Isolation of L-Cell Messenger RNA Which Lacks Poly(adenylate)†

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ABSTRACT: It has been found that centrifugation of (ethylenediamine)tetraacetic acid dissociated L-cell polyribosomes at 25 °C through preformed Cs_2SO_4 density gradients containing 15% dimethyl sulfoxide resolves them into two distinct classes of particles that contain ribosomal and messenger RNA, respectively. Ribosomal RNA bands at a higher density than mRNA, presumably because it is more extensively stripped of protein by the action of Cs_2SO_4 than mRNA. Undegraded RNA can be recovered from the gradients by trapping the particles on nitrocellulose filters and then eluting at 60 °C with a solution containing formamide and sodium dodecyl sulfate. When cells were labeled with [^{3}H]adenosine, either in the presence or absence of a dose of actinomycin D which selectively inhibits rRNA synthesis, and their polyribosomes centrifuged through Cs_2SO_4 density gradients, a large amount of the radioactivity recovered from the messenger-ribonucleoprotein region of the gradient had the size distribution of mRNA, but lacked poly(adenylate) (poly (A)). This material constituted 29–31% of the total mRNA. Histone mRNA was the most prominent component of poly(A)-lacking mRNA. However, 50–65% of the poly(A)-lacking mRNA was of larger size than histone mRNA.

Recently, the investigation of eukaryotic mRNA has focused on molecules that contain poly(A),[1] largely because of the ease with which they can be isolated. Several different kinds of eukaryotic mRNA have been shown to contain poly(A) (for references see the review (Greenberg, 1975)). In fact, the only identified species that has been conclusively shown to lack it is histone mRNA (Adesnik and Darnell, 1972; Greenberg and Perry, 1972). This species constitutes a special case because of its small size and other unique properties, and there is still considerable uncertainty as to the extent of occurrence of other poly(A)-lacking mRNAs.

Some experiments have suggested that in cultured mammalian cells virtually all mRNA except histone mRNA contains poly(A) (Greenberg and Perry, 1972; Adesnik et al., 1972). On the other hand, another recent paper reported that 30% of mRNA in HeLa cells lacks poly(A) (Milcarek et al., 1974). Furthermore, it has been shown that in the early development of sea urchin embryos a large fraction of the newly synthesized mRNA exclusive of histone mRNA lacks poly(A) (40% or more, depending on the developmental stage) (Nemer et al., 1974; Fromson and Duchastel, 1975). The experiments with cultured cells all depended on drugs that inhibit rRNA synthesis in order to detect mRNA and quantitate the relative amounts of poly(A)(+) and poly(A)(−) mRNA. The experiments with sea urchin embryos depended on the fact that they do not synthesize rRNA at the stages examined. Until the present, however, no method for the direct isolation of poly(A)(−) mRNA has been available. In this paper I report a method for the isolation of mRNA that relies on the presence of proteins tightly and specifically bound to mRNA (Blobel, 1973; Brian and Hayashi, 1973; Morel et al., 1973; Lindberg and Sundquist, 1974; Kumar and Pederson, 1975), rather than on the presence of poly(A). It was suggested to me by the observation that hnRNA-containing RNP particles from sea urchin embryos are stable upon centrifugation through Cs_2SO_4 density gradients (Wilt et al., 1973). Utilizing this method, it has been found that 29–31% of the mRNA isolated from mouse L-cells labeled with [^{3}H]adenosine either in the presence or absence of actinomycin D lacks poly(A).

Experimental Procedures

Cell Culture, Labeling, and Fractionation. Culture and labeling of mouse L-cells were carried out as described (Perry and Kelley, 1968a,b). In some experiments actinomycin D was used at 0.08 μg/ml to inhibit rRNA synthesis. Polyribosomes were prepared from cells lysed in isotonic buffer containing 0.1% Triton X-100 (Perry and Kelley, 1968a) by centrifugation through continuous 15–45% (w/w) sucrose gradients containing RSB (0.01 M NaCl, 0.01 M Tris-HCl, pH 7.5, 0.002 M $MgSO_4$) in a Beckman SW27 rotor for 3.5 h at 27 000 rpm (Schochetman and Perry, 1972). The polyribosome region from the sucrose gradient (material sedimenting faster than disomes) was layered over 4 ml of 2 M sucrose containing RSB in polycarbonate tubes that were centrifuged 17 h at 36 000 rpm at 4 °C in the Beckman Ti 60 fixed angle rotor in order to pellet the polyribosomes.

Cs_2SO_4 Density Gradients. Cs_2SO_4 was obtained from Kawecki-Berylco Industries (Revere, Pa.) and purified with bentonite and activated charcoal by the method of Wilt et al. (1973). Cs_2SO_4 density gradients having volumes of 8 ml were made with a linear gradient former from equal volumes of stock solutions made up as if to have densities of 1.30 and 1.50 g cm^{-3} in water. However, the presence of Me_2SO made the actual densities somewhat higher. The gradients contained 15% (v/v) Me_2SO, 0.001 M EDTA, 0.01 M Tris-HCl (pH 7.5), 0.01 M NaCl, and 0.01% Triton X-100, and were centrifuged at 33 000 rpm for 22 h at 25 °C. In most of the experiments described in this paper, cellulose nitrate centrifuge tubes were used. However, it has now been found that polyallomer tubes give much higher recoveries of mRNP. The same relative amounts of poly(A)(+) and poly(A)(−) mRNA are obtained with both types of tube. Furthermore, it is not necessary to preform the gradients. Satisfactory gradients are obtained if the high and low density Cs_2SO_4 solutions are simply layered in the tubes and centrifuged as described. Polyribosome sam-

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[1] Abbreviations used are: poly(A), poly(adenylate); poly(A)(+) and poly(A)(−) mRNA, poly(A)-containing and poly(A)-lacking mRNA, respectively; hnRNA, heterogeneous nuclear RNA; RNP, ribonucleoprotein; EDTA, (ethylenedinitrilo)tetraacetic acid.

