Diversity and Nature of Ribosomal Pools in Hepatoma 7800 and Host Liver

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1. The ribosomal components in the postmitochondrial supernatant of a rat hepatoma (hepatoma 7800) and the corresponding host liver were examined for diversity and functional competence. 2. The 'free' and 'membrane-bound' polyribosomes of both tissues were equally active *in vivo* and had equilibrated with newly synthesized ribosomes 4 hr. after administration of [6-¹⁴C]orotic acid. 3. The inactive monomer-dimer pool in hepatoma 7800 was unattached to membranes and a larger fraction of the polyribosomes was free in hepatoma than in liver. 4. By using sensitivity to puromycin as a criterion, evidence was obtained that most of the polyribosomes in hepatoma 7800 were active *in vivo*. 5. Actinomycin, azaguanine and carbon tetrachloride caused marked conversion of polyribosomes into inactive monomers and dimers in the host liver and moderate conversion in the hepatoma. 6. Significant accumulation of ferritin and shifts in the mean polyribosome size to the lighter species occurred in the host liver of rats bearing large hepatomas.

Morphological and biochemical studies (Palade & Siekevitz, 1956) suggest that the ribosomes of mammalian cells are free in the cytoplasmic matrix or attached to cytoplasmic membranes that form part of the endoplasmic reticulum. There is morphological and physicochemical evidence (Hruban, Swift & Recheigl, 1965; Webb, Blobel, Potter & Morris, 1965; Goldberg & Green, 1967; Kuff & Roberts, 1967) that well differentiated tissues differ from poorly differentiated tissues in that the latter have a higher proportion of free ribosomes. In addition to free and bound pools, the ribosomal components may be further classified on the basis of structure and function into polyribosomes, monomers, dimers and sub-units (Wettstein, Staehelin & Noll, 1963; Vaughan, Warner & Darnell, 1967). It seems probable that the validity of conclusions about the structure and function of ribosomal components in mammalian cells is dependent on the extent to which the various pools can be separated.

The present investigation attempted to characterize the ribosomal pools in the postmitochondrial supernatant of host liver and hepatoma 7800 (Morris, 1965; Morris & Wagner, 1968) with respect to their diversity and functional competence.

Studies of this nature should provide clues about the physiological significance of the various ribosomal components and aid in the detection of subtle differences that may exist between normal and neoplastic liver.

MATERIALS AND METHODS

Animals. Hepatoma 7800 (gen. 31 and 33) was carried intramuscularly in rats of the Buffalo strain (Morris, 1965). The rats weighed 200–300 g. when killed. Rats were starved overnight to decrease liver glycogen content when polyribosomes were to be prepared from the host liver. In several experiments the rats bearing hepatoma 7800 were partially hepatectomized (Higgins & Anderson, 1931) to diminish metabolism by the liver of administered drugs or labelled compounds, and thereby to promote their uptake by the hepatoma.

Preparation and analysis of ribosomes. The liver or hepatoma was quickly removed and chilled, and then homogenized (0.5 g./ml.) in cold 0.25 M-sucrose in a Potter-Elvehjem homogenizer. Approx. 85% of the cells were broken with five strokes of a tight-fitting Teflon pestle (clearance 0.02 mm.) rotating at 680 rev./min. The post-mitochondrial supernatant was prepared by centrifuging the homogenate at 12000 g for 10 min. To separate the ribosomal components according to size, the supernatant, untreated or treated with 1.25% deoxycholate, was layered

over an exponential 10–40% sucrose gradient; the gradient was centrifuged at 63000*g* in an SW25.1 rotor of a Beckman–Spinco model L-2 ultracentrifuge and then monitored in a Beckman DB-G spectrophotometer with a flow cell with a 1 cm. light-path. The amount of supernatant layered on the gradients varied with the nature of the experiment and is therefore specified in the Results section. Where applicable, the ‘free’ and the ‘bound’ ribosomes (Bloemendal, Bont & Benedetti, 1964; Webb *et al.* 1965) were prepared by zonal centrifugation by the procedure of Blobel & Potter (1967) and then separated according to size in sucrose density gradients as described above. The term ‘total ribosomes’ refers to preparations, or density-gradient profiles of preparations, containing all the ribosomes in the deoxycholate-treated postmitochondrial supernatant.

