Some Properties of High Molecular Weight Ribonucleic Acid Isolated from Chick Embryo Polysomes†

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ABSTRACT: Three discrete RNA species having molecular weights of 1,300,000, 1,050,000, and 900,000 are resolved on polyacrylamide gels after two cycles of sucrose gradient fractionation of RNA sedimenting from 28 to 18 S, prepared from polysomes of 14-day-old chick embryos. When labeled for 24 hr, these RNAs each have a base composition very similar to 28S rRNA, but at least two of them have a significantly lower specific activity than 28S rRNA, suggesting they are the result of *in vivo* rather than *in vitro* processing. After a 2-hr label, the radioactivity found in 28S to 18S RNA is DNA like in base composition, and over 60% binds to Millipore filters. The latter is also DNA like except for its high (31–32%) adenosine content. The Millipore-bound rapidly labeled RNA does not coelectrophorese with the three discrete peaks of absorbance and radioactivity seen after a 24-hr label, but has an approximately continuous distribution which peaks at 1,300,000 daltons. The radioactivity but not the absorbance found between 28 and 18 S is unaffected by exposure to 99% formamide, suggesting that these fractions of rapidly labeled RNA are molecularly intact. The appearance of ultraviolet-absorbing RNA peaks as a result of sucrose gradient recycling of RNA sedimenting from 28 to 18 S, and the identification of these peaks as being derived from 28S rRNA, suggests that rRNA fragments may exist in small amounts in slower sedimenting regions of the gradient as well. The possibility of isolating a *pure* mRNA species on the basis of its size alone is therefore very small.

Only 1–2% of the RNA in polysomes is mRNA. Even in cells programmed to make predominantly one protein, such as reticulocyte cells, which have essentially one type of mRNA, the latter is barely visible optically without prior fractionation. Indeed it has become standard procedure to initially fractionate RNA by the repeated sucrose gradient centrifugation of those regions of the gradient which correspond to RNA molecules having the sedimentation constant of interest before proceeding to further purification. Of course this is only possible if the mRNA in question has a sedimentation constant sufficiently different from that of the large or small rRNA component to afford a reasonable separation on such gradients. The best example of the implementation of such fractionation is the preparation of "9S" hemoglobin mRNA in which optical amounts of apparently homogeneous RNA can be readily obtained (Chantrenne *et al.*, 1967). However, it has recently become apparent that even the best of these preparations are heavily contaminated with fragments of rRNA (Lanyon *et al.*, 1972; Morrison *et al.*, 1972). The source of this contamination has been assumed to be RNase action during isolation and purification. Moreover, it has been known for some time that high molecular weight RNA is highly susceptible to specific cleavage by minute quantities of RNase, and in spite of all precautions, it is unlikely that the last trace of RNase activity can be eliminated from most RNA preparations. Thus in concentrating a particular size class of RNA, perhaps 10- to 100-fold, one is likely to concentrate minute amounts of rRNA fragments as well.

We report here our recent studies of relatively large RNA species, sedimenting from 18 to 28 S, isolated from chick embryo polysomes. We found at least three RNA species present in optical density quantities ranging in molecular weight from 1,300,000 to 900,000 which are derived from 28S rRNA, either *in vitro* or *in vivo*. Both stable and rapidly labeled RNA were studied. No evidence for mRNA was found in stable RNA while rapidly labeled RNA contained largely mRNA species.

Materials and Methods

Preparation of ^{32}P-Labeled Polysomes. The 14-day-old chick embryos were labeled with 1–3 mCi of carrier-free $H_3{}^{32}PO_4$ (New England Nuclear) in 50 μl of physiological saline by injection into the surface blood vessel of the chorioallantoic membrane using a fine glass needle. Prior to injection a small rectangle of the shell, about 0.5 × 1 cm, was removed by cutting with a sharp file. After injection, the shell was taped back on the egg, and it was returned to the incubator for the desired time.