ples were resuspended in RSB containing 10% glycerol to which 0.1 M EDTA was added to a concentration of 0.0167 M. The suspensions were spun at 16 000*g* for 5 min to remove aggregated material before layering on Cs_2SO_4 gradients in volumes of up to 2 ml. To fill up the tubes the gradients were overlaid with buffer lacking glycerol. The gradients and buckets were kept ice-cold until the time of centrifugation. However, the rotor was preequilibrated at room temperature. After centrifugation, gradients were collected in fractions of 0.2 ml at a constant flow rate using a peristaltic pump by upward displacement with a dense solution of Cs_2SO_4. This procedure eliminates tailing of rRNA into mRNP during fractionation. Densities were measured by weighing 0.1 ml of every fourth fraction in a disposable capillary pipet. The contents of the pipet were saved for further analysis. Fractions were then assayed for radioactivity in RNP by binding to 25-mm Millipore HAWP filters (Infante and Nemer, 1968). Aliquots of 20 μl were diluted into 1 ml of RNP buffer (0.005 M $MgSO_4$, 0.01 M Tris-HCl, pH 7.5) containing 0.35 A_{260} unit of unlabeled polyribosomes. They were then filtered, and the filters were washed with RNP buffer, dried, and counted in a liquid scintillation counter.

RNA Recovery and Analysis. Pooled gradient fractions were passed through Millipore 25-mm HAWP filters to trap RNP after diluting 1:1 with water and adding a total of 2 A_{260} units of unlabeled polyribosomes as carrier. The filters were then washed with RNP buffer to remove Cs_2SO_4, and placed in scintillation vials. The filters were eluted four times at 60 °C for 5 min with 1 ml of a solution containing 60% formamide, 0.05 M Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, and 0.5% sodium dodecyl sulfate. The four eluates from each filter were combined and 10 ml of ethanol was added. After 2 h or more at −20 °C the precipitated RNP was spun down and dissolved in 2 ml of sodium dodecyl sulfate buffer (0.1 M NaCl, 0.01 M Tris-HCl, pH 7.5, 0.001 M EDTA, 0.5% sodium dodecyl sulfate), then deproteinized by extracting twice with a phenol–chloroform mixture (Perry et al., 1972). The RNA was precipitated with ethanol. For chromatography on oligo(dT)-cellulose T_3 (Collaborative Research, Waltham, Mass.), the RNA was dissolved in 0.5 ml of a buffer containing 0.5 M NaCl, 0.01 M Tris-HCl (pH 7.5), and 0.1% sodium lauroyl sarcosinate and applied to a column containing 0.1 g of oligo(dT)-cellulose. The column was washed with 5–10 ml of this buffer to elute poly(A)(−) RNA, and then with 5 ml of the same buffer lacking NaCl to elute poly(A)(+) RNA. Fractions of 1 ml were collected at 1 ml/min and assayed for trichloroacetic acid insoluble radioactivity. Peak fractions from the column were pooled and ethanol precipitated after addition of NaCl and carrier RNA to the poly(A)-containing fractions. Polyacrylamide gel electrophoretic analysis on 2.7 or 14% ethylene diacrylate-cross-linked gels was carried out as described (Perry and Kelley, 1968b; Perry et al., 1972). The gels were sliced into 2-mm segments that were counted in a liquid scintillation counter after dissolving in 10 ml of Liquifluor (New England Nuclear) containing 3% Protosol (New England Nuclear).

Results

Labeling Conditions. Two different sets of labeling conditions have been used in this study. (a) Cells were preincubated with 0.08 μg/ml of actinomycin D for 30 min, then labeled for 70 min with [^{3}H]adenosine in the presence of the drug. This procedure labels polyribosomal mRNA with very little incorporation of radioactivity into rRNA (Perry and Kelley, 1970). (b) Cells were labeled for 70 min with [^{3}H]adenosine in the absence of actinomycin D. Also, in this case metabolically stable RNA, i.e., rRNA and 4S and 5S RNA, was labeled with ^{14}C by exposing the cells to [^{14}C]uridine for one doubling followed by a chase for two doublings in unlabeled medium before labeling with [^{3}H]adenosine. The reason for using the ^{14}C label was to provide an internal control for possible contamination of the mRNA fractions with stable RNA. It can be calculated that even if mRNA has a mean lifetime (defined as decay to $1/e$) as long as one cell generation (Greenberg, 1972) virtually all of the ^{14}C label should be in stable RNA after following this protocol. After the chase about 0.3–0.7% of the label should be in mRNA if 2–5% of the label was in mRNA at the beginning of the chase. The reason for using cells labeled either in the presence or in the absence of actinomycin D was to establish whether results obtained with drug-treated cells are also valid for untreated cells.

Absence of EDTA-Resistant Radioactivity from Polyribosomes. In order to estimate the relative amounts of poly(A)(+) and poly(A)(−) mRNA, it is first necessary to show that one is truly working with mRNA. The accepted working definition for mRNA (in the absence of demonstrating synthesis of specific polypeptides) is high-molecular-weight RNA of heterogeneous size distribution that can be released from polyribosomes by treatment with EDTA (Milcarek et al., 1974; Penman et al., 1968).