All sucrose solutions were prepared in a buffer containing 25 mM-KCl, 5 mM-MgCl₂ and 50 mM-tris-HCl, pH 7.6 at 25°. Details of methods for the preparation and monitoring of the density gradients, and for the collection and radioassay of fractions from the density gradients, were as described by Webb (1967) and Kwan & Webb (1967). Liver ferritin has a significant absorption at 260 mμ; it was therefore necessary to apply a ferritin correction to the size-distribution profiles to obtain a true estimate of the monomer pool size. The ferritin concentration was obtained by monitoring a duplicate gradient at 320 mμ (Jackson, Munro & Korner, 1964; Wilson & Hoagland, 1965).

Materials. L-[¹⁴C]Leucine (sp. radioactivity 200 mc/m-mole), [6-¹⁴C]orotic acid (sp. radioactivity 2–10 mc/

m-mole) and [³H]orotic acid (sp. radioactivity 250 mc/m-mole) were purchased from the New England Nuclear Corp., Boston, Mass., U.S.A. 8-Azaguanine and puromycin were purchased from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Actinomycin D was a gift from Dr W. Dorion of Merck, Sharpe and Dohme of Canada Ltd., Montreal, P.Q., Canada. The labelled compounds or drugs were administered intraperitoneally or intravenously via the portal vein.

RESULTS

Host liver. Experiments were designed to determine whether the presence of a hepatoma influenced the relative pool sizes and activities of ribosomal components in the postmitochondrial supernatant of rat liver. The size-distribution profile of the total ribosomes in the livers of rats bearing hepatoma 7800 is shown in Fig. 1. Each rat was injected intravenously with 25 μC of L-[¹⁴C]leucine 3 min. before removal of the liver, to label preferentially the ribosomes actively synthesizing protein *in vivo*. Fig. 1(a) shows the size-distribution and specific-radioactivity profiles characteristic of the liver from a rat in which the hepatoma 7800 had been growing for 2 months. The size distribution of the polyribosomes resembled that of normal rat liver (Webb, Blobel & Potter,

Fig. 1. Size-distribution and L-[¹⁴C]leucine-labelling patterns of the ‘total’ ribosomes from the livers of rats bearing hepatoma 7800. Each rat was injected with 25 μC of the labelled compound 3 min. before removal of the tissues. (a) Profiles of ribosomal components prepared from the liver of a rat 2 months after inoculation of hepatoma 7800. (b) Profiles of ribosomal components prepared from the liver of a rat 2.6 months after inoculation of hepatoma 7800. The top of the gradient is to the right. A 0.7 ml. sample of the deoxycholate-treated supernatant was layered on the gradient. E_{260} before (—) and after (....) correction for absorption of ferritin; specific-radioactivity profile (●). A low specific radioactivity is interpreted to mean that the corresponding components are non-functional.

Fig. 2. Size-distribution and L-[^{14}C]leucine-labelling pattern of ribosomal components in the postmitochondrial supernatant of hepatoma 7800. The rats were injected with $50\mu\text{C}$ of the labelled compound 3 min. before removal of the tumour. A 1.0-ml. sample of the supernatant was layered on the gradient. The top of the gradient is to the right. (a) Deoxycholate-treated supernatant. (b) Untreated supernatant. E_{260} before (—) and after (....) correction for the absorption of ferritin; radioactivity profile (●).

1966). The host liver had an abnormally high concentration of ferritin, which contributed to the absorption in the monomer region. After correction for the interference of ferritin (Wilson & Hoagland, 1965), however, the monomer concentration was similar to that of normal liver. Approx. 70% of these monomers were unattached to membranes in rat liver (A. J. Rizzo & T. E. Webb, unpublished work).

Significant changes occurred in the size distribution of ribosomal components in the liver of rats bearing hepatoma 7800 when the hepatoma was sufficiently large to cause a cachexic state. The profiles of ribosomal components from the liver of a rat bearing hepatoma 7800, 2.6 months after inoculation, are shown in Fig. 1(b). There was a marked shift in the mean polyribosome size distribution to the lighter species, in addition to the appearance of an inactive dimer peak; there also appeared to be a further increase in the concentration of ferritin.