Following incubation, the embryo was removed from the shell, decapitated, and then washed repeatedly with ice-cold buffer containing 0.25 M KCl, 0.01 M $MgCl_2$, 0.01 M Tris-Cl (pH 7.4), 0.1 mM dithiothreitol, and 0.1 mM Na_2EDTA (polysome buffer). The embryos were then minced with sharp sterile scissors, and 1 ml of polysome buffer was added per g of wet tissue. The suspension was homogenized at 0° in a loose Dounce homogenizer with three to five strokes. In some experiments, a mechanical press was used to drive the pestle. The homogenate was centrifuged at 100,000*g* for 10 min, and the supernatant was decanted, made 1% in sodium deoxycholate, and then carefully layered on a step gradient consisting of equal volumes of 20 and 40% sucrose in polysome buffer. The postmitochondrial supernatant was then centrifuged for 2 hr at 60,000 rpm in a Beckman 60 Ti rotor. The clear polysome pellet was washed twice with polysome buffer after first removing the sucrose. It was then used immediately, or stored at −70°. About 3–4 mg of polysomes was obtained from one 14-day-old embryo.

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RNA Isolation and Fractionation. To release RNA from proteins, a modification of Marbaix and Burny's (1964) procedure was used. The polysome pellet was covered with 1 ml of 1% sodium dodecyl sulfate–0.01 M Na_2EDTA (pH 7.0) and heated at 37° for 5 min. After measuring the RNA concentration, the solution was diluted with 1% sodium dodecyl sulfate solution to an RNA concentration of about 1 mg/ml, layered on a 5–20% sucrose gradient containing 0.1 M NaCl, 0.01 M sodium acetate (pH 5), and 0.001 M Na_2EDTA, and centrifuged in a Beckman SW 27 rotor for 12 hr at 4°. Fractions sedimenting between 28 and 18 S were pooled and precipitated with two volumes of ethanol. The precipitates were redissolved in 1 ml of distilled water and rerun on identical gradients. Fractions sedimenting between 28 and 18 S were again collected and precipitated as before. Starting with about 12 mg of polysomal RNA before the first fractionation, we obtained about 500 μg of RNA after the second fractionation.

Polyacrylamide Gel Electrophoresis. AQUEOUS GEL ELECTROPHORESIS. RNA was analyzed on aqueous gels according to the procedures developed by Loening (1968) except that the gels were allowed to polymerize overnight, and electrophoresis was carried out for 5 hr at 5 mA/tube. The gels were scanned immediately using a Gilford Model 261 linear gel scanner, frozen, and sliced with a Mickle gel slicer (the Mickle Laboratory Engineering Co., Gomshall, Surrey, England) and the slices were counted in a Beckman Model LS250 scintillation counter using Cerenkov radiation to measure the ^{32}P-labeled RNA.

FORMAMIDE GEL ELECTROPHORESIS. RNA was analyzed on 99% formamide gels using a modification of the method described by Staynov *et al.* (1972) which Dr. W. Gratzer kindly sent us. Formamide (60 ml) was deionized by stirring vigorously with 2 g of wet Dowex AG-501-X8 (20–50 mesh, Bio-Rad) for at least 1 hr at room temperature, filtered, and made 0.016 M Na_2HPO_4 + 0.004 M NaH_2PO_4. Buffered deionized formamide (19 ml) was then added to 0.8 g of acrylamide and 0.12 g of *N,N'*-methylenebisacrylamide. After stirring, 0.04 ml of *N,N,N',N'*-tetramethylethylenediamine and 0.32 ml of freshly prepared ammonium persulfate (75 mg/ml of H_2O) were added and the solution was poured into 10-cm tubes, and allowed to polymerize overnight. Before applying the sample, the gels were covered with buffered formamide.

RNA samples were dissolved in 100 μl of buffered deionized formamide; 50 μl of a Bromophenol Blue–glycerine solution (prepared by combining 5 ml of glycerine, 5 ml of deionized formamide, and 60 μl of 1% Bromophenol Blue) was added, and the sample was carefully layered below the formamide on top of the gel. After filling the tubes to the top with formamide, 0.02 M phosphate buffer was added to the upper and lower reservoirs. The electrophoresis was carried out for a minimum of 4 hr at 5 mA/tube.

Base Composition. Three to five gel slices (1-mm thick) were pooled and extracted with 0.5–1 ml of H_2O/slice overnight at 37°. The extracted RNA was hydrolyzed in 0.3 M KOH at 37° for 18 hr. After being neutralized with $HClO_4$, the precipitate was removed by centrifugation and the supernatant was concentrated to about 200 μl on a rotary evaporator. The sample was applied to Whatman 3MM paper (90 × 40 cm) and the nucleotides were separated by high-voltage electrophoresis (40–55 V/cm) in 0.05 M ammonium formate buffer (pH 3.5) for 40–100 min; 1-cm wide strips were cut and counted in toluene scintillation fluid. The radioactivity in each nucleotide peak was at least 1000 cpm, resulting in a precision of ±2%.

