Determination of the Amount of Small Messenger Ribonucleic Acid-Containing Particles Free in Liver Cytoplasm

By BARBARA M. MULLOCK and RICHARD H. HINTON

*Wolfson Bioanalytical Centre, University of Surrey,
Guildford GU2 5XH, Surrey, U.K.*

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To assess the contribution made by mRNA-containing particles to the heterogeneity previously observed among rat liver 40S ribonucleoprotein particles, the amount of poly(A)-containing RNA in subribosomal particles was determined. RNA was labelled with orotate *in vivo* for 24 h and then for 50 min. Poly(A)-containing RNA was trapped on filters impregnated with poly(U). Very little poly(A)-containing RNA was found in conventionally prepared ribonucleoprotein particles after fractionation in sucrose. However, after preparation of ribonucleoprotein particles by sedimentation through 1 M-sucrose in the presence of 0.15 M-KCl or by precipitation with Mg^{2+} as described by Leitín & Lerman [(1969) *Biokhimiya* 34, 839–849], amounts of poly(A)-containing RNA were similar to amounts of mRNA found by other workers in total ribonucleoprotein particles. Even in such preparations, less than 5% of the total rapidly labelled RNA in native subribosomal-particle fractions was mRNA. It seems that mRNA-containing particles make up only a very small part of the population of subribosomal particles in liver.

The population of hepatic ribonucleoprotein particles that sediments at about 40S has been shown to be heterogeneous. Small ribosomal subunits, both newly synthesized and recycled, are known to sediment in this region; in addition rapidly labelled ribonucleoprotein particles containing non-ribosomal RNA have been reported (Henshaw & Loebenstein, 1970; Mullock *et al.*, 1971a,b; Sugano *et al.*, 1971; Woodcock & Mansbridge, 1971). The latter particles are thought to be mRNA-containing ribonucleoprotein particles and as such would contain poly(A)-rich regions (Edmonds *et al.*, 1971; Adesnik *et al.*, 1972; Mendecki *et al.*, 1972).

In order to assess the contribution made by such mRNA-containing particles to the population of subribosomal ribonucleoprotein particles, we have examined the proportion of poly(A)-containing RNA found in the RNA species from ribonucleoprotein particles of various sizes between 20S and 60S. Adequate amounts of the various sizes of particles were prepared by using a zonal rotor. The various classes of ribonucleoprotein particles sedimenting at less than 80S were either (a) separated directly from the post-mitochondrial supernatant, or (b) separated from a ribonucleoprotein particle fraction prepared by centrifugation through 1 M-sucrose in the presence of 0.15 M-KCl, or (c) separated from a ribonucleoprotein-particle fraction prepared by precipitation with Mg^{2+} by the method of Leitín & Lerman (1969). The results indicated that less than 7% of the rapidly labelled poly(A)-containing RNA was contained in particles smaller than ribosomes.

Materials and Methods

Preparation of ribonucleoprotein-particle fractions

Male hooded rats were injected intraperitoneally with 40 μ Ci of [5- 3 H]orotate (100–1000 mCi/mmol) 24 h before death and with 40 μ Ci of [6- 14 C]orotate (>50 mCi/nmol) 50 min before death. Both 3 H- and 14 C-labelled orotate were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Procedures for the preparation of a 12000g-10 min supernatant fraction from the livers of two rats and its fractionation by centrifugation in a B-XIV zonal rotor have been described (Mullock *et al.*, 1971a). The gradient in these experiments extended from 0.5 to 0.9 M-sucrose and centrifugation time was 3.5 h. Fractions corresponding to subribosomal particles were identified from the u.v. traces, pooled, diluted with 2–3 vol. of 1 mM-MgCl₂ and concentrated by filtration on Diaflo PM30 membranes. Particles from the microsomal region were collected by centrifugation for 1.5 h at 115000g after dilution with 2 vol. of water.