In the present study polyribosomes (aggregates containing three or more ribosomes) were obtained by centrifuging cytoplasmic extracts in continuous sucrose gradients, then pelleting the resulting polyribosome fraction through 2 M sucrose. Portions of the cytoplasmic extracts were also centrifuged in sucrose gradients containing EDTA, and the region of these gradients corresponding to the polyribosome region of the preparative gradients was layered over 2 M sucrose containing EDTA and centrifuged under conditions identical to those used for pelleting polyribosomes. Table I shows that after this two-step purification the amount of radioactivity in EDTA-resistant material of non-polyribosomal origin was negligibly small. In the case of the actinomycin-treated cells, where there was little or no label in rRNA, only 2% of the radioactivity was in EDTA-resistant material. In the case of the untreated cells a similarly small proportion of the radioactivity was in EDTA-resistant material, although in this case the measurement was less meaningful, since the vast majority of the radioactivity was in rRNA. Furthermore, when pelleted polyribosomes were resuspended and centrifuged in EDTA-containing sucrose gradients, virtually all of the radioactivity sedimented slower than 100 S.

Separation of Messenger Ribonucleoproteins from rRNA and Quantitation of Poly(A)(+) and Poly(A)(−) mRNA. The pelleted polyribosomes were resuspended, dissociated with EDTA, and centrifuged in Cs_2SO_4 gradients containing dimethyl sulfoxide to fractionate them into particles containing mRNA and particles containing rRNA. This procedure, which will be described in more detail in a subsequent publication, differentially removes protein from ribosomal subunits and mRNP particles, and separates the resulting derived particles according to buoyant density. Figure 1 shows the results of centrifuging polyribosomes from actinomycin-treated cells in such a gradient. Only radioactivity bound to nitrocellulose filters in low salt is shown, since RNP particles bind to the filters under these conditions, whereas the majority of protein-free RNA, chiefly low-molecular-weight RNA, is not bound (Infante and Nemer, 1968). Three distinct regions, designated A, B, and C, were apparent in the distribution of radioactivity. The fractions in each group were pooled and the

TABLE I: Percent of EDTA-Resistant Radioactivity in Polyribosomes.

	Polyribosome Fraction from Sucrose Gradient		Pelleted Polyribosomes	
	^3H	^{14}C	^3H	^{14}C
Cells labeled in the presence of 0.08 μg/ml of actinomycin D[a]	17.3	–	2.0	–
Cells labeled in the absence of actinomycin D[b]	4.0	1.4	0.89	0.52

[a] For the labeling protocol, see the legend to Figure 1. The cells were labeled with ^3H only. The postmitochondrial supernatants were layered on 15–45% (w/w) sucrose gradients containing either 0.01 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.002 M $MgSO_4$, or the same solvent containing 0.001 M EDTA in place of $MgSO_4$. The gradients were centrifuged for 3.5 h at 27 000 rpm at 4 °C in the Beckman SW27 rotor. The gradients were collected through the flow cell of a recording spectrophotometer. The polyribosome fraction from the $MgSO_4$-containing gradient, or the equivalent region from the EDTA-containing gradient, was assayed for CCl_3COOH-insoluble radioactivity, then layered over 4 ml of 2 M sucrose dissolved in the gradient buffer containing either $MgSO_4$ or EDTA. The samples were then centrifuged for 17 h at 36 000 rpm at 4 °C in the Beckman Ti60 rotor. The pellets were resuspended and assayed for CCl_3COOH-insoluble radioactivity. [b] For the labeling protocol, see the legend to Figure 3. The other details were as described above.

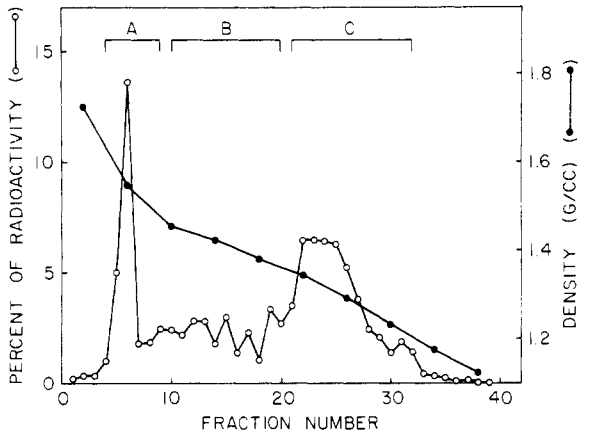

FIGURE 1: Fractionation in a Cs_2SO_4 density gradient of polyribosomes from cells labeled with [^3H]adenosine in the presence of actinomycin D. A 400-ml suspension culture of L-cells at 3×10^5 cells/ml was preincubated for 30 min in medium containing 0.08 μg/ml of actinomycin D, then concentrated to 50 ml in the same medium and labeled for 70 min with 10 μCi/ml of [2-^3H]adenosine (New England Nuclear, 11 Ci/mmol). The polyribosomes were isolated and 2.1 A_{260} units of EDTA-dissociated polyribosomes were layered on each of two preformed Cs_2SO_4 density gradients. After centrifugation and fractionation of the gradients, 1/10 of each fraction was assayed for radioactivity bound to a nitrocellulose filter. The letters A, B, and C designate groups of fractions that were pooled for further analysis.

RNA in them was recovered for analysis by means of oligo(dT)-cellulose chromatography and polyacrylamide gel electrophoresis.

Group A, which corresponds in buoyant density to particles containing rRNA (see below), had the lowest content of radioactivity in poly(A)(+) RNA bound to oligo(dT)-cellulose

[2] Subscripts of nucleotides refer to average residue lengths.