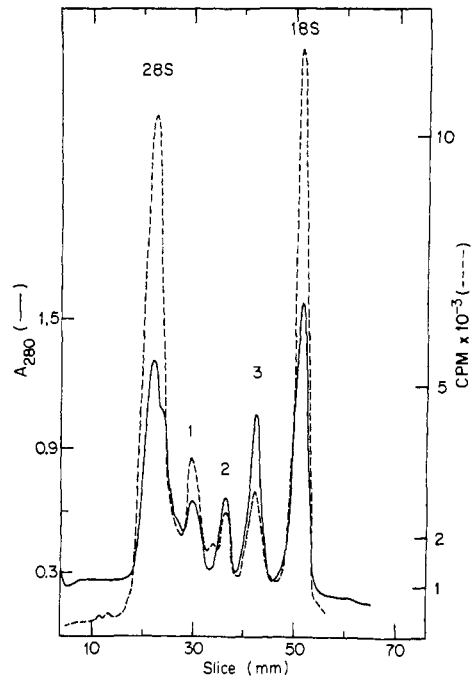

FIGURE 1: Absorbance and radioactivity profile of stable polysomal RNA sedimenting from 28 to 18 S analyzed on polyacrylamide gels. Details in text.

Millipore Binding of RNA. RNA fractions obtained from sucrose gradients were dissolved in 0.5 M KCl, 10 mM Tris-HCl (pH 7.6), and 1 mM $MgCl_2$, and passed through Millipore filters (HA) following the procedure described by Brawerman *et al.* (1972). The fraction that was bound to the filters was released by shaking the filters in 3 ml of 0.1 M Tris-HCl (pH 9)–0.5% sodium dodecyl sulfate for 15 min at room temperature. Both the Millipore-bound RNA and that in the filtrate were analyzed by gel electrophoresis.

Results

Analysis of Stable Polysomal RNA. RNA sedimenting between 28 and 18 S was concentrated by two successive sucrose gradients (see Methods) of polysomal RNA prepared from 14-day-old chick embryos labeled with ^{32}P for 24 hr. When analyzed by polyacrylamide gel electrophoresis, five discrete ultraviolet-absorbing peaks are obtained, as shown in Figure 1. The two major ones are the 28S and 18S rRNA components. The relative amounts of these components do not reflect the amount present in the original preparation, but reflect how the fractions were selected from the gradients. The other three peaks, labeled 1, 2, and 3, correspond to RNA molecules with molecular weights of 1,300,000, 1,050,000, and 900,000 daltons as determined from their mobility on gels. The nearly perfect coincidence between the absorbance and the radioactivity profiles means that only the RNA that accumulates is labeled in detectable amounts under these conditions. The only possible exception is the small peak of radioactivity between peaks 1 and 2.

To establish the origin of these fractions, the base composition and specific activity of each was measured. The results together with published values for 28 and 18 S are given in Table I. Although the base composition of peaks 1, 2, and 3 are not identical with that of 28S rRNA, they are sufficiently similar to it, and different from that of 18S rRNA to establish that each of these fractions is derived predominantly from

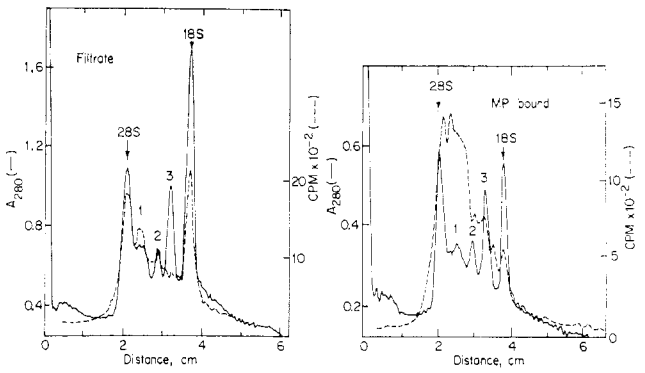

FIGURE 2: Absorbance and radioactivity profile of rapidly labeled RNA sedimenting from 28 to 18 S: (a) RNA in filtrate; (b) RNA bound to Millipore filters.

