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Unexpectedly Large Size of Globin Messenger Ribonucleic Acid

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ABSTRACT The globin messenger RNA of rabbit reticulocytes is identified on the basis of its presence in polysomes, absence from single ribosomes, and sedimentation coefficient of 9 S. Analysis of this RNA by electrophoresis in polyacrylamide gels provides evidence that its size is unexpectedly large (approximately 650 nucleotides) for a messenger coding for the 141 and 146 amino acids of a globin polypeptide.

Reticulocyte RNA molecules were labeled *in vivo* with [^{32}P]orthophosphate. Globin messenger RNA and transfer RNA appear to be synthesized late in erythropoiesis, whereas ribosomal RNA and 5S RNA are synthesized at the beginning stages of cytodifferentiation. At least one fragment of ribosomal RNA accumulates in erythrocytes in the terminal stages of maturation.

Although much information about growth and development has been obtained by studying the protein products of cytodifferentiation, the available information about messenger RNA is sparse and questionable. For example, theories concerning mRNA stability have been largely based on studies of protein synthesis inhibition with actinomycin D. However, this drug causes modifications of RNA stability and has other side effects (1–5). It was the purpose of this study to learn about mRNA in differentiating cells by direct observations on the RNA molecule.

Our studies were made possible by the observation of Evans and Lingrel that a mouse reticulocyte RNA that sediments at 9 S is specifically located on polysomes but is absent from single ribosomes (6). Furthermore, this 9S RNA is the messenger since it can direct the synthesis of mouse globin chains in a cell-free extract that synthesizes proteins from rabbit reticulocytes (7). Presumptive mRNAs for histone (8), antibody (9), and myosin (10) syntheses are also specifically localized on polysomes but are absent from single ribosomes.

Using rabbit reticulocytes, we have confirmed the observation that a 9S RNA is specifically localized on polysomes. However, in confirmation of the results of several groups (11–14), we find this RNA to be contaminated with other RNA molecules that sediment between 8–15 S and which may be breakdown products of ribosomal RNA. The mRNA can be purified away from the contaminants by polyacrylamide gel electrophoresis. However, its electrophoretic mobility in the gels suggests that the globin mRNA has an unexpectedly large size.

MATERIALS AND METHODS

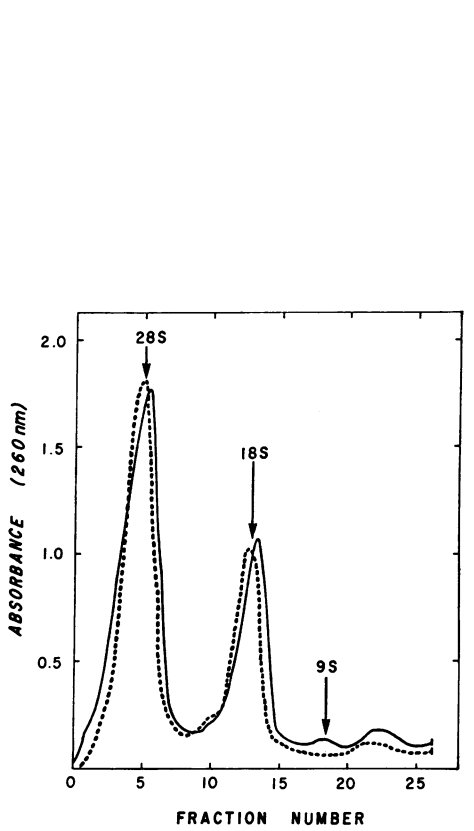
Methods used for producing severe anemia in rabbits, for removal of leukocytes, and for purification of reticulocyte polyribosomes and single ribosomes have been described (15, 16). Injection of 1 mCi of [^{32}P]phosphate (New England Nuclear Corp., NEX-054) was into the ear vein of an anemic rabbit. For sedimentation analysis of ribosomal RNA, the ribosomes were dissolved in buffer containing 0.5% sodium dodecyl sulfate–5 mM EDTA–20 mM sodium acetate–40 mM Tris·HCl, pH 7.8. The samples were then layered onto 29-ml, 10–30% linear sucrose gradients in the same buffer, and the tubes were centrifuged for 20 hr at 23,500 rpm at 20°C in the Spinco SW 25.1 rotor. The gradients were pumped through a Gilford model 222 spectrophotometer and the various fractions were collected into flasks. The RNA sedimenting between 6–16 S was pelleted by centrifugation at 65,000 rpm for 12 hr in the Spinco 65 rotor. Extraction of RNA from ribosome preparations was by the chloroform–isoamyl alcohol method of Oda and Joklik (17). This method of extraction gives a very high yield of undegraded RNA (15) and has been used to successfully extract intact mRNA from reovirus-infected (mouse) L cells (18). Electrophoresis of RNA on 2.4 or 4% polyacrylamide gels is described elsewhere (11, 15). In one experiment, the electrophoresis buffer was adjusted to 0.15 M NaCl in order to test the effect of ionic strength on the RNA mobilities. Acrylamide was recrystallized from chloroform; methylene bisacrylamide was recrystallized from water, and then from acetone (11). The gels were sectioned into 1-mm sections with a gel slicer (Brinkman Instruments); two adjacent gel sections were counted together on planchets in a low background gas flow counter. The background was 1.5 cpm.