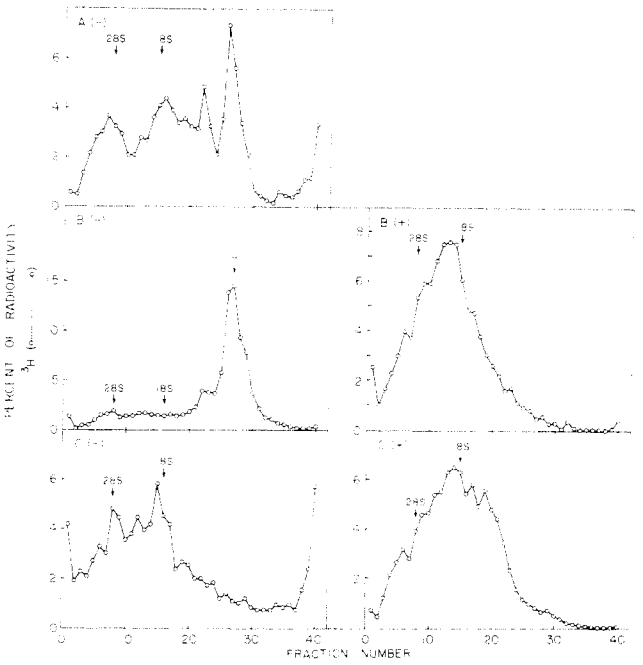

FIGURE 2: Polyacrylamide gel electrophoretic analysis of poly(A)(−) and poly(A)(+) RNA from Cs_2SO_4 density gradient-fractionated polyribosomes of cells labeled in the presence of actinomycin D. The RNA was recovered from the groups of fractions designated A, B, and C in Figure 1 and resolved into poly(A)-containing and -lacking species by means of chromatography on oligo(dT)-cellulose. The resulting RNA fractions were then subjected to polyacrylamide gel electrophoresis. The letters A, B, and C refer to the groups of fractions in Figure 1 from which the RNA was obtained. Poly(A)(−) RNA is displayed on the left, and poly(A)(+) RNA on the right. The poly(A)(+) RNA of group A was not analyzed because of insufficient radioactivity. The arrows labeled "28S" and "18S" refer to the positions of rRNA markers run on a separate gel. The arrow labeled "H" in Figure 2B(−) refers to the position of histone mRNA.

(29.1%). Group B had an intermediate poly(A) content (42.7%), and group C had the highest poly(A) content (77.0%). The results of polyacrylamide gel electrophoretic analysis of the poly(A)(+) and poly(A)(−) RNA (RNA not bound to oligo(dT)-cellulose) from these groups are shown in Figure 2. The poly(A)(+) RNA of groups B and C had the size distribution typical of L-cell poly(A)(+) mRNA (Perry et al., 1973). The poly(A)(−) RNA of group B had a prominent peak at an apparent molecular weight of 150 000. This is the expected size for histone mRNA, which has previously been shown to lack poly(A) (Adesnik and Darnell, 1972; Greenberg and Perry, 1972). In addition, there was labeled RNA of heterogeneous size distribution that migrated more slowly than histone mRNA, and which was presumably poly(A)(−) mRNA of larger size. The poly(A)(−) RNA of group C appeared to be virtually devoid of histone mRNA, but contained heterogeneously migrating RNA of higher molecular weight, also presumably poly(A)(−) mRNA. Some of this material was similar in size to rRNA. However, it was unlikely to have been rRNA, since (1) actinomycin D was used to inhibit rRNA synthesis, (2) the vast majority of rRNA was confined to region A of the gradient, and (3) the presence of poly(A)-lacking messenger-like RNA has been demonstrated by a method which does not require inhibiting rRNA synthesis (see below). In addition, about 15% of the radioactivity in this gel was in low-molecular-weight RNA that was probably not mRNA. The poly(A)(−) RNA of group A had a peak at the position of histone mRNA, but most of the radioactivity was in RNA of higher molecular weight, possibly including some rRNA synthesized in spite of the use of actinomycin D.

TABLE II: Percent of mRNA Which Lacks Poly(A) in Polyribosomes from Cells Labeled in the Presence of 0.08 μg/ml of Actinomycin D.

Region of Cs_2SO_4 Gradient[a]	cpm[b]	% cpm in Poly(A) (+) mRNA[c]	% cpm in Poly(A) (−) mRNA[d]	cpm in PolyA(+) mRNA	cpm in PolyA(−) mRNA	% of Total mRNA Which Lacks Poly(A)[e]
A	416	29.1	-	-	-	-
B	317	42.7	57.3	135	182	15.7
C	869	77.0	20.2	669	176	15.1
Total						30.8

[a] Gradient shown in Figure 1. [b] Total cpm in region bound to Millipore filter. 1/10 of each fraction assayed. [c] From results of chromatography on oligo(dT)-cellulose. [d] From results of chromatography on oligo(dT)-cellulose corrected for radioactivity in low-molecular-weight RNA on the basis of gel electrophoresis data (Figure 2). [e] For regions B and C only. It is uncertain what proportion of radioactivity in region A was in mRNA.

The results of the experiment are summarized in Table II. After allowing for the presence of 15% of the label in the poly(A)(−) RNA of group C in low-molecular-weight RNA, 30.8% of the radioactivity in groups B and C was in RNA that resembled mRNA in size distribution, yet lacked poly(A), since it did not bind to oligo(dT)-cellulose. About 65% of the poly(A)(−) messenger-like RNA was of larger size than histone mRNA. To the extent that the poly(A)(−) RNA of group A consisted of messenger-like RNA, the overall percentage of radioactivity in poly(A)(−) messenger-like RNA would have been higher; it could have been as high as 41% if all of the labeled poly(A)(−) RNA in group A had been in messenger-like RNA.