In other experiments ribonucleoprotein particles were collected by pelleting as described below before separation by zonal centrifugation. The livers were homogenized in 0.25 M-sucrose containing 0.025 M-KCl, 0.05 M-Tris-HCl buffer, pH 7.4, and 5 mM-MgCl₂, so that 4 ml of homogenate contained material from 1 g of liver. Lysosomes and smaller particles were removed by centrifugation for 15 min at 16000g in a M.S.E. High-Speed 18 centrifuge. A sufficient volume of 3 M-KCl and 20% (v/v) Triton X-100 was

added to the supernatant to give final concentrations of 0.15M-KCl and 2% Triton X-100, and the mixture was maintained at 0°C for 30min. Large polyribosomes were removed by layering 33ml portions of the supernatant over 7ml of 1M-sucrose containing 0.15M-KCl, 0.05M-Tris-HCl, pH7.6, and 5mM-MgCl₂ in the tubes of a M.S.E. 8×50ml rotor and centrifuging for 90min at 130000g in a Super-Speed 65 ultracentrifuge. The supernatants were decanted and 35ml portions layered over 10ml of 1M-sucrose, 0.15M-KCl, 0.05M-Tris-HCl, pH7.4, and 5mM-MgCl₂ in the tubes of an 8×50ml angle-head rotor. Ribonucleoprotein particles were collected by centrifugation for 13h at 130000g.

Alternatively, the ribonucleoprotein particles were precipitated by 0.05M-MgCl₂ (Leitin & Lerman, 1969). For these experiments, particles were prepared from the livers of four to six rats. Details of the method used in our laboratory are given by Hinton & Dessev (1973).

Analysis of separated particles

Re-centrifugation of particles on sucrose gradients was carried out in a 3×23ml swing-out rotor as described by Mullock *et al.* (1971a). RNA was measured by the method of Fleck & Begg (1965) and protein as described by Hinton & Norris (1972). Fractions were counted in a Triton X-100-containing scintillant and the counts were corrected for interference by sucrose (Dobrota & Hinton, 1973).

RNA extraction and estimation of poly(A) content

RNA was extracted by the method of Perry *et al.* (1972). Size distributions were examined by layering RNA dissolved in 0.01M-Tris-HCl (pH7.4) over 0.5–1.0M-sucrose gradients, which also contained 0.01M-Tris-HCl (pH7.4), and centrifuging in a 3×23ml swing-out rotor at 66000g for 16–17h.

The proportion of mRNA with an attached poly(A) sequence in the total RNA was estimated by trapping such RNA on fibre-glass filters impregnated with poly(U) as described by Sheldon *et al.* (1972). In later experiments, 0.5% sodium dodecyl sulphate was present both in the RNA solution applied and in the buffer-salt mixture (Sheldon *et al.*, 1972) used to equilibrate and wash the filters, since this appeared to decrease the non-specific adsorption of labelled RNA to filters lacking poly(U). All results were corrected for such non-specific adsorption. Total radioactivity in RNA was measured by drying small amounts of RNA solution on fibre-glass filters; the absence of significant label in smaller molecules was checked in one experiment by washing the discs thoroughly with trichloroacetic acid and ethanol.

Subribosomal particles separated directly from the post-lysosomal supernatant are contaminated by considerable amounts of cytosol proteins which can

interfere with the binding of poly(A)-containing RNA to the filters. Such contaminating proteins are absent from subribosomal fractions separated from ribonucleoprotein particles concentrated by pelleting or by Mg²⁺ precipitation. In such cases it proved unnecessary to extract the RNA with phenol before estimation of the proportion of RNA containing poly(A) sequences. The concentrates of ribonucleoprotein particles were simply adjusted to 0.5% sodium dodecyl sulphate–0.01M-Tris-HCl (pH7.4)–0.12M-NaCl and kept at room temperature (18°C) for 1–2min before passage through the poly(U)-impregnated filters. In such experiments, the salt-buffer mixture used for equilibration and washing always contained 0.5% sodium dodecyl sulphate. Comparison of measurements of poly(A)-containing RNA content made by this method on polyribosomes and on RNA extracted from the same polyribosomes gave values of 14.86±2.29% and 2.43±0.37% for the proportion of short- and long-labelled RNA respectively bound from sodium dodecyl sulphate-treated ribonucleoprotein and 13.04±1.68% and 2.55±0.61% for short- and long-labelled RNA bound from isolated RNA.

Dried filter discs were finally counted for radioactivity as described by Scherrer (1969).