Hepatoma 7800. As shown by Webb & Potter (1966), cell extracts prepared from the rapidly growing Novikoff hepatoma contain an inactive monomer-dimer ribosome pool. As shown in Fig. 2(a), a monomer-dimer ribosome pool was also present in the highly differentiated hepatocellular carcinoma, hepatoma 7800 (Morris, 1965); the

dotted line represents the 'total ribosome' pattern after a small ferritin correction was applied. Only the polyribosomes showed significant incorporation of L-[^{14}C]leucine into nascent protein; the monomers and dimers were virtually inactive. As a control, the polyribosomes were isolated under conditions where the ribonuclease inhibitor was made limiting by dilution of the postmitochondrial supernatant; the density-gradient profiles indicated that the polyribosomes were partially degraded and that label from polyribosomes prelabelled with L-[^{14}C]leucine was transferred to the monomer pool (S.-W. Kwan & T. E. Webb, unpublished work).

Fig. 2(b) shows the profiles of another portion of the supernatant from hepatoma 7800 that was not treated with deoxycholate. A comparison of Figs. 2(a) and 2(b) suggests that there was interaction between the membranes and most of the polyribosomes. That a large proportion of this interaction is weak is suggested by results reported below, showing that most of the polyribosomes could be separated from the membranes by zonal centrifugation. A comparison of the two patterns also showed that virtually all the inactive monomers and dimers in the postmitochondrial supernatant were unattached to cytoplasmic membranes. In six individual experiments the ratio of the heights of the monomer peak and dimer peak in the

Fig. 3. Size-distribution and L-[^{14}C]leucine-labelling patterns of 'free' and 'bound' ribosomes from hepatoma 7800. The rats were injected with $25\mu\text{C}$ of the labelled compound 3 min. before removal of the tumour. The ribosomes were obtained from 1.5 ml. of post-mitochondrial supernatant. The top of the gradient is to the right. Corrected E_{260} (—); radioactivity (●).

deoxycholate-treated supernatant to the corresponding peaks in an equal volume of the untreated supernatant were 1.16 ± 0.50 and 1.04 ± 0.30 (mean $\pm$ s.d.) respectively.

A further separation and characterization of the ribosomal pools in hepatoma 7800 was undertaken. The ribosomal components were first separated into two fractions operationally defined as 'free' and 'bound' (Webb *et al.* 1965) by using the modified procedure of Blobel & Potter (1967). The two fractions were then separated according to size on density gradients after the bound fraction had been treated with deoxycholate. The size-distribution profiles of the 'free' and the 'bound' ribosomes recovered from the postmitochondrial supernatant of hepatoma 7800 are shown in Fig. 3. The 'bound' ribosomes represented only a small fraction of the total ribosomal components in the postmitochondrial supernatant. A much larger proportion of the ribosomes was recovered in the 'bound' fraction prepared from the supernatant of the host liver (S.-W. Kwan & T. E. Webb, unpublished work). Though it is possible that a fraction of ribosomes became detached from the membranes during zonal centrifugation, there was a significant

Table 1. Relative labelling of 'free' and 'bound' ribosomes with [6- ^{14}C]orotic acid and L-[^{14}C]leucine in hepatoma 7800 and host liver

[6- ^{14}C]Orotic acid ($50\mu\text{C}$ per rat) was injected intraperitoneally at 4 hr., or L-[^{14}C]leucine ($30\mu\text{C}$ per rat) was injected intravenously at 3 min., before removal of the host liver and hepatoma 7800; the rat had been inoculated 2 months earlier with the tumour. The 'free' and the 'bound' polyribosomes prepared from the postmitochondrial supernatant (Blobel & Potter, 1967) were subjected to density-gradient centrifugation. The specific radioactivity ratio refers to the ratio of the average specific radioactivity (counts/min./ E_{260} unit) of the free polyribosomes to that of the bound polyribosomes; the specific radioactivities were estimated from the extinction and radioactivity profiles (see the Materials and Methods section).

Labelled compound	Sp. radioactivity ratio	
	Host liver	Hepatoma 7800
[6- ^{14}C]Orotic acid	0.98	1.21
L-[^{14}C]Leucine	1.02	0.95

difference between the proportion of tightly bound polyribosomes in the two tissues. The data in Table 1 show that the ratios of the specific radioactivities of the 'free' and the 'bound' ribosomes in the two tissues were approximately equal after 4 hr. labelling with [6- ^{14}C]orotic acid. This suggests that the 'free' and 'bound' pools were in equilibrium with newly synthesized ribosomes 4 hr. after administration of the labelled compounds. Further, the specific radioactivities of the free and bound polyribosomes were approximately equal after 3 min. pulse-labelling with L-[^{14}C]leucine. Therefore both the 'free' and the 'bound' polyribosomes were active in protein biosynthesis in the hepatoma as well as in the host liver.

