Vol. 81, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1978 Pages 504-511

RIBOSOME BOUND RIBONUCLEASE; ITS PREFERENTIAL ASSOCIATION WITH SMALL POLYSOMES

Anthony B. Bransgrove and Chontal L. Cosquer

The SAND Institute,
Stella Close, Killara, Australia

Received January 3,1978

SUMMARY: Ribonuclease of the total rat liver ribosome fraction proved to be considerably more active than the same enzyme of the polysome fraction. This diminished polysomal activity was caused by exclusion of the enzyme-rich small polysomes and monosomes from discontinuous sucrose gradient preparations. An incidental finding was the demonstration that regenerating liver ribosomes appear to carry some of this enzyme in a dormant state not normally revealed during autodegradation.

INTRODUCTION

A wealth of information exists on the biological specificities and functions of bacterial and eukaryotic ribonucleases. Selective changes in the activity of these enzymes and their inhibitors have been reported to accompany numerous and diverse growth conditions (1-3). Unfortunately,the literature shows a marked lack of agreement between authors reporting the magnitude and direction of these changes as well as the identity of the responsible enzymes and the part played by their natural inhibitors. An outstanding exception to the inconsistent nature of these findings however, is the ribosome bound ribonuclease. This enzyme responds in a most definite fashion by decreasing almost completely in liver during regeneration (4) carcinogenesis (5) and frank tumour formation as well as in the spleen during immune response (6) and also in bacteria during the growth phase (7). Nevertheless, several reports (8,9,13) of ribosome autodegradation present results which are at variance with this apparent accord. Since each of the latter studies utilized procedures yielding polysomes rather than those more commonly used which yield monosomes the object of this investigation was to identify any aspects of ribosome preparation which might modify or bias the definitive enzyme activity.

0006-291X/78/0812-0504$01,00/0

MATERIALS AND METHODS

Mature Wistar strain male rats weighing between 180 and 230 gm were used as a source of native or regenerating liver and foetal Wistar rats were obtained at the 17th day. Regenerating liver was obtained 24 hours after a 60% partial hepatectomy (10) under ether anaesthesia. All tissue was taken from animals previously fasted for 12 hours. Animals were killed by exsanguination under ether anaesthesia. Tissue for ribosome or polysome preparation was removed, washed in distilled water and chilled under crushed ice, after which all operations were performed at 0.4°C. The tissue was minced with scissors then homogenised with a Potter Elvehjem glass/teflon homogenisor rotating at 450 rpm and a clearance of 0.2mm. Five strokes were found to be optimal. Homogenates were prepared in T-K-M sucrose buffer (25mM Tris-maleate, 50mM KCl, 5mM $MgCl_2$, 250mM sucrose pH 7.6, 0.4°C.) A post mitochondrial supernatant was obtained from a 25% homogenate by centrifuging at 12,000 x g for 15 min with a Sorvall RC-2 refrigerated centrifuge.

Monoribosomes were prepared from the 25% homogenate according to the procedure of Tashiro and Siekevitz (11) and then suspended in T-M buffer (1mM $MgCl_2$, 1mM Tris-maleate pH 7.6) at required concentration using an A_{260nm} 1% = 240/cm (26). Polysomes were prepared by the procedure of Wettstein et al (12) from a 25% post mitochondrial supernatant, suspended in buffer, and adjusted to the required concentration using an A_{260nm} 1% = 122/cm (26). The remaining methods appear in legends.

RESULTS AND DISCUSSION

Yield: The average yield for six preparations each of native liver monosomes was 3.1 $\pm$ 0.34mg/gm liver and native liver polysomes was 2.5 $\pm$ 0.36 mg/gm liver. Inoue et al (9) reported that a 0.1% solution of foetal rat liver polysomes in the presence of 2mM EDTA at pH 7.6 released only 1.33% acid soluble material after 90 min incubation at 37°C. Avadham and Buetow (8) using polysomes from Euglena gracilis also reported an unusually low ribonuclease activity as demonstrated by acrylamide gel electrophoresis and Roth (13) failed to detect any ribonuclease activity in rat liver polysomes Table I lists the ribonuclease activity of both the polysome and monosome preparations from native. foetal and regenerating rat liver. The monosomes are markedly more active than the polysomes in all cases except regenerating liver where both preparations appear to be inactive. Thus the seemingly deficient enzyme activity evident in some studies appears to be an expression of the fact that polysomes prepared by sedimentation through 2.0M sucrose are considerably less active than monosomes when assayed by autodegradation. The question therefore arises are polysomes inherently resistant to ribonuclease

TABLE I: A comparison of ribonuclease activity of monosomes and polysomes from rat liver under differing growth conditions.