Since the use of Cs_2SO_4–Me_2SO density gradients for isolating messenger-like RNA is a novel procedure, it seemed desirable to compare results obtained with this procedure to those obtained using a more conventional procedure, namely, simple analysis of RNA from cells labeled in the presence of actinomycin D without prior centrifugation in Cs_2SO_4–Me_2SO density gradients, adsorption to Millipore filters, or exposure to 60% formamide at 60 °C. Therefore, a preparation of labeled polyribosomes was divided into two parts. One part was centrifuged in Cs_2SO_4–Me_2SO density gradients to isolate messenger-like RNA that was recovered as described and analyzed by means of chromatography on oligo(dT)-cellulose and electrophoresis in 2.7% polyacrylamide gels. The other part was deproteinized, then analyzed directly by means of oligo(dT)-cellulose chromatography and polyacrylamide gel electrophoresis. This analysis is thus comparable to previous measurements of the proportion of poly(A)(−) mRNA (Greenberg and Perry, 1972; Perry et al., 1973; Milcarek et al., 1974; Nemer et al., 1974; Fromson and Duchastel, 1975). The messenger-like RNA recovered from Cs_2SO_4–Me_2SO density gradients was estimated to be 31.1% poly(A)(−). The messenger-like RNA that was analyzed without prior separation from rRNA was estimated to be 26.4% poly(A)(−). These values were corrected for the presence of low-molecular-weight RNA on the basis of gel electrophoresis data. Thus, the two procedures gave similar results.

When polyribosomes from cells labeled in the absence of actinomycin D were centrifuged in Cs_2SO_4 gradients (Figure 3), most of the radioactivity (both ^3H and ^{14}C) was found in a single sharp peak at 1.55 g cm^{-3} that contained rRNA (see below). The remainder of the radioactivity was distributed heterogeneously at lower densities (1.25–1.50 g cm^{-3}). Again, only radioactivity bound to nitrocellulose filters is shown. The fractions in the groups designated A, B, and C (corresponding in density to groups A, B, and C in Figure 1) were pooled and

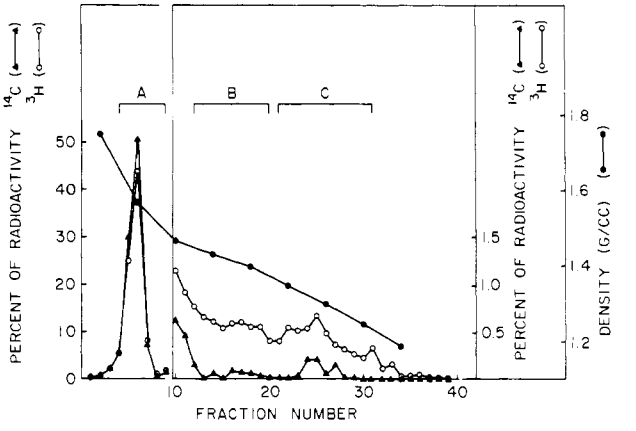

FIGURE 3: Fractionation in a Cs_2SO_4 density gradient of polyribosomes from cells labeled with [^{14}C]uridine and [^3H]adenosine in the absence of actinomycin D. A 100-ml suspension culture of L-cells at a density of 1.4 × 10^5 cells/ml was grown to a density of 3.7 × 10^5 cells/ml in the presence of 0.05 μCi/ml of [2-^{14}C]uridine (New England Nuclear, 50 mCi/mmol). The [^{14}C]uridine was quantitatively incorporated into CCl_3COOH-insoluble form. The cells were then spun down and resuspended in 400 ml of unlabeled medium containing 0.3 mM uridine. When they had grown to 3.5 × 10^5 cells/ml (approximately two doublings), they were concentrated to 50 ml and labeled for 70 min with 10 μCi/ml of [2-^3H]adenosine (New England Nuclear, 11 Ci/mmol). The polyribosomes were isolated and 3.4 A_{260} units of EDTA-dissociated polyribosomes were layered on each of two preformed Cs_2SO_4 density gradients. Other details were as described in the legend to Figure 1.

the RNA was recovered for analysis. The RNA in group A had a very low content of radioactivity in poly(A)(+) RNA, less than 1% of both the ^3H and ^{14}C radioactivity. In groups B and C 34.9% and 79.1% of the ^3H radioactivity were in poly(A)(+) RNA, respectively. There was no detectable ^{14}C radioactivity in the poly(A)(+) RNA of these groups.

The results of polyacrylamide gel electrophoretic analysis of the poly(A)(−) and poly(A)(+) RNA of groups A, B, and C are shown in Figure 4. The poly(A)(−) RNA of group A consisted largely, if not entirely, of rRNA. The distributions of ^3H and ^{14}C radioactivity were nearly identical. The ^{14}C label in the poly(A)(+) RNA of group A consisted largely of contaminating rRNA. The ^3H label appeared to be in a mixture of rRNA and heterogeneously migrating RNA, presumably poly(A)(+) mRNA. The ^3H-labeled poly(A)(+) RNA of groups B and C appeared to be typical L-cell poly(A)(+) mRNA. The ^3H-labeled poly(A)(−) RNA of group B had a prominent peak at the position of histone mRNA. There was also considerable high-molecular-weight heterogeneously migrating RNA, as well as small amounts of rRNA. The ^{14}C

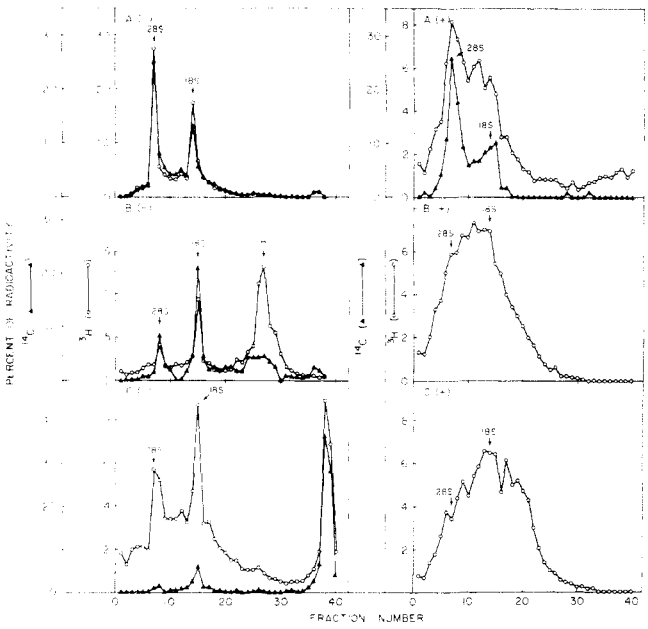

FIGURE 4: Polyacrylamide gel electrophoretic analysis of poly(A)(−) and poly(A)(+) RNA from Cs_2SO_4 density gradient-fractionated polyribosomes from cells labeled in the absence of actinomycin D. The groups of fractions designated A, B, and C in Figure 3 were pooled and the RNA was recovered for analysis. Additional details were as described in the legend to Figure 2.

