×10^6 and 0.89×10^6) and *Euglena gracilis* (1.3×10^6 and 0.85×10^6). L-RNA from both of these organisms is unstable, as in *C. oncopelti*, and the S-RNA in all three organisms is larger than usual. It is possible that L-RNA lability in these organisms reflects differences in rRNA maturation or ribosome structure from other protists.

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