	Activity (A_{260nm})					
	Monosomes			Polysomes		
Incubation Time (min)	Native Liver	Foetal Liver	Regenerating Liver	Native Liver	Foetal Liver	Regenerating Liver
30	0.21	0.15	0.00	0.00	0.00	0.00
60	0.43	0.23	0.01	0.05	0.02	0.00
90	1.11	0.48	0.01	0.06	0.05	0.00
120	1.25	0.63	0.03	0.08	0.05	0.00

Ribosome bound ribonuclease was assayed by autodegradation of the particle after activation with EDTA (K^+ salt). The reaction mix contained 0.5ml ribosome suspension (14.0 A_{260nm}/cm), 0.25ml of 125mM EDTA and 0.25ml of 100mM phosphate buffer pH 7.6. Samples were incubated in sealed glass tubes at 37°C for periods increasing by 30 min increments up to 2 hours, then precipitated with 1ml of cold acid alcohol (conc.HCl and ethanol 1:4) centrifuged (1500 x g for 10 min, 0-4°C) and the A_{260nm} of the supernatant determined. Blanks taken at the beginning of incubation were similarly treated and ribonuclease activity was expressed as the mean A_{260nm} of acid soluble material generated by duplicate preparations over the period of incubation.

or do they in fact carry less of the enzyme than their monosome counterparts. In support of the former possibility it is known that polysomes are resistant to dissociation by concentrated salt solution because of the bound peptidyl tRNA (14-16) and dissociation into subunits is a prerequisite for autodegradation (26). Accordingly tRNA-induced tighter binding between polysome subunits may be responsible for retarding their autodegradation even in the presence of a normal complement of particle bound ribonuclease. To test this possibility polysome preparations from native and regenerating rat liver (4mg/ml) were first reduced to single particles by aging over a seven day period at 0-4°C, then exposed to puromycin to release peptidyl tRNA (14) and eliminate any hindrance to dissociation of subunits.

Sucrose gradient analysis (Fig.1) confirms that aging alone (of native or regenerating liver polysomes) causes an almost total conversion to monosomes. It also demonstrated that these same monosomes after treatment

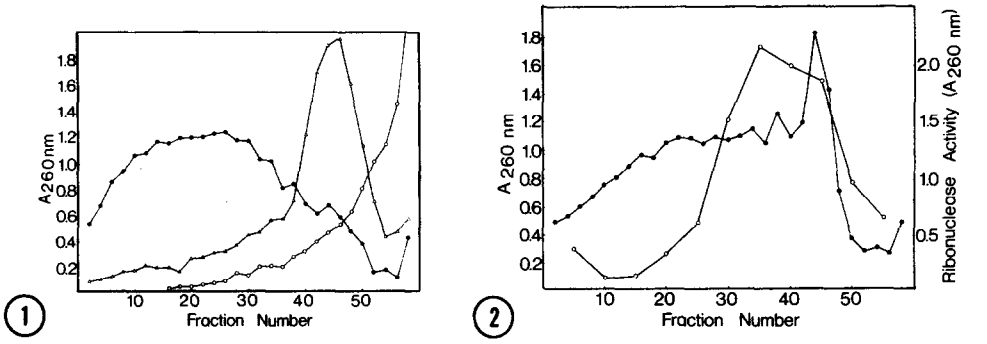

Fig. 1. The size distribution profiles of polysomes; from native liver (●——●), following aging (Δ——Δ) and following treatment with puromycin (○——○). Polysomes in T-K-M buffer were aged by storage at 0-4°C under sterile conditions over a seven day period. Puromycin treated polysomes were first aged and then incubated with 10mM puromycin at 37°C for 20 min. Each preparation (4mg/ml) was layered as a 4ml volume on a 55ml 10-40% linear sucrose gradient of high ionic strength (0.4MKCl, 5mM $MgCl_2$, 25mM Tris-maleate, pH 7.6 0-4°C,) and centrifuged for 3 hours at 24,000 rpm in a Spinco SW 25.2 rotor. Following centrifugation 2ml fractions were collected and A_{260nm} determined. Sedimentation is from right to left.

Fig. 2 The size distribution profile of native liver polysomes prepared by the modified discontinuous sucrose gradient technique described in the text (●——●), together with the associated ribonuclease activity of the pooled fractions (○——○). The conditions of gradient analysis are described in the legend to Fig. 1. However the high ionic strength was reduced to that of T-K-M buffer. Three gradients were run and following A_{260nm} determinations corresponding fractions were combined to give twelve 13.0ml portions. These were centrifuged at 105,000 x g in a 40' rotor for 4 hours and the resultant pellets suspended in a T-M buffer and adjusted to a concentration of 1.0mg/ml. Ribonuclease activity was determined as described in the legend of Table I. Assay fractions 1,2, and 12 contained less than 1mg/ml of ribosome material but activity has been adjusted accordingly.

with puromycin readily dissociate to subunits (unlike polysomes) in the presence of 0.4M KCl and so should be susceptible to autodegradation.