label in the fraction was largely in rRNA, although there was some ^{14}C radioactivity at the position of histone mRNA. The ^{3}H-labeled poly(A)(−) RNA of group C was a mixture of heterogeneously migrating high-molecular-weight RNA, low-molecular-weight RNA, and small amounts of rRNA. Histone mRNA appeared to be absent. The ^{14}C-labeled RNA of this fraction was predominantly low-molecular-weight RNA, although there was some rRNA.

The results of the experiment are summarized in Table III. It is possible, by making use of the ^{14}C label, to estimate the amount of radioactivity in poly(A)(−) messenger-like RNA in regions B and C. This estimate makes the assumption that all of the ^{14}C label was in contaminating stable RNA rather than in mRNA. It furthermore assumes that all of the labeled RNA in region A is stable RNA, so that this $^{14}C/^{3}H$ ratio corresponds to 100% stable RNA, whereas a $^{14}C/^{3}H$ ratio of zero corresponds to 100% messenger-like RNA. The gel electrophoresis data were used to calculate the $^{14}C/^{3}H$ ratios. The poly(A)(+) RNA of regions B and C contained no detectable ^{14}C label, and was therefore essentially pure mRNA. The poly(A)(−) RNA of region B was 68% messenger-like RNA. Similarly, the poly(A)(−) RNA of region C was 21% messenger-like RNA, and the poly(A)(−) RNA of groups B and C combined was 56% messenger-like RNA. Overall, the proportion of messenger-like RNA that lacked poly(A) in regions B and C was 29%. Most of this was in region B. About 50% of the poly(A)(−) messenger-like RNA was of larger size than histone mRNA.

In a separate experiment a portion of the same preparation of doubly labeled polyribosomes was centrifuged in a Cs_2SO_4–Me_2SO density gradient and RNA was recovered for analysis from the entire mRNA-containing region (corresponding to both regions B and C of Figure 3). The proportion of poly(A)(−) messenger-like RNA was calculated to be 31% in this experiment. Thus, there was no difference between actinomycin-treated and untreated cells with respect to the proportion of messenger-like RNA that lacked poly(A).

Absence of Poly(A) from RNA not Bound to Oligo(dT)-Cellulose. It is important to establish a limit on the amount of poly(A) present in the messenger-like RNA that did not bind to oligo(dT)-cellulose. It has consistently been found that 90% or more of poly(A)(−) RNA fractions from oligo(dT)-cellulose columns rechromatograph on oligo(dT) as poly(A)-(−) RNA. Similarly, 90% of poly(A)(+) RNA rechromatographs as poly(A)(+) RNA. Therefore, the failure of 30% of the messenger-like RNA to bind on oligo(dT)-cellulose was not an artifact of chromatography. However, these data do not exclude the possibility that the unbound RNA contained segments of poly(A) too small to allow binding to occur. In order to investigate this possibility, poly(A) segments were isolated from poly(A)(+) and poly(A)(−) messenger-like RNA by means of digestion with RNases A and T_1 in the presence of 0.5 M NaCl, and analyzed by means of electrophoresis in 14% polyacrylamide gels. The results are shown in Figure 5. The digestion products of poly(A)(+) mRNA showed a major peak of poly(A) at fraction 11 and minor peaks of mono- and oligonucleotides in fractions 31–41. The relative size of these peaks does not accurately reflect the proportion of radioactivity in poly(A) in the mRNA molecule, since some of the nucleotides were selectively lost as a result of ethanol precipitation. The digestion products of poly(A)(−) messenger-like RNA contained major peaks of mono- and oligonucleotides and a small amount of radioactivity at the position of poly(A). This amount was consistent with the RNA being about 90% free of poly(A). Some of the radioactivity in oligonucleotides overlapped with markers of synthetic rA_{18}[2] and oligo(A) isolated from nuclear RNA that is believed to have a length of about 20 nucleotides (Nakazoto and Edmonds, 1974). However, it has been shown in other experiments that the oligonucleotides do not bind to oligo(dT), whereas nuclear oligo(A) of 20 nucleotides does bind (Nakazoto and Edmonds, 1974; Greenberg, unpublished results). Since there was virtually no radioactivity migrating between poly(A) and oligonucleotides, it is concluded that poly(A)(−) messenger-like RNA does not contain short poly(A) segments larger than 20 nucleotides. The data do not rule out the existence of shorter segments of oligo(A).

Discussion

Fractionation of Polyribosomes in Cs_2SO_4 Density Gradients. When EDTA-dissociated polyribosomes are centrifuged through Cs_2SO_4 gradients that contain 15% Me_2SO they are fractionated into two distinct classes of particles that contain rRNA and messenger-like RNA, respectively. Presumably, rRNA and messenger-like RNA band at different densities under these conditions because rRNA is largely stripped of protein by the action of Cs_2SO_4, whereas messenger-like RNA remains associated with considerable amounts of protein. Data in support of this explanation will be presented elsewhere. The presence of Me_2SO is necessary to prevent precipitation of RNP that can result in heavy contamination of the messenger-like RNA-containing fractions with rRNA.

