The Presence of a High-Molecular-Weight (Guanine-plus-Cytosine)-Rich Segment at the 3' End of Rabbit 28S Ribosomal Ribonucleic Acid

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The 3' hydroxyl end of 28S L-rRNA (major RNA species of the larger subribosomal particle) was labelled by coupling its 2-hydroxy-3-naphthoic acid hydrazine with diazotized [³H]aniline. The RNA was hydrolysed partially with ribonuclease T₁ and fractionated on Sephadex G-200. The results show that a highly structured segment with 78% G+C content and a number-average molecular weight of at least 1.0×10^5 – 1.8×10^5 is located at the 3' hydroxyl end of the 28S rRNA molecule.

The non-random distribution of guanine and cytosine residues along the chain of liver rRNA was deduced from results on its hydrolysis with snake-venom exonuclease (Hadjiolov *et al.*, 1966, 1967, 1968) or RNAase* T₁ (Delihas & Bertman, 1966; Delihas, 1967; Hadjiolov & Milchev, 1967) and from spectrophotometric measurements (Cox, 1966). In particular, limited hydrolysis with RNAase T₁ resulted in the formation of (G+C)-rich high-molecular-weight segments, 28S L-rRNA (Delihas, 1967; Hadjiolov & Milchev, 1967; Wikman *et al.* 1969; Cox *et al.*, 1973). Recently, characteristic loops, resistant to partial denaturation by formamide-urea, were observed by electron microscopy of 28S rRNA from HeLa cells (Wellauer & Dawid 1973), L cells (Wellauer *et al.*, 1974) and *Xenopus laevis* (Wellauer & Dawid, 1974). Isolated (G+C)-rich fragments of rabbit L-rRNA were shown by Wellauer to have the characteristic looped structure when detected by electron microscopy (Godwin *et al.*, 1974). On the basis of results with partial degradation with exonuclease it was concluded that part of the characteristic secondary structure loops are located at the 3' end of 28S rRNA (Wellauer & Dawid, 1973; Wellauer *et al.*, 1974). Because of the hazards of endonucleolytic attack during the action of exonucleases on high-molecular-weight RNA it was desirable to use an independent approach in solving this problem. Here, we report results supplying evidence that a high-molecular-weight rRNA segment with a high G+C content is located at the 3' end of reticulocyte 28S rRNA.

* Abbreviations: RNAase, ribonuclease; L-rRNA, major (23–28S) RNA species of the larger subribosomal particle; S-rRNA, RNA moiety of the smaller subribosomal particle; L3-rRNA, approx. 5S rRNA species of eukaryotes that is attached by non-covalent bonds to L-rRNA (cf. Pene *et al.*, 1968).

Experimental

Rabbit reticulocytes were prepared and lysed as previously (see Cox *et al.*, 1973). All subsequent operations were carried out at 4°C. The lysate was adjusted to 0.25M-sucrose, 25mM-KCl and 3mM-MgCl₂ and centrifuged at 6000*g* for 10min. Sodium dodecyl sulphate was added to the supernatant (final concn. 0.5%), and RNA was extracted by treatment with an equal volume of phenol [saturated with 0.14M-NaCl plus 0.1% 8-hydroxyquinoline and adjusted to pH6.0 (glass electrode) with 0.1M-sodium acetate buffer, pH7] for 30min at 4°C. The water phase was deproteinized two more times with phenol as above and precipitated overnight with 2vol. of 96% (v/v) ethanol containing 1% (w/v) potassium acetate. To remove traces of nuclease contaminants the RNA precipitate was dissolved in a small volume of 0.14M-NaCl and deproteinized successively with phenol, phenol-chloroform (15:1, v/v) and chloroform. The RNA from the final water phase was precipitated as above and stored at –30°C.

The reticulocyte RNA was fractionated by zonal centrifugation with the zonal rotor B XIV of an MSE Superspeed 65 refrigerated ultracentrifuge. A linear density gradient was prepared with 15–50% (w/v) sucrose in 0.1M-sodium acetate and 0.05M-NaCl. RNA (30–60mg) was loaded on top of the gradient and the centrifugation was carried out at 43000rev./min for 4h. About 35–40 10ml fractions were collected and the *E*₂₆₀ was recorded. The average *E*₂₆₀ L-rRNA/*E*₂₆₀ S-rRNA ratio from five independent fractionations was 2.62. The clearly delineated 28S rRNA peak fractions were pooled, and the RNA was precipitated and stored as above.