Autodegradation however, of both native and regenerating liver polysomes was not promoted by prior aging and puromycin facilitated disassembly of the particles (Table II) and hence polysomes appear to actually lack the ribonuclease. Polysomes prepared by sedimentation through 2.0M sucrose are known to be selectively enriched, because the monosomes and the slightly faster sedimenting small polysomes are not able to move through the heavy sucrose (17,25). The lower yield gained from the sucrose gradient preparation

TABLE II: Ribonuclease activity of native and regenerating liver monosomes and polysomes following aging or combined aging and puromycin treatment.

Treatment Prior to Assay	Activity (A_{260nm})			
	Monosomes		Polysomes	
	Native Liver	Regenerating Liver	Native Liver	Regenerating Liver
Nil	1.21	0.00	0.00	0.00
Aged	1.15	0.85	0.00	0.00
Aged + Puromycin	1.11	0.83	0.07	0.00

Native and regenerating liver monosomes and polysomes were prepared as described in text, aged and treated with puromycin as described for Fig.1 then adjusted to 1mg/ml with T-M buffer prior to assay by autodegradation over a 2 hour period. Activity is the mean of duplicates and conditions of assay are described in the legend to Table I.

in this study does suggest the exclusion of some of the total ribosome complement. If so it may be these smaller elements which carry the ribonuclease normally found in the total ribosome preparations. This possibility was examined by modifying the preparation. The 2.0M sucrose was replaced by 1.5M sucrose and the centrifugation time was increased from 4 to 12 hours. Fig.2 shows the size distribution profile of the resultant polysomes from native liver and by comparison with Fig.1 the proportion of small polysomes and monosomes can be seen in fact to have increased quite considerably as also did the yield (3.3mg/gm). In addition, the procedure appears to still exclude ribosome subunits. Rat liver polysomes prepared by the modified procedure were assayed for ribonuclease activity and found to possess an activity similar to that of the monoribosome preparations of that tissue. Moreover, by assaying the various sucrose gradient fractions (Fig.2) the specific enzyme activity of the smallest polysomes was seen to be considerably higher than that of monoribosome preparations, the most active fractions being associated with the monomer, dimer and trimer-peaks.

Regenerating liver monosomes are deficient in ribonuclease (4).

TABLE III: The partial restoration of ribonuclease activity to regenerating liver ribosomes at 37°C and the counteraction of GSH.

Time of Aging at 37°C (hours)	Activity (A_{260nm}) Incubation with GSH	Incubation without GSH
2	0.05	0.00
4	0.00	0.44
6	0.12	0.60
8	0.10	0.72
10	0.14	0.74

Monosomes from 24 hour regenerating liver (12ml, 1mg/ml) were incubated at 37°C with agitation over a 10 hour period. At 2 hour intervals duplicate 0.5ml aliquots were removed and assayed for ribonuclease as described in the legend to Table I. A second preparation was similarly incubated but in the presence of 5mg/ml of GSH. The values given are the mean of duplicates.

Their inclusion as controls during the aging of polysomes quite unexpectedly restored some degree of enzyme activity to the particle (Table II). Furthermore, it was found possible to accelerate this process by simple incubation at 37°C. The time course of enzyme restoration at 37°C in the presence and absence of GSH* is indicated in Table III.

Ribosomal ribonuclease activity of rat liver showed two orders of magnitude the difference depending on whether the preparations assayed were polysomal or monosomal. A selective removal of ribonuclease-containing particles during centrifugation through heavy sucrose and the variable efficiency of this removal probably explains why some authors have found a low but definite activity for rat liver polysomes (9) while others have found none (13). The modified preparation procedure as might be expected gave the highest yield of the three methods used in this study. It also probably offers some advantage over the more common practice of applying the post mitochondrial supernatant directly to a continuous sucrose gradient when analysing the 'total ribosome' (polysome and monosome) population because

* Abbreviation: GHS, reduced glutathione.

the sample is freed from the soluble nucleases of the cell sap which otherwise diffuse and contaminate the upper portion of the gradient. The absence of ribosome subunits and degraded material from the modified preparation is inconsistent with the view (13) that ribonuclease is an artifact of ribosome preparation and is merely contaminating the subribosomal particles of the upper gradient. On the contrary, results of this study indicate that ribonuclease is associated with either monosomes or small polysomes or both and is not consequent on the presence of enzyme-contaminated subribosomal material. This conclusion also corroborates the view that ribosome populations of both animal and bacterial cells are heterogenous with respect to their ribonuclease content (18,27). Under growth conditions associated with diminished or absent ribosomal ribonuclease, such as regenerating liver, the larger polysomes are known to increase at the expense of the smaller ones (19,20). Under these in vivo conditions the loss of smaller entities might also account for the diminished ribosomal ribonuclease.

