Comparison between the Ribosomal Ribonucleic Acids from Free and Membrane-Bound Ribosomal Fractions of HeLa Cells

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The rRNA species from the total cytoplasmic, free and membrane-bound fractions of HeLa cells were compared. With the use of T₁ ribonuclease and combined T₁ ribonuclease plus pancreatic ribonuclease 'fingerprinting' procedures, no significant differences were found between the rRNA species from the different subcellular fractions.

Ribosomes in mammalian cells occur either free in the cytoplasm or bound to membranes. The membrane-bound ribosomes are attached by the large (60S) subunit (Sabatini *et al.*, 1966; Baglioni *et al.*, 1971; Harrison *et al.*, 1974). It is believed that ribosomes that are bound to the endoplasmic reticulum synthesize proteins that are destined to be secreted from the cell, whereas free ribosomes synthesize proteins that are retained within the cell (Hicks *et al.*, 1969; Redman, 1969). To distinguish between hypotheses as to how ribosomes become functionally segregated in this way (Baglioni *et al.*, 1971; Harrison *et al.*, 1974) it would be useful to know whether 'free' and 'bound' ribosomes differ structurally.

In reticulocytes there are differences between the proteins of free and membrane-bound ribosomes (Burka & Bulova, 1971; Fehlmann *et al.*, 1975a,b), though in this case binding of ribosomes is to the plasma membrane and not the endoplasmic reticulum. The large number of genes coding for rRNA in mammalian cells (Jeanteur & Attardi, 1969) might permit the presence of different rRNA sequences, which might in turn provide a basis for functional differentiation between classes of ribosomes. Here we examine this possibility for the high-molecular-weight rRNA species of free and endoplasmic-reticulum-bound ribosomes from HeLa cells, after separation by the method of Attardi *et al.* (1969), and by the use of RNA 'fingerprinting' methods.

Methods

Cell growth and labelling

HeLa cells were cultured in monolayers. Growth medium and labelling with [³²P]P_i and [*Me*-¹⁴C]-methionine were as described elsewhere (Maden & Salim, 1974; Robertson & Maden, 1973).

Subcellular fractionation

Free and membrane-bound ribosomes were frac-

tionated according to the method of Attardi *et al.* (1969). Membrane-bound ribosomes in HeLa cells constitute only about 15% of the total ribosomal population (Attardi *et al.*, 1969; Rosbash & Penman, 1971). To minimize the possible aggregation of free polyribosomes with membranes *in vitro* the Mg²⁺ concentration in the homogenate was lowered to the minimum compatible with the stability of polyribosomes, namely 0.15 mM (Girard *et al.*, 1965; Attardi & Attardi, 1967). For the preparation of rRNA from membrane-bound ribosomes, only 8100g membrane fractions were used. These contained the bulk of mitochondria and rough endoplasmic reticulum as well as smooth-membrane components (Attardi *et al.*, 1969). A further purification of the membrane fraction was achieved by re-running it through a linear 10–40% (w/v) sucrose gradient (Attardi & Attardi, 1967).

Extraction of RNA and 'fingerprinting'

The RNA extraction method and 'fingerprinting' by the combined T₁ ribonuclease plus pancreatic ribonuclease technique were as described elsewhere (Maden & Forbes, 1972; and in detail by Khan & Maden, 1976). The ribonuclease procedure was as described by Brownlee (1972).

Results

'Fingerprints' of digests of 28S rRNA species from free and membrane-bound ribosomes were prepared as follows.

(1) ³²P-labelled 28S RNA was subjected to digestion by T₁ ribonuclease followed by long separations in both dimensions of the 'fingerprinting' system. This procedure displayed many unique oligonucleotides in the penta- and larger oligonucleotide size range, principally among sequences containing one uridylylate residue. The results (Plate 1, top panels) revealed no obvious qualitative or major quantitative differences between the two patterns.