28S rRNA. Moreover since the specific activities of peaks 2 and 3 are significantly lower than that of either 28 or 18 S, it is unlikely that they are caused by degradation during isolation, but must be formed *in vivo*. A similar conclusion has been reached about peak 3, or "22S" RNA, by Nair and Knight (1971) and by Judes *et al.* (1972), as will be discussed below. By contrast, the difference in the specific activity of peak 1 and 28S rRNA is not sufficient to permit us to differentiate between its appearance due to *in vivo* or *in vitro* degradation.

Analysis of Rapidly Labeled Polysomal RNA. Uv-absorbing profiles, identical with those shown in Figure 1, were obtained after sucrose gradient fractionation of polysomal RNA prepared from chick embryos labeled for only 2 hr. The 28S–18S RNA species were further fractionated by passing the RNA through Millipore filters, as described in Methods. Of the 500 μg of RNA applied, 22% was Millipore bound, but this 22% included 50% of the radioactivity. The absorbance and radioactivity profiles of the unbound and bound RNA are shown in Figure 2a,b, respectively. Clearly rRNA is retained on filters, although 18S rRNA binds relatively less than 28S rRNA, there being more than 50% less 18S rRNA on the filters than in the filtrate relative to the amount of 28S RNA found in each. The amount of peaks 1, 2, and 3 retained on the filter is proportional to the amount of 28 S retained. This again suggests that these peaks have sequences in common with 28S RNA.

The radioactivity profile for the unbound RNA is similar to that of the original sample and is approximately coincident with the absorbance. But the radioactivity profile of the Millipore-bound RNA is quite different, peaking between

TABLE I: Base Composition and Specific Activity of Stable Polysomal RNA.

	28[a]	28 S	1	2	3	18 S	18 S[a]
C	28	30	29	27	29	24	25
G	34	37	33	33	35	29	29
A	19	17	20	20	18	23	24
U	18	16	18	20	17	23	22
G + C	62	67	62	60	64	53	54
Specific activity[b]		2.20	1.80	1.36	1.19	1.90	

[a] Base composition reported by Turner *et al.* (1963). [b] Specific activity determined from total cpm in peak divided by weight of peak in mg.

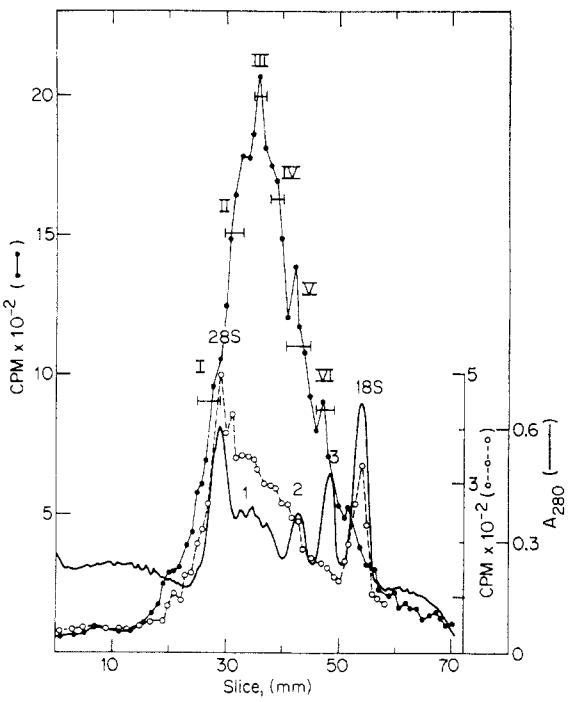

FIGURE 3: Absorbance and radioactivity profile of rapidly labeled RNA sedimenting from 28 to 18 S: (—) absorbance of unbound RNA; (O- - -O) radioactivity of unbound RNA; (●—●) radioactivity of RNA bound to Millipore filters.

28S rRNA and fragment peak 1. Both the bound and the unbound 28S rRNA have a higher specific activity than the 18S rRNA. Since the latter is labeled more rapidly (Greenberg and Penman, 1966), the high specific activity cannot reflect labeling of rRNA but of mRNA species which migrate with the same mobility.