The development of ribonuclease activity following aging of regenerating liver ribosomes signifies the survival of the enzyme in a dormant form distinct from the latency normally associated with this particle-bound enzyme. Furthermore, this form appeared to be stabilized in the presence of GSH. Gavard and de Lamirande (7) have experienced a similar phenomemon in which this same enzyme increased in activity by 25% during its extraction and they concluded that some ribosomal ribonuclease is present in an inactive form which cannot be attributed to the well known natural inhibitor of ribonuclease. Since the inhibitor is not found in the ribosomal fraction (4,18) the effect of GSH in this study may point to an alternate mechanism of inactivitating the enzyme. In this regard bovine pancreatic ribonuclease is known to be specifically sensitive to reductive inactivation by GSH (21). The liver ribosome-bound ribonuclease resembles this enzyme in many respects and so might also be susceptible to inactivation

by GSH, increased levels of which are found in liver during regeneration (24).

REFERENCES

1. Shortman, K. (1962) Biochim. Biophys. Acta, 61,50-55.
2. Maor, D. and Alexander, P. (1968) Biochim. Biophys. Acta, 157, 627-629,
3. Roth, J.S. and Hilton, Sylvia and Morris, H.P. (1964) Cancer Res., 24, 204-301.
4. Arora, D.J.S. and De Lamirande, G. (1967) Can.J. Biochem. 45, 1021-1026.
5. Arora, D.J.S. and De Lamirande, G. (1968) Cancer Res., 28, 225-227.
6. Willis, Dawn B. and Star, J.L. (1972) Biochim. Biophys. Acta, 262, 181-188.
7. Gavarde, D. and De Lamirande, G. (1972) Biochim. Biophys. Acta, 277,284-289.
8. Avadhani, N.G. and Buetow, D.E. (1973) Biochem. Biophys. Res. Comm., 50,443-451.
9. Inoue, T., Yamaguchi, H. and Umemura, Y. (1975) Biochem. Biophys. Res. Comm.,62,176-183.
10. Higgins, C.M. and Anderson, R.M. (1931) Ach.Pathol., 12,186-202.
11. Tashiro, Y. and Siekevitz, P. (1965) J. Mol.Biol.,11, 149-165.
12. Wettstein, F.O., Staehelin, T. and Noll, H. (1963) 197, 430-435.
13. Roth. J.S. (1967) 'Methods in Cancer Research', H. Busch, ed., Acad.Press, N.Y. Vol.3, 220-224.
14. Infante, A.A. and Graves, P.N. (1971) Biochim. Biophys. Acta, 246, 100-110.
15. Schlessinger, D. Mangiarotti, G. and Apirion, D. (1967) Proc. Nat. Acad,Sci.U.S. 58, 1782-1789.
16. Zylber, E.A. and Penman, S. (1970) Biochim. Biophys. Acta, 204, 221-229.
17. Webb, T.E. and Potter, V.R. (1966) Cancer Res., 26,1022-1025.
18. Krechetova, D.G., Chudinova, I.A. and Shapot, V.S. (1972) Biochim. Biophys. Acta, 277, 161-178.
19. Cammarano, P. Giudice, G. and Lukes Bessie (1965) Biochem. Biophys. Res.Comm., 19, 487-493.
20. De Lamirande, G. and Arora, D.J. (1969) Cancer Res., 29,795-799.
21. Kim, S. and Paik, W.J. (1968) Biochem. J., 106,707-710.
22. Barker, G.R. and Pavlik, J.G. (1967) Nature, 215,280-281.
23. Fontaniere, B. and Daoust, R. (1973) Cancer Res., 33,3108-3111.
24. Smith, H.M. (1962) Proc. Soc.Exp.Biol. 109,182-183.
25. Noll, H. (1969) 'Techniques in Protein Biosynthesis' P. Campbell and J. Sargent, eds., Acad.Press, N.Y. Vol.2, 171-174.
26. Bransgrove, A. (1976) PhD. thesis, Univ.of Sydney, Sydney, Australia.
27. Mangiarotti, G. and Turco, E. (1973) Eur.J. Biochem., 38,507-515.