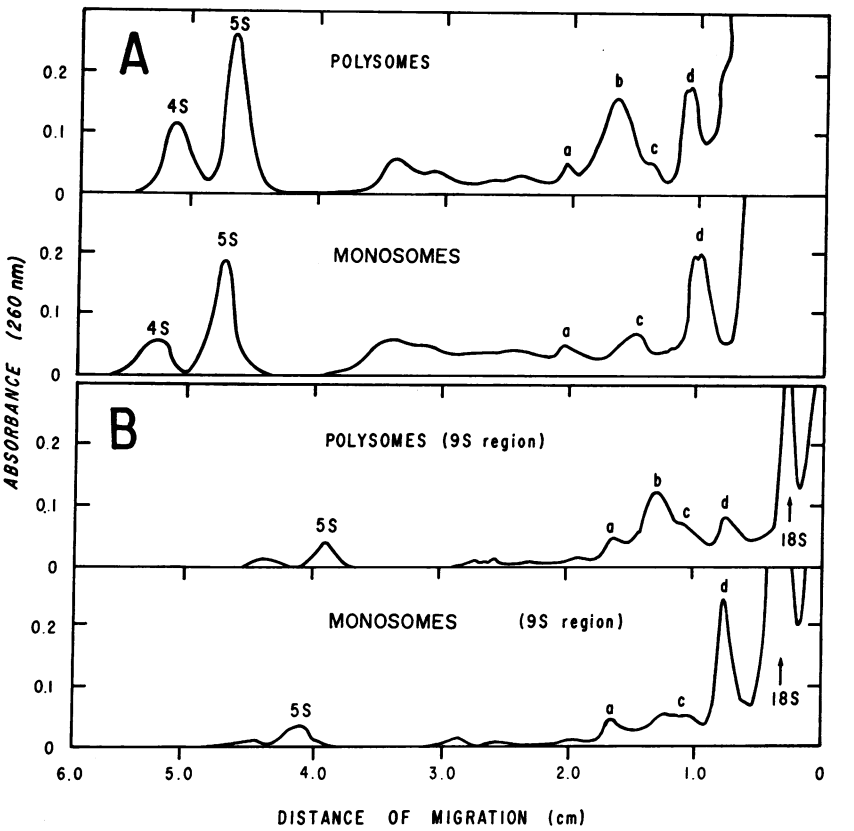
RESULTS

Sucrose gradient analysis of RNA from polysomes and from single ribosomes is shown in Fig. 1. In confirmation of the results of Evans and Lingrel (6), we find that a small RNA peak, with a sedimentation coefficient of 9 S, is specifically located on polysomes but is absent from single ribosomes.

