Hemoglobin Messenger Ribonucleic Acid. Distribution of the 9S Ribonucleic Acid in Polysomes of Different Sizes*

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ABSTRACT: Reticulocyte polysomes contain a ribonucleic acid (9S ribonucleic acid) having many of the properties proposed for the hemoglobin messenger ribonucleic acid. If this 9S ribonucleic acid is the hemoglobin messenger ribonucleic acid its distribution in polysomes of different sizes should vary in a manner predicted by the polysome model. Polysomes are structures composed of various numbers of ribosomes attached to a single messenger ribonucleic acid. Thus a disome is two ribosomes and a messenger ribonucleic acid, a trisome is three ribosomes and a messenger ribonucleic acid, etc. The polysomal distribution of 9S ribonucleic acid was determined by isolating various ribosome fractions, namely- tri- and tetrasomes, penta- and hexasomes, and free 80S ribosomes by sucrose density gradient centrifugation. These fractions were treated with sodium dodecyl sulfate to dissociate the ribonucleic acid and protein, and the quantities of the various ribonucleic acids were analyzed by density gradient centrifugation. The tri- and tetrasomes contained an average of 3.1 ribosomes/9S ribonucleic acid and the penta- and hexasomes contained an average of 4.7 ribosomes/9S ribonucleic acid. This is in close agreement to the calculated values of 3.6 for the tri- and tetrasomes and 5.4 for the penta- and hexasomes. In the free 80S ribosome fraction there was 10.7 ribosomes/9S ribonucleic acid. The observed distribution is in accord with the 9S RNA being the hemoglobin RNA.

Previously we reported the isolation of an RNA from rabbit reticulocyte ribosomes which enhances amino acid incorporation into protein when added to an *Escherichia coli* cell-free system (Drach and Lingrel, 1964, 1966a). The material synthesized in this system, however, resembled *E. coli* protein rather than globin (Drach and Lingrel, 1966b). It was concluded from this study that the RNA added was not acting as a mRNA but rather enhancing the translation of endogenous mRNA. Hunt and Wilkinson (1967) reached a similar conclusion when they added RNA extracted from reticulocyte ribosomes, bone marrow cells, and liver to a reticulocyte cell-free system and found in each instance, including the addition of liver RNA, that not only was enhanced amino acid incorporation into protein observed but also an increase in hemoglobin synthesis was noted. Again the added RNA appeared to be serving a function other than a messenger function. These studies point out the difficulties of identifying mRNA on the basis of enhanced amino acid incorporation. Therefore emphasis has been placed on using indirect criteria for identifying the hemoglobin mRNA. Marbaix and Burny (1964), Burny and Marbaix (1965), Huez *et al.* (1967), and Chantrenne *et al.* (1967) have isolated an RNA with many of the characteristics expected for the hemoglobin mRNA. These properties include: (1) its presence in small quantities (approximately 2% of the total RNA), (2) its size (9 S) which would allow for the coding of either the α or β chains of hemoglobin, (3) its rapid degradation when polyribosomes are treated with quantities of ribonuclease which dissociate polysomes but do not degrade rRNA, (4) its release from polyribosomes at low magnesium ion concentrations, and (5) its higher specific radioactivity than rRNA when rabbits are injected with ^{32}P 10–20 hr prior to the collection of reticulocytes. On the basis of these observations it was proposed that this RNA is the hemoglobin mRNA.

If the 9S RNA is the hemoglobin mRNA then its presence in polysomes should vary according to the size of the polysome. This communication describes the realization of this unequal distribution.

Experimental Section

Production of Anemia. Circulating reticulocytes were obtained from rabbits made anemic by six daily subcutaneous injections of 0.3 ml/kg of 2.5% phenylhydrazine-HCl in 0.15 M sodium acetate (pH 7.0).

Collection of Cells. Blood was removed by cardiac puncture using a heparinized syringe and cooled to 2°.

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All further manipulations were performed at this temperature. The blood was filtered through cheesecloth and the cells were collected by centrifugation at 600*g* for 10 min. The cells were washed four times with four volumes of a saline solution containing 0.13 M NaCl, 0.005 M KCl, and 0.0074 M $MgCl_2 \cdot 6H_2O$. The "buffy coat" which is composed mainly of white cells was removed after the second wash.