The 3' hydroxyl end of 28S rRNA was labelled as described by Russev (1969) by oxidation with NaIO₄, followed by reaction with 2-hydroxy-3-naphthoic acid hydrazide to which was coupled diazotized aniline. The procedure was carried out as

described by Russev except that [*ring* (G)-³H]aniline at a specific radioactivity of about 4.7mCi/mmol was used in the reaction. In this way both the E_{512} of the pink azo-dye-linked RNA and the ³H label from [³H]aniline could be used as markers of the 3' end of L-rRNA. Agar-gel electrophoresis of this RNA showed one single E_{260} peak coinciding with the radioactivity peak, thus indicating that no degradation of RNA had taken place during this treatment.

The 3'-terminal-labelled 28S rRNA (about 4mg) was dissolved in 0.01M-KCl and hydrolysed for 2 or 5min at 25°C with 33.8units of RNAase T₁ (Sigma, Kingston-upon-Thames, Surrey, U.K.; grade III). The reaction was stopped by the addition of cold phenol, and the RNA extracted, deproteinized and precipitated as above. The pink RNA precipitate was dissolved in about 3ml of 0.01M-potassium acetate (pH7.0) and fractionated on a column (120cm×1.2cm) of Sephadex G-200 at 4°C with the same solvent as eluent at a flow rate of 0.25ml/min. Fractions (2.65ml) were collected and their E_{260} and E_{512} values recorded. The radioactivity was determined in a Beckman LS 230 liquid-scintillation spectrometer with a toluene (500ml), Triton X-100 (250ml), 2,5-diphenyloxazole (3g) phosphor at about 38% efficiency.

Several adjacent fractions were pooled, concentrated and their nucleotide compositions determined as described by Katz & Comb (1963).

Results and discussion

Fig. 1 shows that L-rRNA contains a substantial amount of high-molecular-weight segments which are partially resistant to RNAase T₁. These segments are excluded from Sephadex G-200 and are characterized by a G+C content of about 78%, a value markedly higher than the average G+C content of 28S rRNA (Table 1). Since RNAase T₁ splits GpN phosphodiester bonds (where N is a purine or pyrimidine nucleoside) it is obvious that the observed resistance is due to the highly ordered structure of the (G+C)-rich segments in L-rRNA.

The comparison of the results of 2min and 5min hydrolyses with RNAase T₁ (Table 1) shows that the peak of high-molecular-weight segments is heterogeneous, the (G+C)-rich components being markedly more resistant. The [³H]aniline-labelled 3' end of L-rRNA was located completely within and moved slightly ahead of the (G+C)-rich peak. The ³H-labelled peak was fully resistant to longer treatment with RNAase T₁, a finding which confirms its high G+C content and highly ordered structure. The pale-pink colour of the (G+C)-rich fractions coincides with the position of the ³H-labelled RNA segment, which further supports the conclusion that [³H]aniline was solely at the 3' end of

the azo-dye-linked rRNA segment. A rough estimate of the length of the 3'-terminal (G+C)-rich (28S) L-rRNA segment (based on the correlation between ³H labelling and E_{260}) yielded a number-average molecular weight in the range 1.0×10^5 – 1.8×10^5 . As shown previously (Cox *et al.*, 1973) the L-rRNA fraction excluded from the Sephadex G-200 column is polydisperse and further separation may be achieved either by polyacrylamide-gel electrophoresis or by zone centrifuging. When the excluded L-rRNA fraction (see Fig. 1) was further fractionated by either of these techniques the radioactivity followed the E_{260} [see Godwin *et al.* (1974) for a brief report]. In a typical experiment L-rRNA had a specific radioactivity of 5500c.p.m./ E_{260} unit before hydrolysis. After partial hydrolysis with RNAase T₁ approx. 35% of the radioactivity was associated with the fraction excluded from the column (pools A and B, Fig. 1); the specific radioactivity of this fraction was 3000c.p.m./ E_{260} unit.

Several fractions (e.g. A in Fig. 1) were pooled and fractionated further by zone centrifuging. A 5–20% (w/v) sucrose gradient made 0.01M in potassium phosphate buffer, pH7.6, was used (Beckman SW 25.1 rotor) and was centrifuged at 5°C for 27h at 24000rev./min. Fractions (1.5ml) were collected and the radioactivity and E_{260} were measured; the radioactivity generally followed the extinction. The peak of the extinction was located in the middle of the gradient, and gel electrophoresis (cf. Cox *et al.*, 1973) indicated a mass of approx. 0.3×10^6 daltons for this fraction. The specific radioactivity was found to be 2500c.p.m./ E_{260} unit at the peak fraction. This confirmed that 30% or more of the radioactivity was associated with the (G+C)-rich fragments.