To gain further insight into the nature of rapidly labeled polysomal RNA, a second fractionation of RNA labeled for 2 hr was carried out. The results shown in Figure 3 are very similar to those shown in Figure 2. The main difference is that the Millipore fractionation was more efficient this time. Of the 300 μg applied to Millipore filters, 13% was bound and this included 63% of the radioactivity. As a result there was a threefold increase in the specific activity of the bound compared to the unfractionated RNA, and a tenfold increase in the specific activity of the bound compared to the unbound RNA in the filtrate. As before, the rapidly labeled RNA that does not bind to filters follows the absorbance profile approximately, but the Millipore-bound RNA does not. The latter has a bell-shaped distribution centering around peak 1, or 1,300,000 daltons. There is very little label at 18 S and even less 18 S was bound in this experiment (data not shown) than in the first. Although the difference in the efficiency of Millipore binding in the two experiments is not readily explicable, it could be a result of differences in the amount of residual protein bound to rRNA in our preparations. The use of sodium dodecyl sulfate–EDTA without subsequent phenol extraction may well leave more protein bound to RNA, but it has been widely used to prepare mRNA from polysomes (Marbaix and Burny, 1964; Evans and Lingrel, 1969; Rhoads *et al.*, 1971; Prichard *et al.*, 1971; Gross *et al.*, 1973). Whether or not protein contamination is responsible for nonspecific binding of rRNA to Millipore filters, we have found we can reduce the amount of rRNA bound to less than 6% of the RNA applied to filters by simply reducing the RNA concentration to 0.05 mg/ml.

TABLE II: Base Composition of Rapidly Labeled Polysomal RNA.

	Millipore-Bound RNA						Unbound RNA in Filtrate	DNA[a]
	I	II	III	IV	V	VI		
C	20	18	19	19	18	21	22	22
G	24	25	25	26	28	25	25	22
A	32	31	32	31	32	32	26	28
U	24	25	24	23	22	22	27	28
G + C	44	43	44	45	46	46	47	44

[a] Base composition reported by Turner *et al.* (1963).

The Millipore-bound RNA was divided into size fractions corresponding to approximate molecular weights of 1,600,000, 1,300,000, 1,200,000, 1,100,000, 1,000,000, and 900,000, designated by roman numerals I–VI. The base composition of each fraction was determined and the results are summarized in Table II. The rapidly labeled RNA bound to Millipores is DNA like in respect to its G + C content. As expected, however, it is substantially enriched in A.

The rapidly labeled RNA which is not bound to Millipores had too low a specific activity to permit us to measure the base composition of individual peaks isolated from gels. However, an unfractionated sample of unbound RNA was analyzed and its base composition is very similar to that of DNA as shown in Table II. We do not know whether this RNA is mRNA which has lost its poly(A) segment, or whether it is a contamination arising from nuclear leakage of heterogeneous nuclear RNA, or simply mRNA that never had poly(A) regions. However it is clearly not rRNA, which is apparently not labeled enough to be detected after a 2-hr label.

The roughly continuous distribution of radioactivity which peaks at about 26 S for the bound RNA might not represent molecularly intact mRNA molecules but aggregates. To test this, bound and unbound RNA were examined on formamide gels, under conditions where RNA is fully denatured. The results, shown in Figure 4, show that uv-absorbing peaks 2 and 3 are largely lost when the RNA is denatured. Thus the *in vivo* breakdown products appear to contain hidden breaks, and thus they are selectively removed when the RNA is denatured. In contrast to this, the radioactivity profile in formamide clearly resembles that observed on aqueous gels indicating that rapidly labeled RNAs with molecular weights from 900,000 to greater than 1,500,000 survive our isolation and fractionation procedure. Thus, while we do not claim that there is no loss of RNA due to degradation in our preparations, we have been able to demonstrate that molecularly intact high molecular weight RNA molecules can be isolated.

Discussion

The enrichment of polysomal RNA prepared from 14-day-old chick embryos for species sedimenting between 28 and 18 S by successive sucrose gradient fractionation results in the appearance of three distinct, optically visible species of RNA having molecular weights of about 1,300,000, 1,050,000, and 900,000. While these uv-absorbing peaks can be identified as being derived largely *in vivo* from 28S rRNA, the region between 28S and 18S RNA also contains small amounts of