(2) Combined T₁ ribonuclease plus alkaline

Table 1. Sequences and molar yields of selected products from combined T_1 ribonuclease plus pancreatic ribonuclease 'fingerprints' of 28S and 18S rRNA

The quantitative determination of combined T_1 ribonuclease plus pancreatic ribonuclease products has been described in detail elsewhere (Khan & Maden, 1976). Observed 'free' and 'bound' molar yields of products 1-11 are means from two separate determinations each with ^{14}C -labelled RNA and ^{32}P -labelled RNA. The values for total cytoplasmic rRNA are from Khan & Maden (1976). Suggested molar frequencies are in parentheses. The notes for the final column are as follows. (a) The 28S-RNA product mU-U, and to a smaller extent the 18S-RNA product Um-U, gave higher and variable yields in ^{32}P 'fingerprints' than in ^{14}C 'fingerprints'. This was probably due to traces of unhydrolysed non-methylated material in this region of the 'fingerprint', and these values are therefore somewhat unreliable. Results obtained with the T_1 ribonuclease system indicate that the 'parent sequences' to the methylated spots [T_1 ribonuclease system spots 63 (28S RNA) and 86 (18S RNA)] occur once per molecule (Maden & Salim, 1974). (b) Since these methylated products migrate very close to other, non-methylated, products, quantitative determination is based on ^{14}C -labelled RNA only. (c) The nature of the hypermodified nucleoside X was described by Saponara & Enger (1974) and Maden *et al.* (1975). Part of this modification occurs late during ribosome maturation. (d) These two sequences are methylated late during ribosome maturation. The 18S-RNA spot 57 also contains a late-methylated sequence, but the latter product co-migrates with other methylated material in this 'fingerprinting' system.

	Sequence	Molar yield			Notes
		Free	Bound	Total	
28S RNA					
1	Um-Gm-Up + Um-Gm-Ψp	1.96	2.12	2.07	(1 of each)
2	mU-Up	1.82	1.29	1.15	(1) (a)
3	Um-Gp + Gm-Up	4.55	5.12	5.34	(5)
4	Gm-Gp	8.77	8.67	8.98	(9)
5	A-Gm-Gp	1.06	0.93	0.98	(1)
7a	A-A-Um-Gp	1.79	1.89	1.83	(1 of each)
7b	A-A-Ψm-Gp				
8	Cm-A-Gm-Up	0.91	0.99	1.01	(1)
9	Gm-A-Am-A-Gp	0.91	0.73	0.88	(1)
10	A(A,Gm-A)Gp	0.96	0.79	0.86	(1)
11	Am-Gm-Cm-A-A-A-Up	0.77	0.64	0.85	(1)
22	A-Am-Up	0.34	0.50	0.45	(0.5) (b)
18S RNA					
2	Um-Up	1.36	1.23	1.27	(1) (a)
3	Um-Gp + Gm-Up	3.82	4.08	4.54	(4)
4	Gm-Gp	3.07	3.08	2.98	(3)
5	A-Gm-Gp	2.75	2.61	2.76	(3)
6	A-Um-Up	1.09	1.03	1.05	(1)
7'	Um-A-A-Up	1.18	1.06	1.07	(1)
8'	A-Gm-A-Gp	0.49	0.45	0.52	(0.5)
9'	A(A,Um-A)Cm-A-Up	0.87	0.79	0.86	(1)
50	X-Cp	1.00	0.80	0.88	(1) (c)
54	m ⁶ ₂ A-m ⁶ ₂ A-Cp	0.84	1.03	1.01	(1) (b), (d)
65	m ⁷ G-A-A-Up	1.02	1.01	1.06	(1) (d)
	A ₅ -Up	0.95	0.78	0.76	(1)
	A ₆ -Ψp	0.82	0.71	0.69	(1)

phosphatase digests of ^{32}P -labelled 28S RNA, followed by long separations in both dimensions, also revealed no obvious differences on inspection, this system providing information mainly on sequences containing two or more uridyate residues (result not

of penta- and larger oligonucleotides (shaded, above and to right of interrupted line), and also some short methylated products (black). First dimension, 5h at 4.7kV; second dimension, 40h at 1.2kV. The middle and lower panels show combined T_1 ribonuclease plus pancreatic ribonuclease 'fingerprints' of ^{32}P -labelled rRNA: (d) free 28S RNA; (e) membrane-bound 28S RNA; (f) 28S-RNA key, showing positions of methylated products (black), Ψ -containing products (shaded) and 2':3'-cyclic mononucleotides (dotted circles); (g) free 18S RNA; (h) membrane-bound 18S RNA; (i) 18S-RNA key, conventions as for 28S RNA. The 18S-RNA product 9' sometimes streaks. Amounts of cyclic nucleotides vary somewhat between runs. For further details see Table 1 and Khan & Maden (1976).