Interconversion of ribosomal pools. A study was made of the interconversion of the various pools of ribosomal components in host liver and hepatoma by certain inhibitors of protein biosynthesis. Except when carbon tetrachloride was used, the rats were partially hepatectomized 15 hr. before administration of the drugs.

Preliminary experiments showed that a portion of the polyribosomes in hepatoma 7800 and the host liver was converted into monomers and dimers by puromycin, as would be predicted from its effect on normal rat liver (Villa-Treviño, Farber, Staehelin, Wettstein & Noll, 1964). Since puromycin catalyses the breakdown of polyribosomes *in vivo* by accelerating the rate of translation, it was used to determine the proportion of polyribosomes in the supernatant of hepatoma 7800 that was active in protein biosynthesis. Complete breakdown of the polyribosomes did not occur in the tumour even with high doses of puromycin; therefore the ribosomal

Fig. 4. Size-distribution and $[^3\text{H}]$ orotic acid-labelling pattern of ribosomal components from hepatoma 7800 after prelabelling and then treatment of the rat with puromycin. Orotic acid ($200\mu\text{C}$) was injected at 5.5 hr., followed by puromycin (50 mg.) at 30 min. and again at 20 min., before removal of the tumour. A 0.5 ml. sample of the deoxycholate-treated postmitochondrial supernatant was layered on the gradient. The top of the gradient is to the right. Corrected E_{260} (—); counts/min. (●—●); specific radioactivity (●...●).

RNA was prelabelled with $200\mu\text{C}$ of $[^3\text{H}]$ orotic acid/rat to obtain a second parameter for assessing the functional competence of the polyribosomes. Since the ribosomes of non-functioning polyribosomes do not become detached from the messenger RNA (Wettstein *et al.* 1963), they should not equilibrate with newly formed ribosomes; therefore the presence of a significant concentration of the inactive species would affect the specific radioactivity profile. Two doses of 50 mg. of puromycin/kg. were given 30 min. and 20 min. before the removal of the tumour and 4 hr. after injection of the labelled compound. As shown in Fig. 4, most of the heavy polyribosomes in the hepatoma were broken down to lighter species by the puromycin (cf. Fig. 2a). Further, since the specific radioactivity did not vary significantly throughout the polyribosome region, it is concluded that most of the polyribosomes in the postmitochondrial supernatant of hepatoma 7800 were functional *in vivo*.

Polyribosome breakdown can also be induced in rat liver by the base analogue 8-azaguanine (Webb, 1967; Kwan & Webb, 1967), by actinomycin D (Staehelin, Wettstein & Noll, 1963), and by the hepatotoxin carbon tetrachloride (cf. Webb *et al.* 1965). The results in Table 2 indicate that all three drugs induced marked breakdown of the polyribosomes to monomers and dimers in the host

Table 2. Functional competence and interconversion of ribosomal components in hepatoma 7800 and host liver

The 'total' ribosomes in the deoxycholate-treated postmitochondrial supernatant, or the purified 'free' and 'bound' ribosomes, were subjected to density-gradient analysis. Each rat was injected intravenously with $30\mu\text{C}$ of $\text{L-}[^{14}\text{C}]\text{leucine}$ 3 min. before being killed, and the specific radioactivities (counts/min./ E_{260} unit) of the ribosomal components were estimated from the extinction and radioactivity profiles. Actinomycin D was administered intraperitoneally 4.5 hr. (1.0 mg./kg.) and 4 hr. (0.5 mg./kg.) before removal of the liver and tumour. 8-Azaguanine (100 mg./kg.) was injected intraperitoneally at 4.5 hr. and again at 4 hr. before removal of the liver and tumour, and carbon tetrachloride [2.5 ml. of a 1:1 (v/v) mixture in mineral oil] was intubated 20 min. before removal of the tissues. M-D and PR refer to the monomer-dimer pool and the polyribosome pool respectively. In all cases, the liver and hepatoma were obtained from rats 2 months after inoculation with the tumour.