RNA from polysomes and single ribosomes was also analyzed by electrophoresis on polyacrylamide gels. As can be seen in Fig. 2*A*, there is indeed an RNA which is present on polysomes but which is absent from single ribosomes. This RNA band is labeled "*b*" in Fig. 2. However, several other RNA molecules are present both in polysomes and monosomes. Furthermore, as is shown in Fig. 2*B*, the same RNA components are present in purified 9S RNA obtained from sucrose gradients. All of these absorbance bands are degraded by ribonuclease but not by pronase or by deoxyribonuclease.

The molecular weight of RNA can be measured on the basis of its electrophoretic mobility in polyacrylamide gels (19). For many RNAs that have been analyzed, there is a linear relationship between electrophoretic mobility and the





(*Left*) FIG. 1. Sucrose density gradient analysis of RNA from reticulocyte polysomes and from single ribosomes. The solid line is polysomes; the dotted line is single ribosomes.

(*Right*) FIG. 2. Electrophoresis of RNA on 4% polyacrylamide gels. The two upper tracings are part *A*, and show the RNA from polysomes and from monosomes. 100 μg of extracted RNA was applied to each gel. The two lower tracings are part *B*, and contain the 9S material of polysomes and monosomes. The ribosomes from 3 ml of reticulocytes were fractionated in sucrose gradients (see Fig 1) and the 6–16S material was pooled for analysis on the gels. Electrophoresis is toward the left. *A* and *B* are independent experiments.

logarithm of the molecular weight. Analysis by this method (see Fig. 3) indicates that the presumptive globin mRNA has a molecular weight of about 2.2×10^5, and therefore contains about 650 ribonucleotides. Our estimates of the molecular weight have ranged between $2.1–2.35 \times 10^5$. This same estimate was obtained when the electrophoresis was performed in a high ionic strength buffer containing 0.15 M NaCl.

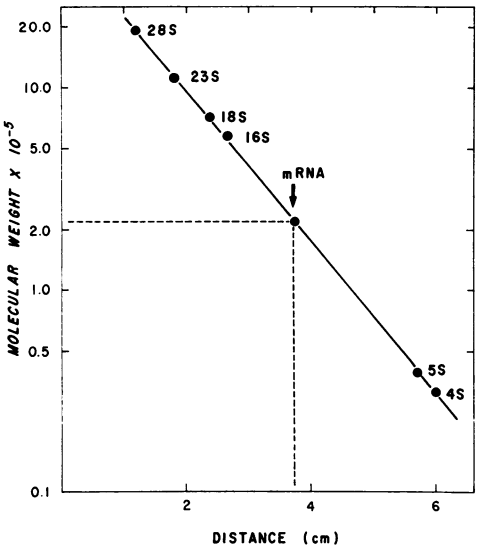
Further evidence for the identification of RNA peak *b* (see Fig. 2) as globin mRNA was obtained from a metabolic study. We injected an anemic rabbit with [^{32}P]phosphate and subsequently analyzed the labeling of the various reticulocyte RNAs. The results of this experiment are shown in Fig. 4. The 4S RNA and mRNA bands are labeled almost as heavily at 16 hr as at 38 hr, whereas the 18S, 28S, and 5S RNA bands are labeled at least four times more heavily after the 38-hr labeling. The distinctive labeling kinetics of the polysome-specific presumptive globin mRNA clearly distinguishes this RNA from all of the contaminating high molecular weight RNAs, and also demonstrates conclusively that this RNA is not a breakdown product of ribosomal RNA.