EXPLANATION OF PLATE I

T_1 ribonuclease and combined T_1 ribonuclease and pancreatic ribonuclease 'fingerprints' of ^{32}P -labelled rRNA species

The upper panels show T_1 ribonuclease 'fingerprints' of ^{32}P -labelled rRNA: (a) free 28S RNA; (b) membrane-bound 28S RNA; (c) 28S-RNA key, showing positions

shown; the T_1 ribonuclease plus phosphatase 'fingerprint' of unfractionated 28S RNA has been shown by Robertson & Maden, 1973).

(3) 28S RNA, ^{32}P -labelled or $\text{Me-}^{14}\text{C}$ -labelled in separate experiments, was subjected to digestion by combined T_1 ribonuclease plus pancreatic ribonuclease. This system provides information on many short, but distinctive, chemically modified sequences (Maden & Forbes, 1972; Khan & Maden, 1976). The middle panels of Plate 1 show the 'fingerprints' of ^{32}P -labelled 28S RNA. Again no differences are evident between the 28S RNA species from free and membrane-bound ribosomes. Quantitative determination of products in 'fingerprints' of ^{32}P -labelled and $\text{Me-}^{14}\text{C}$ -labelled 28S RNA revealed no significant differences between the respective molar yields. Because of their essentially negative nature, the results are not presented exhaustively. However, Table 1 presents the molar yields of the well-resolved products 1–11 from the rRNA species from free and membrane-bound ribosomes, and also the values for total cytoplasmic 28S RNA (Khan & Maden, 1976). The molar yield of product 22, A-Am-U, is also included. This product is recovered in fractional yield from total cytoplasmic 28S RNA. The present result shows that the product is recovered in approximately similar yield from free and membrane-bound 28S ribosomes, i.e. that the fractional yield in total cytoplasmic rRNA does not reflect a functional differentiation in which (for example) only one class of ribosome might contain this modified sequence.

Analysis of 18S RNA, ^{32}P -labelled and $\text{Me-}^{14}\text{C}$ -labelled, was confined to the T_1 ribonuclease plus pancreatic ribonuclease procedure. The 'fingerprints' of the ^{32}P -labelled 18S RNA are shown in the lower panels of Plate 1. As with 28S RNA, no significant differences were found between the patterns for free and membrane-bound 18S RNA. Table 1 gives the molar yields of the well-resolved products 1–9', including another sequence that occurs in fractional yield, namely A-Gm-A-G (spot 8'). This is present in similar yield in free and membrane-bound 18S ribosomes. Also included are the yields of three products (50, 54 and 65) derived from sequences that are modified late during ribosome maturation (Maden & Salim, 1974; Maden *et al.*, 1975). These sequences are equally represented between free and membrane-bound 18S RNA species, each in approximately integral molar yield in both cases. Finally, the molar yields of the two longest oligo(A)-containing sequences ($A_5\text{-Up}$ and $A_6\text{-Up}$) are included. These were recovered in slightly sub-molar yields, probably due to slight overdigestion under the vigorous conditions used to obtain complete hydrolysis of all susceptible bonds (Maden & Forbes, 1972), but again the products are equally represented between free and membrane-bound subunits.

Discussion

Because ribosomes are attached to membranes by their large subunits, one might perhaps expect any structural differences between their rRNA species to affect preferentially the 28S RNA (of the large subunit) rather than the 18S RNA (of the small subunit). None of the 'fingerprinting' systems used here revealed any obvious differences between the 28S RNA species of free and membrane-bound ribosomes. The findings resemble the negative results of a search for differences between rRNA species encoded by the X and Y chromosomes of *Drosophila melanogaster* (Maden & Tartof, 1974). Analysis of the 18S RNA species was confined to the T_1 ribonuclease plus pancreatic ribonuclease procedure. Here negative results were also obtained. Use of this method to demonstrate structural differences between sequences of vertebrate 18S rRNA species has been described elsewhere (Khan & Maden, 1976). Two examples of 'fractional-yield' products (28S-RNA spot 22, A-Am-U; 18S-RNA spot 8', A-Gm-A-G) revealed approximately equal partition of the RNA species containing these products between free and membrane-bound ribosomes, implying that 'point' structural heterogeneities within rRNA (affecting methylation sites in these cases) do not necessarily reflect functional differentiation between the respective rRNA sequences.

In summary, the results do not encourage the view that there are major structural differences between the rRNA species of free and membrane-bound ribosomes. They are consistent with models for ribosome function that envisage exchange of ribosomal subunits between free and membrane-bound situations, possibly as a consequence of the particular polypeptide that is being synthesized at the time (Harrison *et al.*, 1974).

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