Several laboratories have recently reported the existence of proteins that remain associated with mRNA in the presence of high salt concentrations, e.g., 0.5 M KCl (Blobel, 1973; Brian and Hayashi, 1973; Morel et al., 1973; Pederson and Kumar, 1975). Some of these proteins have been shown to be associated with poly(A)(+) mRNA (Blobel, 1973; Morel et al., 1973; Lindberg and Sundquist, 1974; Pederson and Kumar, 1975), or with poly(A) itself (Blobel, 1973; V. M. Kish and T. Pederson, personal communication). In the present study it was

TABLE III: Percent of mRNA Which Lacks Poly(A) in Polyribosomes from Cells Labeled in the Absence of Actinomycin D.

Region of Cs_2SO_4–Me_2SO Density Gradient	^{14}C cpm[a]	% of ^{14}C cpm in Poly(A)(+) RNA[b]	^{3}H cpm[a]	% of ^{3}H cpm in Poly(A)(+) mRNA[b]	^{3}H cpm in Poly(A)(+) mRNA	^{3}H cpm in Poly(A)(−) RNA	$^{14}C/^{3}H$[c]	Proportion of Poly(A)(−) RNA ^{3}H cpm in mRNA[d]	^{3}H cpm in Poly(A)(−) mRNA	% of mRNA Which Lacks Poly(A)
A	6283	0.4	18 894	≤0.9	–[e]	18 894	0.282	–[e]	–[e]	–[e]
B	18	0	997	34.9	348	649	0.0914	0.676	439	26.1
C	46	0	1 070	79.1	847	223	0.224	0.206	46	2.7
B + C	54	0	2 067	57.8	1195	872	0.125	0.557	485	28.9

[a] cpm Bound to Millipore filter. 1/10 of each fraction assayed. Gradient shown in Figure 3. [b] From results of oligo(dT)-cellulose chromatography. [c] Calculated from polyacrylamide gel electrophoresis data. [d] Calculated as $1 - (^{14}C/^{3}H)_i/(^{14}C/^{3}H)_A$, where A signifies region A, and i signifies regions B, C, or B + C. [e] Assumed to be nil. Region A contained predominantly rRNA.

found that histone mRNA, and possibly other poly(A)(−) mRNAs as well, exist as RNP particles stable in Cs_2SO_4. Since these particles tend to band at a higher density than particles which contain poly(A)(+) mRNA, they may have a lower ratio of protein to RNA. It remains to be seen if the proteins associated with poly(A)(−) mRNA are different from those associated with poly(A)(+) mRNA.

Identification of Poly(A)(−) mRNA. A substantial proportion of the labeled RNA from the mRNA-containing region of the gradients had the size distribution of mRNA, yet seemed to lack poly(A), since it failed to bind to oligo(dT)-cellulose. In cells labeled in the presence of 0.08 μg/ml of actinomycin D, 31% of the messenger-like RNA lacked poly(A). In untreated cells, 30% of the messenger-like RNA lacked poly(A). In order to reasonably conclude that the messenger-like RNA that did not bind to oligo(dT)-cellulose is truly poly(A)(−) mRNA, it is necessary to establish (1) that it is derived from polyribosomes rather than from cosedimenting ribonucleoproteins that could represent nuclear contamination or some other nonmessenger material, (2) that it is not rRNA or degraded rRNA, (3) that it lacks poly(A), and (4) that it is not a degradation product of poly(A)(+) mRNA. The data of Table I established that the labeled RNA that cosedimented with polyribosomes in a two-step purification procedure that involved centrifuging in a continuous sucrose gradient followed by pelleting through 2 M sucrose was 98% or more EDTA-releasable, and was therefore actually derived from polyribosomes. That the messenger-like RNA that did not bind to oligo(dT)-cellulose is not rRNA or degraded rRNA has been shown in two ways. First, its presence has been demonstrated in cells labeled in the presence of actinomycin D so as to inhibit rRNA synthesis. Second, in the experiment in which rRNA synthesis was not inhibited, but stable RNA was selectively labeled with ^{14}C, it was shown that the RNA that did not bind to oligo(dT) recovered from regions B and C of a Cs_2SO_4–Me_2SO density gradient had a large excess of ^{3}H over ^{14}C compared with rRNA. The poly(A)(−) messenger-like RNA was shown to be about 90% free of large poly(A) by means of rechromatography experiments and by means of electrophoretic analysis of the RNAase-resistant segments. Furthermore, there was no evidence for the presence of small poly(A) segments. The presence of segments smaller than 20 nucleotides has not been ruled out. However, the absence of larger segments makes this RNA qualitatively different from poly(A)(+) mRNA. Finally, there is the question of whether the poly(A)(−) messenger-like RNA could have arisen by degradation of poly(A)(+) mRNA. From inspection of the electrophoresis data (Figures 2 and 4), it is apparent that the poly(A)(+) mRNA recovered from Cs_2SO_4–Me_2SO density gradients has a size distribution similar to previously published data for L-cell poly(A)(+) mRNA (Perry et al., 1973) and a mean molecular weight greater than that of 18S rRNA. Therefore, it is unlikely that extensive degradation has occurred. Furthermore, the poly(A)(−) messenger-like RNA shown in Figures 2C and 4C appears to be slightly larger in mean size than the corresponding poly(A)(+) mRNA. This observation is not consistent with the poly(A)(−) messen-