Results

After a short period of labelling with orotic acid the ribonucleoprotein particles on the more slowly sedimenting side of the 40S peak, separated by centrifugation of whole liver cytosol, appear to be more highly radioactive than those on the fast-sedimenting side (Mullock *et al.*, 1971a). This labelling difference still remained after the 40S region from a zonal separation had been concentrated and subjected to a second centrifugation in a zonal rotor (Fig. 1). Such a second centrifugation gives better resolution of the 40S fraction from the soluble region, which inevitably merges into the 40S region to some extent during zonal centrifugation of post-mitochondrial supernatant. Agar-gel electrophoresis (Tsanev *et al.*, 1969; Hinton & Dessev, 1973) of RNA from different regions on the slow- and fast-sedimentation sides of the peak showed that the change in radioactivity across the peak was due to change in the specific radioactivity of 18S RNA, little or no RNA of other sizes being detectable. However, some of this approximately 18S RNA might be mRNA.

Assays of poly(A)-containing labelled RNA present in each of the major subribosomal regions, separated directly from the post-mitochondrial supernatant by the zonal rotor, and in microsomal fractions (Table 1) indicated that the proportion of labelled RNA present as mRNA was extremely low. However, the values for the microsomal fraction are far too low

Fig. 1. Re-centrifugation of 40S particles separated from the 12000g-10min supernatant fraction of a rat liver homogenate

(a) Two rats were injected with [^{14}C]orotate 50 min before death; 40S ribonucleoprotein particles were separated as described by Mullock *et al.* (1971a). In brief, the livers were homogenized in 0.25M-sucrose containing 1mM-MgCl₂ and 5mM-Tris-HCl, pH 7.4. Large particulates were removed by centrifugation for 10 min at 12000g. The supernatant was loaded on to a B-XIV zonal rotor and centrifuged for 3.5 h at 47000 rev./min. The fractions corresponding to the 40S particles were pooled and concentrated as described in the text. The concentrated suspension was loaded on to a B-XIV zonal rotor containing a 0.5–0.9M-sucrose gradient and centrifuged for 3.5 h at 47000 rev./min. Groups of fractions were pooled as shown on the inset diagram and RNA was precipitated and analysed by electrophoresis in sodium dodecyl sulphate-containing agar gels as described by Hinton & Dessev (1973). The distribution of radioactivity through the gels was determined by radioautography and the specific radioactivity of the 18S RNA in each fraction calculated as described by Tsanev *et al.* (1966). The specific radioactivities are given in arbitrary units. Scans of the gel strips and corresponding radioautograms from two of the pooled fractions (ii and iv) are shown in Figs. 1(b) and 1(c). Unlabelled 4S carrier RNA has been added in (c). —, E_{260} ; ----, radioactivity, E_{550} .

Table 1. Poly(A)-containing RNA in ribonucleoprotein fractions prepared by zonal centrifugation of post-mitochondrial supernatant in sucrose solution

Each value is the mean of the results of two experiments, each using a pair of rats. In each experiment, the label present as poly(A)-containing RNA in each fraction was determined at two different amounts of RNA/filter (80 and 160 μg respectively). The '50S' fraction here and in Table 3 contains the material taken from the region between the 40S- and 60S-subunit regions.

Labelling time	Percentage of total label in each fraction bound to poly(U)-impregnated filter				Microsomal region
	Pre-40S	40S	50S	60S	
50 min	1.34	0.99	0.40	1.74	0.71
24 h	1.08	1.13	0.33	0.93	0.19

to account for the proportion of mRNA known to be present in polyribosomes, and it was clear that poly(A) was being detached from mRNA by ribonuclease activity during manipulation of the ribonucleoprotein particles. Once the poly(A) region has been detached, mRNA would, of course, no longer be detectable by this assay, although the remainder of the mRNA molecule, complete with label, might still be present more-or-less intact. A method for preparing ribonucleoprotein particles which removed ribonuclease from the system as quickly as possible seemed necessary.