Lysis of Cells and Separation of Polysomes. The washed cells were lysed by the addition of an equal volume of 0.005 M $MgCl_2$ and the cell debris were removed by centrifugation at 12,000*g* for 10 min. The lysate was diluted with three volumes of cold standard buffer (0.01 M KCl, 0.0015 M $MgCl_2$, and 0.01 M Tris-HCl, pH 7.4). This preparation (2.5 ml) was layered on a 37-ml 15–30% sucrose gradient and centrifuged for 3 hr at 27,000 rpm (95,000*g*) in a Spinco SW27 rotor at 2°.

After centrifugation the contents of the tubes were analyzed by pumping 40% sucrose into the bottom of the tube and continuously monitoring the solution emerging from the top at 260 mμ.

Analysis of 9S and rRNA. The pooled fractions of monosomes and polysomes were centrifuged for 10 hr at 76,000*g* at 2°. To the pellet, which was taken up in a minimum amount of 0.005 M Tris-HCl (pH 7.4), was added enough 10% sodium dodecyl sulfate to give a final concentration of 0.5%. This solution was incubated at 37° for 5 min. A volume equivalent to 1.8 mg of ribosomes was removed, layered on a 12-ml 5–20% sucrose gradient, and centrifuged for 14 hr in a SW40 Spinco rotor at 40,000 rpm.

Sucrose Density Gradients. Exponential sucrose gradients were prepared according to the method of Noll (1967). For separation of polysomes a 37-ml 15–30% sucrose gradient was used (Spinco SW27 rotor). These were prepared using a mixing chamber volume of 34 ml of 15% sucrose and a reservoir filled with 40% sucrose. The sucrose solutions were w/w in standard buffer. For the analysis of RNA 12-ml 5–20% sucrose gradients (Spinco SW40 rotor) were prepared by using a mixing chamber containing 14 ml of 5% sucrose and a reservoir filled with 30% sucrose. These sucrose solutions were w/w in 0.005 M Tris-HCl (pH 7.4).

Results

According to current concepts a disome consists of two ribosomes held together by a single strand of mRNA; likewise a trisome is three ribosomes held together by a single strand of mRNA, etc. (Wettstein *et al.*, 1963; Warner *et al.*, 1963). Thus various sizes of polysomes should exhibit different ribosome to mRNA ratios depending upon the number of ribosomes in the polysomal structure. For example, the ratio of ribosomes to mRNA in disomes would be 2:1, while that of pentasomes would be 5:1. Therefore, if the 9S RNA is the hemoglobin mRNA its presence in polysomes should vary in this manner. However, if the 9S RNA is a rRNA its quantity per ribosome should be constant regardless of the number of ribosomes in a polysome. To decide between these two possibilities various sizes of polysomes were prepared and the ratio of ribosomes to 9S RNA was determined.

Figure 1 shows the polyribosomal profile observed in these studies. The ribosomes migrating in the three areas indicated on the figure were pooled. These fractions correspond to monosomes, tri- and tetrasomes, and penta- and hexasomes. Disomes were not taken because of the possibility of contamination from monosomes. The ribosomes were concentrated, dissociated with sodium dodecyl sulfate, and subjected to sucrose density gradient analysis. The RNA profiles

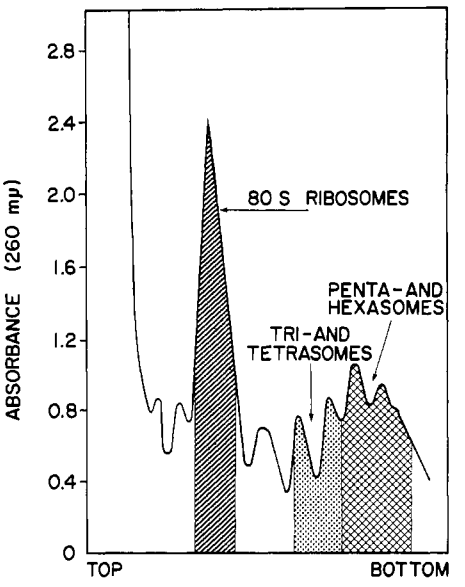

FIGURE 1: Sucrose density gradient separation of polysomes. Lysate (2.5 ml) was layered on a 15–30% sucrose gradient and centrifuged for 3 hr at 95,000*g* in a SW27 rotor.

TABLE I: Ratio of Ribosomes to 9S RNA in 80S Ribosomes and Polysomes.