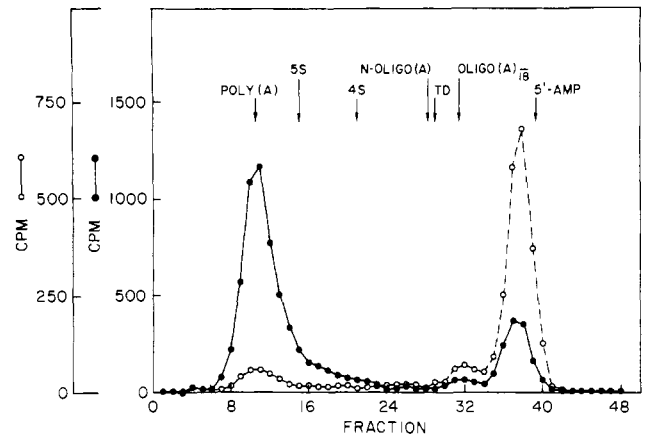

FIGURE 5: Polyacrylamide gel electrophoretic analysis of RNAase digests of poly(A)(+) and poly(A)(−) messenger-like RNA. Polyribosomes from cells labeled with [^{3}H]adenosine in the presence of 0.08 μg/ml of actinomycin D were centrifuged in Cs_2SO_4–Me_2SO density gradients. The labeled RNA in regions B and C of the gradients was recovered, deproteinized, and resolved into poly(A)(−) and poly(A)(+) fractions by means of chromatography on oligo(dT)-cellulose. These fractions were ethanol-precipitated, dissolved in 0.5 ml of 0.5 M NaCl, 0.01 M Tris-HCl (pH 7.5), and 0.001 M EDTA, and digested for 45 min at 37 °C with 20 units of RNase T_1 and 5 μg of RNase A (Sigma). They were then made 0.5% in sodium dodecyl sulfate, extracted once with phenol-chloroform, and ethanol-precipitated after addition of 100 μg of carrier RNA. The precipitates were redissolved and electrophoresed in 14% acrylamide gels (Perry et al., 1972) for 3.5 h at 5 mA/gel. The arrows indicate the positions of poly(A) and of various markers that were run in separate gels. These included [^{14}C]uridine-labeled 5S and 4S RNA prepared from L-cell polyribosomes, [^{3}H]adenosine-labeled oligo(A) prepared from L-cell nuclear RNA by means of RNAase digestion (Nakazoto and Edmonds, 1974), unlabeled synthetic $oligo(A)_{18}$ (obtained from Collaborative Research), and [5′-^{14}C]AMP (obtained from New England Nuclear). The unlabeled $oligo(A)_{18}$ was located by scanning the gel at 260 nm. Other markers were located by slicing and counting. The arrow "TD" indicates the position of the tracker dye, bromophenol blue. (X—X) Poly(A)(+) RNA. (O—O) Poly(A)(−) RNA.

ger-like RNA having arisen from poly(A)(+) mRNA by degradation. Since the poly(A)(−) messenger-like RNA was derived from polyribosomes, was not rRNA or degraded rRNA, lacked poly(A), and was probably not derived from poly(A)(+) mRNA by degradation, then it is most likely to be true mRNA which lacks poly(A).

The finding in this study that 29–31% of mRNA in L-cells labeled in the presence or absence of actinomycin D lacked poly(A) agrees well with the results of Milcarek et al. (1974), who found that 30% of mRNA in HeLa cells labeled in the presence of actinomycin D or 5-fluorouridine lacked poly(A).

Although the most prominent component of poly(A)(−) mRNA is histone mRNA, it seems likely that there are other species of poly(A)(−) mRNA as well. In actinomycin-treated cells about 65% of the poly(A)(−) mRNA was of larger size than histone mRNA, whereas in untreated cells about 50% was of larger size. It is of interest to consider why poly(A)(−) mRNA other than histone mRNA was not detected in some previous investigations with cultured cells. In previous experiments with polyribosomes from L-cells labeled for 1 h with [^{3}H]uridine in the presence of 0.08 μg/ml of actinomycin D (Greenberg and Perry, 1972; Perry et al., 1973) there was a considerable amount of poly(A)(−) RNA of heterogeneous size distribution that migrated more slowly than histone mRNA in polyacrylamide gels. This material was presumed to originate from non-polyribosomal RNP. However, there was no direct measurement of the extent of contamination of the polyribosomes by poly(A)(−) RNA of non-polyribosomal origin. A study with HeLa cells also concluded that most if not all mRNA other than histone mRNA contains poly(A) (Adesnik et al., 1972). This conclusion was based on the nearly complete inhibition of the appearance of labeled mRNA in polyribosomes of cells treated with 3′-deoxyadenosine. It was assumed that this drug specifically inhibited the entrance of poly(A)(+) mRNA into polyribosomes. However, another study has shown that this drug also strongly inhibits the entrance of poly(A)(−) mRNA into polyribosomes (Milcarek et al., 1974).

Possible Significance of Poly(A)(−) mRNA. In HeLa cells and sea urchin embryos poly(A)(−) mRNA was found in hybridization experiments using labeled DNA complementary to poly(A)(+) mRNA to lack nucleotide sequence homology with poly(A)(+) mRNA, and therefore may code for different proteins (Milcarek et al., 1974, Nemer et al., 1974), although proof of this conclusion must await identification of the products of translation of poly(A)(−) mRNA. Presumably, this conclusion also applies to L-cells. The idea that there is a considerable amount of poly(A)(−) mRNA in addition to histone mRNA is not necessarily in conflict with the fact that most of the specific mRNAs that have been examined in this respect contain poly(A). Most, if not all, of these poly(A)(+) mRNAs code for proteins that function in the cytoplasm or outside of the cell. With the exception of histone mRNA, nothing is known about the poly(A) content of mRNAs that code for proteins that function partly or wholly within the nucleus, and these proteins constitute a significant proportion of cellular protein with respect to both mass and diversity.

Now that a method for the isolation of poly(A)(−) mRNA is available, more extensive analysis of its chemical and biological properties should be possible. At present, the purity with respect to mass of the Cs_2SO_4–Me_2SO density gradient-purified poly(A)(−) mRNA cannot be estimated because of uncertainty about the proportion of polyribosomal RNA which is poly(A)(−) mRNA. However, it is apparent from Table III that it is possible to separate the poly(A)(−) mRNA from as much as 99% of the stable RNA, and this degree of purification should be adequate for some purposes.

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