	Host liver			Hepatoma 7800		
	M-D (% of total)	Sp. radioactivity		M-D (% of total)	Sp. radioactivity	
		M-D	PR		M-D	PR
Control						
Total	20	130	1200	30	20	320
Free	15	95	1200	35	45	200
Bound	15	80	1150	20	25	210
Actinomycin						
Free	60	90	1100	45	20	160
Bound	75	250	1230	45	30	200
Azaguanine						
Free	40	300	2200	50	30	100
Bound	20	575	1800	25	55	111
Carbon tetrachloride						
Total	120	100	2400	50	45	130

liver and somewhat less breakdown in the hepatoma. The monomers and dimers were inactive *in vivo*, as indicated by their low specific radioactivities after pulse labelling *in vivo* with L-[¹⁴C]leucine. Though both actinomycin D and 8-azaguanine probably affect messenger-RNA formation and function, the former appears to increase the monomers and dimers in both the 'free' and the 'bound' ribosomal pools, whereas the latter appears to affect only the free ribosomal pool. The significance of this difference is not clear. The sensitivity of hepatoma 7800 to carbon tetrachloride further confirms the close resemblance of this tumour to liver.

DISCUSSION

The ribosomal pools in the cell are influenced by controls that operate at the transcriptional and translational levels. Because of their unique structure and function, the polyribosomes in particular form a focal point for cellular controls that regulate the amount and type of proteins being synthesized. Comparative studies of the diversity and functional competence of ribosomal components from normal and neoplastic tissue should therefore provide a means of detecting any major abnormalities that may exist in these basic controls in tumours. A number of ribosomal pools have been identified in liver and hepatoma by physicochemical separation and differential labelling with L-[¹⁴C]leucine. The present results and those presented earlier (Webb *et al.* 1965; Webb & Potter, 1966) suggest that, with the exception of the inactive dimer pool in hepatomas, the ribosomal pools appear to be qualitatively similar in both tissues. However, quantitative differences appear to exist in the degree of association between ribosomes and the endoplasmic reticulum. Whereas 36% of the ribosomes in the postmitochondrial supernatant of the regenerating liver of the host rat were recovered as 'bound' polyribosomes, only 18% were in the 'bound' fraction of hepatoma 7800; the total amount of ribosomes in the post-mitochondrial supernatants of both tissues was the same. These probably represent relative rather than absolute values, since a constant fraction of the rough endoplasmic reticulum appears to be lost during the preparation of the postmitochondrial supernatant (Howell, Loeb & Tomkins, 1964; Blobel & Potter, 1967). Though there is the possibility that a fraction of the ribosomes are stripped from the endoplasmic reticulum by the rigorous procedures required for the separation of the 'free' and the 'bound' pools, morphological studies also suggest that an increased proportion of free ribosomes is a characteristic of tumour cells (Hruban *et al.* 1965).

The polyribosomes of the hepatoma were converted into inactive monomers and dimers by a number of drugs known to cause polyribosome breakdown in rat liver. The lower sensitivity of the hepatoma to actinomycin and azaguanine compared with that of the host liver cannot be interpreted in terms of messenger RNA stability, since it is probably due, in part, to a difference in the relative availability of the drug at the two sites. The conversion of polyribosomes into monomers and dimers by puromycin is a result of accelerated translation, i.e. a decrease in the time necessary to charge the acceptor site on the ribosome with the correct aminoacyl transfer RNA (Villa-Treviño *et al.* 1964). By using sensitivity to puromycin as a criterion, most of the polyribosomes recovered in the postmitochondrial supernatant of hepatoma 7800 appeared to be functional *in vivo*. However, the possibility that puromycin activates non-functional polyribosomes cannot be ruled out. The question of whether all polyribosomes are active *in vivo* is of interest, since not all proteins normally synthesized by rat liver are synthesized by the hepatomas (Potter, 1964; Schreiber, Boutwell, Potter & Morris, 1966). The results of the present study suggest that the 'free' and the 'bound' polyribosomes are equally active *in vivo*.

The livers of rats bearing hepatoma 7800 have an abnormally high concentration of ferritin. The reason for this accumulation of ferritin is not clear. Few changes in polyribosome size distribution occurred in the host liver until the tumour produced cachexia, at which time significant polyribosome breakdown was observed; similar breakdown was not observed in the tumour. The degree of conversion of polyribosomes into monomers and dimers was greater than that induced by prolonged starvation (Webb *et al.* 1966) and may have resulted from amino acid imbalance or other deficiencies that accompany cachexia (Costa, 1963). These findings, which were also observed in the liver of rats bearing hepatoma 7794B (S.-W. Kwan & T. E. Webb, unpublished work), are in accord with the report that the ultrastructure of the liver undergoes marked changes during the terminal stages (Parry & Ghadially, 1966).

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