Two methods appeared suitable for separating ribonucleoprotein particles without risk of degradation. The first was direct pelleting of the ribonucleoprotein particles through 1M-sucrose after dissolving the membranes in Triton X-100. The risk of this method was that some RNA might be degraded during the long centrifugation needed to pellet subribosomal particles. This risk may be lessened, but not eliminated, by the addition of 0.15M-KCl to the medium to prevent the non-specific binding of basic proteins (including ribonucleases) to the ribonucleoprotein particles. Much more rapid separation of the ribonucleoprotein particle could be obtained by the second method used, the precipitation of ribonucleoprotein particles from the Triton X-100-treated post-mitochondrial supernatant by 0.05M-MgCl₂ (Leitin & Lerman, 1969). The advantage of this method is that the ribonucleoprotein particles are practically free of ribonuclease; the disadvantage of the method is that, although a considerable proportion of the subribosomal particles is precipitated, important subfractions may be lost.

To assess these various risks we compared the amount of poly(A)-containing RNA in ribonucleoprotein particles prepared by direct pelleting through 1M-sucrose with the amount in ribonucleoprotein

Table 2. Poly(A)-containing RNA in directly pelleted ribonucleoprotein particles, in the Mg^{2+} -precipitated preparation and in the supernatant therefrom

Numbers of determinations are given in parentheses. RNA was extracted from the Mg^{2+} -precipitated ribonucleoprotein before the final dialysis step. RNA was extracted from the supernatant after the addition of sufficient EDTA to bind all the high concentration of Mg^{2+} present in the supernatant; the extracted RNA was passed through a Sephadex G-25 column before assay to remove any low-molecular-weight contaminants.

Labelling time	Percentage of total label in each fraction bound to a poly(U)-impregnated filter		
	Directly pelleted ribonucleoprotein	Washed Mg^{2+} -precipitated ribonucleoprotein	Supernatant from Mg^{2+} -precipitated ribonucleoprotein
50 min	15.65 (4)	16.96 ± 2.13 (12)	6.63 (4)
24 h	2.32 (4)	3.84 ± 1.06 (12)	1.69 (4)

Fig. 2. Centrifugation for 3.5 h at 47000 rev./min in a B-XIV zonal rotor of ribonucleoprotein particles prepared by the method of Leitin & Lerman (1969)

—, E_{260} ; ○, ^{14}C radioactivity (50 min labelling); ●, 3H radioactivity (24 h labelling). For further details see the text.

particles prepared by precipitation with 0.05M- $MgCl_2$ and in the ribonucleoprotein particles that remained in the supernatant after precipitation with 0.05M- $MgCl_2$. The results (Table 2) show that there is no significant degradation of poly(A)-containing RNA when ribonucleoprotein particles are separated by direct pelleting and that there is no evidence of any significant loss of particles rich in poly(A)-containing RNA during precipitation with $MgCl_2$. Analysis of the ribonucleoprotein particles precipitated by 0.05M- $MgCl_2$ showed that about 50% of the native small ribosomal subunits and almost all the native large ribosomal subunits were present in the fraction. The small subunits containing rapidly labelled RNA sediment slightly faster than the bulk of the small subunits (Fig. 2). This was not observed with 40S particles separated directly from the post-mitochondrial supernatant owing to dimerization of the particles

containing slowly labelled RNA (Wettenhall *et al.*, 1973; Mullock & Hinton, 1974).

It seemed therefore that representative samples of subribosomal particles could be prepared either by the pelleting of ribonucleoprotein particles through 1M-sucrose, or by precipitation with 0.05M- $MgCl_2$. The proportion of labelled RNA that contained poly(A) in various fractions of subribosomal particles was therefore studied (Table 3). Comparison of the percentage of poly(A)-containing RNA in ribonucleoprotein particles separated by the two methods gives no evidence for any loss of mRNA-containing particles on precipitation by Mg^{2+} . Very little poly(A)-containing RNA was found in any of the subribosomal fractions. This was not due to loss during centrifugation, for the expected amounts could be recovered after fractionation of subunits prepared by the EDTA treatment of polyribosomes

Table 3. *Poly(A)-containing RNA in subribosomal particles separated from purified ribonucleoprotein-particle preparations*

The ribonucleoprotein particles were prepared either by precipitation with $MgCl_2$ or (*) by direct pelleting through 1 M-sucrose. Numbers of determinations are given in parentheses. Native subunit fractions were prepared by zonal centrifugation from either directly pelleted ribonucleoprotein particles or dialysed $MgCl_2$ -precipitated ribonucleoprotein particles. Derived subunit fractions were prepared by treating dialysed $MgCl_2$ -precipitated ribonucleoprotein particles with 2.5 μ mol of EDTA/mg of ribonucleoprotein and were separated on a sucrose gradient containing 5 mM-EDTA instead of 1 mM- $MgCl_2$.