Fraction	Ribosomes:9S RNA				
	Expt 1	Expt 2	Expt 3	Av	Theor[a]
80S ribosomes	12.0	8.6	11.5	10.7	
Tri- and tetrasomes	3.3	2.9	3.2	3.1	3.6
Penta- and hexasomes	4.5	4.3	5.2	4.7	5.4

[a] In moles per mole.

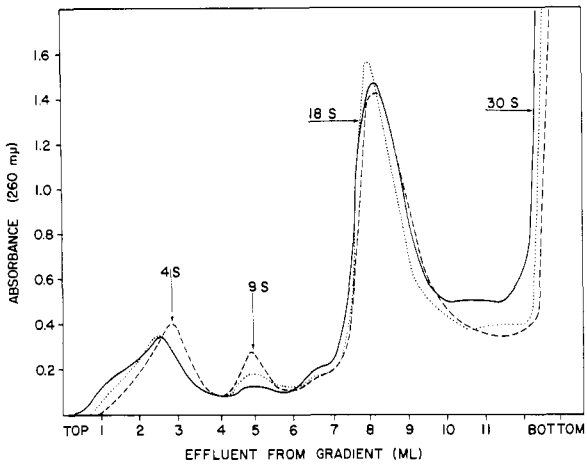

FIGURE 2: Sucrose density gradient centrifugation of sodium dodecyl sulfate treated ribosome fractions. Monosome and polysome fractions (1.8 mg) were treated with sodium dodecyl sulfate and centrifuged for 14 hr at 40,000 rpm in a SW40 rotor. The run was made at 2°. (—) Monosomes, (---) tri- and tetrasomes, and (····) penta- and hexasomes.

from one such experiment are shown in Figure 2. The monosome fraction contains little 9 S while the tri- and tetrasomes contain more 9 S than the penta- and hexasomes and considerably more than the monosomes. In order to calculate the number of ribosomes per 9S RNA the area under the 9S and 18S RNA peaks was estimated graphically. The moles of each of these RNAs were then calculated using a molecular weight of 150,000 and 500,000 for 9S and 18S RNA, respectively (Burny and Marbaix, 1965). Since there is one 18S RNA per ribosome, the moles of 18S RNA equal the moles of ribosomes. Quantitation of 30S RNA was not possible because it was largely pelleted; however, in shorter runs where both rRNAs were well resolved the ratio of the 18S to 30S RNA was found to be identical regardless of the polysome size. The molar ratios of ribosomes to 9S RNA from three such experiments are summarized in Table I.

The number of ribosomes per 9S RNA in the tri- and tetrasomes is 3.1 compared with 3.5 calculated on the basis of the amounts of trisomes and tetrasomes in the pooled fractions analyzed. Similarly 4.7 ribosomes/9S RNA were present in the penta- and hexasomes compared with a theoretical value of 5.4. The difference in the observed and theoretical values is probably due to the difficulty in quantitating the amount of 9S RNA. Nevertheless the agreement is quite good and readily depicts the expected variation of 9S RNA in polysomes on the basis of its being the hemoglobin mRNA.

Discussion

Polysomes are thought to be multiple ribosomes attached to a single mRNA molecule. By knowing the number of ribosomes in a particular polysome one can calculate the ratio of ribosomes to mRNA. Thus if the 9S RNA is the hemoglobin messenger its ratio should vary accordingly. However, if the 9S RNA is an rRNA which is present in every ribosome, then the ratio of ribosomes to 9S RNA should be constant regardless of the polysome size. The observed ratio was found not to be constant and varied in a manner predicted for mRNA. In the 80S ribosome fraction only one out of ten ribosomes contain a 9S RNA. The theoretical ribosome to mRNA ratio cannot be calculated because the mRNA content of the 80S ribosomes is unknown. Lamfrom and Knopf (1964) have compared the synthetic activity of 80S ribosomes and polysomes and found the 80S or free ribosomes to be less active than polysomes in hemoglobin synthesis.

The finding that 9S RNA varies in polysomal fractions in a manner expected for mRNA adds to the growing list of indirect evidence supporting the hypothesis that the 9S RNA is the hemoglobin mRNA. Direct proof will only come when this RNA is shown to direct the synthesis of hemoglobin. A preliminary account of such a study with a 9S reticulocyte RNA has recently appeared (Schapira *et al.*, 1968).

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