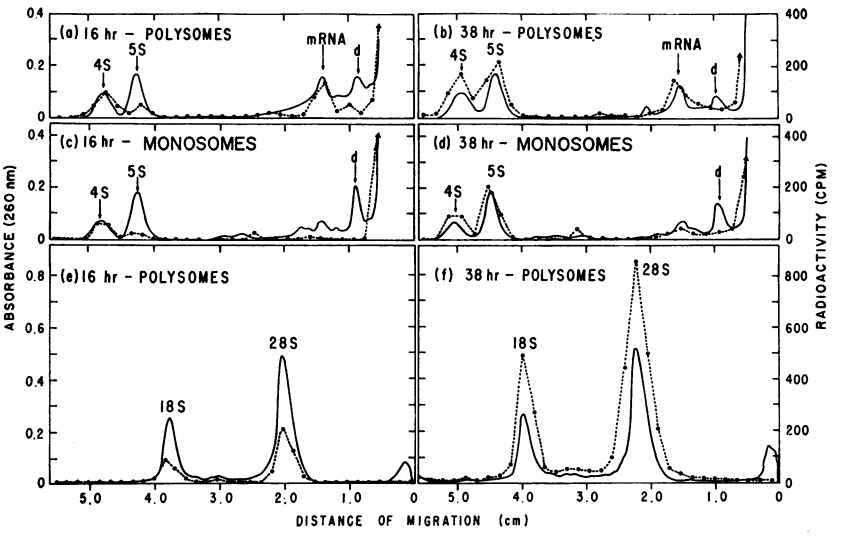
DISCUSSION

Identification of globin messenger RNA

The 9S RNA molecule on polysomes has several characteristics that support its identification as the globin messenger RNA. It is the only RNA in reticulocytes that we find to be specifically associated with polysomes. Several workers have obtained evidence for a specific polysomal localization of various mRNAs (6, 8–10). Furthermore, the RNA sediments in sucrose gradients at 9 S and it is labeled relatively extensively 16 hr after an injection of [^{32}P]phosphate into anemic rabbits. It has been shown previously that 9S RNA from mouse reticulocytes is specifically associated with polysomes and can direct the synthesis of mouse globin in a cell-free extract from rabbit reticulocytes (6, 7). Furthermore, the 9S RNA was relatively highly labeled shortly after injection of [^{3}H]uridine into anemic mice (20). Although these labeling results agree almost exactly with our own observations (Fig. 4), Lingrel and co-workers did not purify their RNA on polyacrylamide gels; they were presumably examining the labeling of a mixture of RNA molecules. It is now quite well established that the 9S region of sucrose gradients contains several RNA molecules in addition to the messenger RNA (Fig. 2; see also ref. 11–14). Some of these may be breakdown products of ribosomal RNA. It is well-known that ribosomes are being degraded rather rapidly during erythrocyte maturation (21).

Chantrenne *et al.* (22) have also reported a 9S mRNA in rabbit reticulocytes. Although they did not report a specific polysomal localization for the presumptive mRNA, it is





(*Left*) FIG. 3. A plot of the logarithm of molecular weight vs. electrophoretic mobility in 2.4% polyacrylamide gels. The curve obtained with the dilute 2.4% gels is linear. Calibration was made using the known molecular weights (19, 23) 1.9×10^6, 1.1×10^6, 0.71×10^6, 0.56×10^6, 3.9×10^4, and 2.6×10^4 for 28S, 23S, 18S, 16S, 5S, and 4S RNA, respectively. The *E. coli* ribosomes were a generous gift of Dr. E. T. Young, II.

(*Right*) FIG. 4. Electrophoresis of radioactive RNA on 2.4 and 4% polyacrylamide gels. The RNA was labeled *in vivo* with [^{32}P]-phosphate for 16 or 38 hr. 60 μg of RNA was analyzed on 4% gels, whereas only 7 μg was analyzed on the 2.4% gels. The 4S and 5S bands are seen on the 4% gels, whereas the 18S and 28S RNA bands are shown on the 2.4% gels. The polysomes and monosomes gave the same results on 2.4% gels; only the polysome data is presented. Electrophoresis is toward the left. (*a*) 16-hr polysomes (4% gel), (*b*) 38-hr polysomes (4% gel), (*c*) 16-hr monosomes (4% gel), (*d*) 38-hr monosomes (4% gel), (*e*) 16-hr polysomes (2.4% gel), (*f*) 38-hr polysomes (2.4% gel).