Subunit	Labelling time	Percentage of total label in each fraction bound to a poly(U)-impregnated filter				Dialysed total ribonucleoprotein particles
		Pre-40S	40S	50S	60S	
Native	50min	7.66 (4)	1.77 (4)	2.78 (4)	1.23 (4)	12.26 $\pm$ 1.75 (14)
		0.50* (2)	1.22* (2)	—	—	—
Native	24h	6.06 (4)	1.41 (4)	1.20 (4)	0.35 (4)	4.06 $\pm$ 1.07 (14)
		0.88* (2)	0.88* (2)	—	—	—
Derived	50min	2.13 (2)	6.60 (2)	12.28 (2)	4.67 (2)	—
	24h	2.27 (2)	1.76 (2)	1.60 (2)	0.16 (2)	—

(Table 3). We therefore conclude that there are very few particles, free in the liver cytosol, that contain mRNA and are smaller than monomeric ribosomes.

Discussion

From the results in Table 3 it is considered that very little of the rapidly labelled RNA of the subribosomal particles is mRNA containing poly(A). As there was no significant difference between the results obtained with 40S particles when ribonucleoprotein particles were collected by precipitation with Mg^{2+} and when the particles were collected by direct centrifugation, it seems unlikely that our results are due to degradation during pelleting, and examination of the supernatant remaining after Mg^{2+} precipitation indicates there is no preferential loss of mRNA. Further, the proportion of poly(A)-containing RNA demonstrated in the total particle preparation agrees reasonably with the estimates of other workers (Lindberg & Persson, 1972; Singer & Penman, 1973). As discussed in the introduction, it appears that all non-histone mammalian mRNA molecules contain poly(A) sequences. It is unlikely that much degradation of mRNA occurs during separation of the subribosomal particles, in view of the agreement between our results on the distribution of mRNA after dissociation of polyribosomes with EDTA and the results of Henshaw (1968).

It therefore seems that less than 5% of the radioactivity found in RNA after 50min labelling, in any one of the 40S–60S fractions, is in mRNA. By using values from Mullock *et al.* (1971a) for distribution of short-term RNA label among the various classes of ribonucleoprotein particles, it can be calculated that about 7% of the rapidly labelled mRNA is found outside the polyribosomes in rat liver. This is much less than the 28% of 2h-labelled poly(A)-containing

RNA found outside KB-cell polyribosomes by Lindberg & Persson (1972) or the 20–25% of rapidly labelled poly(A) found outside HeLa-cell polyribosomes by Jelinek *et al.* (1973). The difference suggests that less mRNA is found outside polyribosomes in normal non-growing tissue than in tumour-cell cultures.

Since such a small proportion of short-term label is contained in poly(A)-containing RNA in rat liver, the presence of mRNA-containing particles is insufficient to explain the heterogeneity observed in the 40S particle region. Sugano *et al.* (1971) describe ribonucleoprotein particles of buoyant density 1.39–1.44 g/cm³ which contained about 15% of the RNA label of the 40S fraction after 40min and which they suggest are mRNA-containing particles. It is possible that this much larger estimate of mRNA content arises from the 24h starvation that the rats had previously undergone. However, it also seems likely that much of this low-density ribonucleoprotein does not contain true mRNA, since low-density ribonucleoprotein can easily arise as an artifact of fixation (Woodcock & Mansbridge, 1971). The correct explanation of the heterogeneity observed among particles sedimenting at about 40S appears to be the existence of two forms of the small ribosome subunit (Ayuso-Parilla *et al.*, 1973; Mullock & Hinton, 1974). These two forms differ in their sedimentation rates. In addition, one form is more rapidly labelled than the other after the injection of radioactive orotate (Mullock & Hinton, 1974). The presence of these two forms of the subunit suffices to explain the variation of specific radioactivity observed in the 18S RNA of particles sedimenting at about 40S.

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