The lower specific radioactivity of the (G+C)-rich fragments compared with the original specific radioactivity of L-rRNA is partly attributed to the hydrolysis of L3-rRNA, which in the intact molecule is hydrogen-bonded to L-rRNA (Pene *et al.*, 1968; King & Gould, 1970). L3-rRNA has a terminal 3' nucleotide residue that is oxidized by sodium metaperiodate and is labelled by our procedure (Hunt, 1970). Thus half the specific radioactivity (approx. 2750c.p.m./ E_{260} unit) is attributable to this group. When, after partial hydrolysis with RNAase T₁, the treatment with phenol was omitted and the whole partial digest was applied to the column of Sephadex G-200 approx. 40% of the radioactivity was found in the oligonucleotide region (C, in Fig. 1). This radioactivity is absent after extraction with phenol etc. (see under 'Experimental') to remove RNAase T₁, as would be expected if L3-rRNA and the products separated on a sucrose gradient were hydrolysed to small fragments.

When L-rRNA was first heated to 50°C to dissociate L3-rRNA (King & Gould, 1970) and the

Fig. 1. Fractionation of 28S rRNA after partial hydrolysis with RNAase T_1

Reticulocyte 28S L-rRNA labelled at the 3' end by [^3H]aniline was hydrolysed for 2 min (a) or 5 min (b) with 33.8 units of RNAase T_1 at 25°C. The reaction was stopped, and the partly digested fragments were extracted by phenol treatment at 4°C and precipitated by the addition of ethanol. The precipitate of RNA fragments was dissolved in 0.01 M-potassium acetate buffer, pH 7, and fractionated on a column of Sephadex G-200 with 0.01 M-potassium acetate (pH 7.0) as eluent. —, E_{260} ; ····, radioactivity. The E_{512} peak (of about 0.1) coincided with the radioactivity peak. The letters on the abscissae designate the pooled fractions for nucleotide composition analysis. For further details see the text. When the partial hydrolysate was applied directly to the column omitting the phenol extraction step a second peak of radioactivity was found in the region 'C' and approx. 40% of the radioactivity was recovered in this zone compared with approx. 35% of the radioactivity associated with pools A plus B. The radioactivity of the RNA excluded from the column was not greatly altered by first heating L-rRNA to 50°C to dissociate L3-rRNA.

products were separated by centrifuging through a sucrose gradient the specific radioactivity of L-rRNA decreased from 440 c.p.m./ E_{260} unit before heating to 270 c.p.m./ E_{260} unit after heating. After RNAase T_1 treatment the radioactivity was still associated with the high-molecular-weight fraction excluded from Sephadex G-200; the specific radioactivities of the (G+C)-rich fractions were approx. 334 c.p.m./ E_{260} unit for the heated sample compared with approx. 410 c.p.m./ E_{260} unit for the control. It is unlikely that the labelled 3' terminus of L3-rRNA makes a significant contribution to the radioactivity of the (G+C)-rich fragments; the radioactivity of the latter is attributed to the 3' terminus of L-rRNA. The 3'-terminal sequence of

L-rRNA is known to be pGpUpUpUpGpU compared with pGpUpCpGpCpU for L3-rRNA (Hunt, 1970). The resistance of the 3'-terminal residue to hydrolysis by RNAase T_1 indicates that secondary structure might play a protective role.

Our results give strong evidence that a high-molecular-weight (G+C)-rich and highly structured segment is located at the 3' hydroxyl end of reticulocyte L-rRNA. They are in complete agreement with the electron-microscopic studies of Wellauer & Dawid (1973, 1974) and Wellauer *et al.* (1974) with other L-rRNA of animal origin. A different conclusion was reported by Hackett & Sauerbier (1975) on the basis of the effect

Table 1. Nucleotide composition of the (28S) L-rRNA segments obtained by partial hydrolysis with RNAase T₁

The (28S) L-rRNA was hydrolysed with RNAase T₁ for 2 or 5min and fractionated on Sephadex G-200 as described in the text. The eluted fractions, designated A, B and C in Fig. 1, were pooled, concentrated and their nucleotide compositions determined.

Fraction	Nucleotide composition (mol/100mol)				G+C content (mol/ 100mol)
	AMP	UMP	GMP	CMP	
2min hydrolysis					
A	8.1	15.2	38.9	37.8	76.7
B	12.6	17.9	32.8	36.7	69.5
C	18.6	20.9	31.4	29.1	60.5
5min hydrolysis					
A	7.3	14.4	44.3	34.0	78.3
B	7.5	14.2	41.5	36.8	78.3
C	22.1	20.3	29.1	28.5	57.6
Total 28S rRNA*	16.9	15.5	35.9	31.7	67.6

* Data were taken from Cox *et al.* (1973).

of u.v. irradiation on rRNA biosynthesis. The evolutionary implications of the presence of long (G+C)-rich segments in animal L-rRNA were discussed by Cox *et al.* (1973) and by Godwin *et al.* (1974).

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