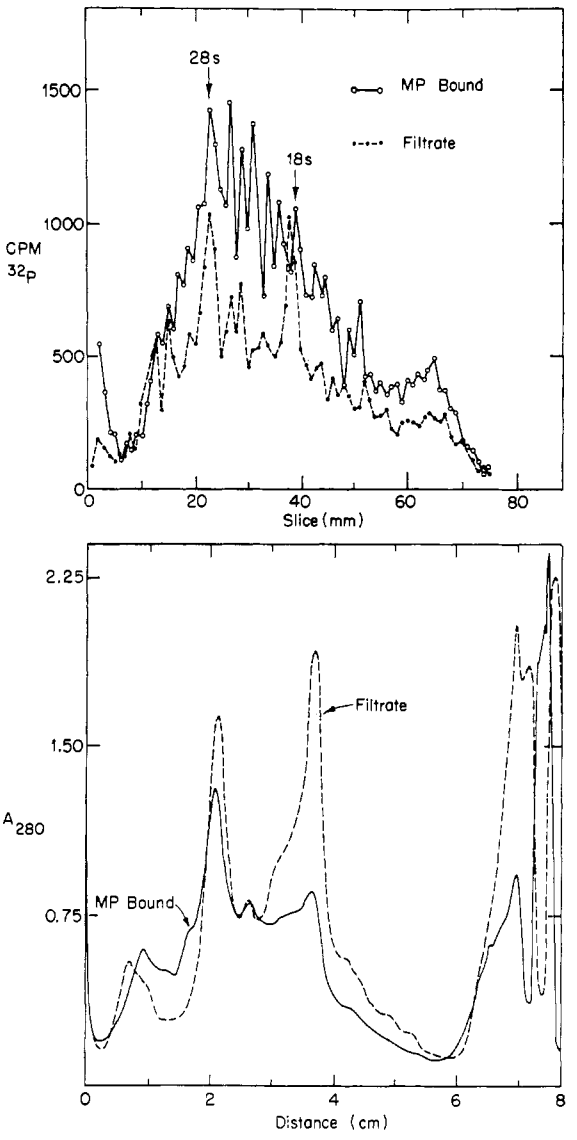

FIGURE 4: Absorbance and radioactivity profile of rapidly labeled RNA sedimenting from 28 to 18 S analyzed on 99% formamide gels. Upper frame: radioactivity of unbound RNA (●--●) and Millipore-bound RNA (○--○) Lower frame, absorbance of unbound RNA (- - - -) and Millipore-bound RNA (—).

rapidly labeled RNA which is DNA like in its G + C content, and which contains a large fraction of adenine-rich molecules.

Of the three uv-absorbing peaks we isolated, one corresponding to peak 3 is visible in all our polysomal RNA preparations even prior to fractionation. This RNA species is also found in HeLa cells (Nair and Knight, 1971), and in chick liver and brain. (Judes *et al.*, 1972). Detailed studies, including the kinetics of labeling, its origin in the 60S ribosomal subunit, its base composition, methylation, and competition hybridization measurements, leave little doubt that the "22S" RNA component found in HeLa and chicks is derived from 28S rRNA *in vivo*. Our results on the kinetics of labeling, the composition, and the retention on Millipore filters confirm this identification of the origin of the "22S", or peak 3, RNA. But we also find two larger uv-absorbing peaks which closely resemble "22 S" in base composition and retention on Millipores, but whose specific activity is higher than that of the 22S peak. The slightly higher specific activity and the slightly lower G + C content of peaks 1 and 2 compared with peak 3 ("22 S") could be explained by the

former being a mixture of 28S fragment and mRNA. Alternatively, the gradual decrease in specific activity with decreasing size of the large 28S fragments could be the result of the *in vivo* processing of 28S rRNA.

The preponderance of rRNA found in the 28–18S region of polysomal RNA appears at first to imply a selective degradation of mRNA during our isolation procedures. There is certainly no evidence for the survival of a stable polysome-bound mRNA. However, the rapidly labeled polysome-bound RNA is not affected by exposure to denaturing conditions. This shows that mRNA can survive our preparative procedures.

Although our data do not permit us to conclude that the degradation of 28S rRNA *in vivo* proceeds by successive excision of fragments several hundred nucleotides long or by specific cleavage at a limited number of sites, either mechanism should produce rRNA fragments having a molecular weight of approximately 200,000. Therefore it is not surprising that preparations of 9S hemoglobin mRNA are usually heavily contaminated with rRNA. The accumulation of rRNA fragments after successive sucrose gradient fractionations of RNA sedimenting between 28S and 18S observed by us is likely to occur, perhaps to an ever greater extent, in regions of the gradient sedimenting below 18 S. The purification of mRNA based on size fractionation alone is clearly not practical. Fortunately most mRNAs contain poly(A), thus providing an alternative method of separating mRNA from rRNA.

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