quite likely that they were observing the same RNA molecule studied here. These workers also first reported that the 9S RNA of reticulocytes was highly labeled shortly after an injection of anemic rabbits with [^{32}P]phosphate. We must point out, however, that ribosomal phosphoproteins have recently been discovered in reticulocytes (16). These phosphoproteins are relatively highly labeled shortly after an injection of [^{32}P]PO_4 into rabbits, or after reticulocyte incubation with [^{32}P]PO_4 *in vitro* (16), and they partially sediment at 9 S under the gradient conditions employed by Chantrenne *et al.* (22) and by Evans and Lingrel (6, 20). Although the labeling of these phosphoproteins is responsible for some of the earlier results (22), it is interesting that the conclusions of these workers have been subsequently substantiated nonetheless. Unlike the situation when nucleic acids are labeled with [^{32}P]PO_4, the phosphoprotein radioactivity remains precipitable after treating with 10% trichloroacetic acid at 90°C for 20 min. The extraction procedure used here for RNA completely removes the phosphoproteins.

The globin messenger RNA appears to have an unexpectedly slow mobility on polyacrylamide gel electrophoresis (Fig. 3). An RNA encoding a globin chain of 146 amino acids would be expected to contain approximately 440 nucleotides and to have a molecular weight of approximately 1.5×10^5. On the contrary, the globin mRNA migrates as expected for an RNA with 650 nucleotides and a molecular weight of 2.2×10^5. Although it is possible that a small RNA with an open, extended configuration would migrate relatively slowly in the gels, we think it most likely that the globin mRNA contains considerably more than 440 nucleotides. The mobilities of many RNA molecules are linearly related to the logarithms of their molecular weight (19), and only slight deviations have been observed. Furthermore, the molecular weight estimation for the globin mRNA is independent of ionic strength, suggesting that the mRNA configuration is not appreciably more extended than that of the calibrating RNAs. It is possible that the additional nucleotides perform a regulatory function in protein synthesis.

Messenger RNA synthesis during cytodifferentiation

The reticulocyte RNAs can be divided into three classes on the basis of their labeling (Fig. 4) *in vivo* with [^{32}P]PO_4: *Class I.* These RNA molecules are at least four times more highly labeled after 38 hr than after 16 hr of labeling. This group consists of 5S rRNA and of 18S and 28S rRNA molecules. *Class II.* These RNA molecules are almost as highly labeled at 16 hr as at 38 hr. This class consists of 4S RNA and globin mRNA. *Class III.* This RNA is labeled only weakly, even after 38 hr, and consists of the band labeled "*d*" (see Fig. 4).

The rather slow labeling of the *Class I* RNA molecules indicates that they are synthesized in the immature, nucleated, erythroid cells of the bone marrow. Nuclear extrusion at the orthochromatic erythroblast stage, and subsequent maturation in the marrow, must occur before the labeled reticulocytes are released into the circulation. It is interesting that erythropoiesis occurs mainly by reductive cellular divisions in which the cells arising from any division are about half the volume of the parent cells (24), and that nucleoli are visible only in the earliest developmental stages. The erythroblast proteins are degraded during erythropoiesis and are replaced with hemoglobin molecules. It is therefore reasonable to conclude that ribosomes are synthesized in the

early erythroblasts and that cytodifferentiation occurs without further ribosome production.

The relatively very rapid labeling of the *Class II* reticulocyte RNA indicates that they are at least partially synthesized late in the process of erythropoiesis. Perhaps this synthesis occurs in cells of the bone marrow just prior to nuclear extrusion. However, there are other possibilities. For example, the apparent late labeling of 4S RNA might partially reflect the well-known slow turnover of terminal -CCA nucleotides that occurs in reticulocytes (25). Similarly, the apparent late globin mRNA synthesis could also be due to a cytoplasmic synthesis or modification subsequent to nuclear extrusion.

The very low labeling of *Class III* RNA molecules, even at 38 hr, is clearly documented only for the RNA labeled "*d*" in Fig. 4. This RNA might be a breakdown product of ribosomal RNA that accumulates in the terminal stages of erythrocyte maturation in the blood. One would expect such rRNA degradation products to become labeled only very slowly compared with the labeling of the *Class I* RNAs.

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