A Protein of Molecular Weight 78,000 Bound to the Polyadenylate Region of Eukaryotic Messenger RNAs

(L cells/rat hepatocytes/rabbit reticulocytes/polysomes)

GÜNTER BLOBEL

The Rockefeller University, New York, N.Y. 10021

Communicated by George E. Palade, January 2, 1973

ABSTRACT   Two distinct proteins were found to be tightly bound to the heterogeneous messenger RNAs associated with polysomes in mouse L cells and rat hepatocytes. The molecular weight (78,000) of the larger of these two proteins is identical to that of the protein previously found associated with rabbit globin messenger RNA. This protein is shown to be bound to the adenylate-rich region of messenger RNA. The molecular weight of the smaller protein associated with messenger RNAs from hepatic and L-cell polysomes is similar to that found in globin messenger ribonucleoprotein.

Globin mRNAs released from rabbit reticulocyte polysomes by the puromycin–KCl procedure (1) contain two proteins, with molecular weights of 78,000 and 52,000 (2). These proteins appear to be tightly associated with globin mRNA, since they remain bound to the mRNA at 500 mM KCl in the absence of magnesium, conditions under which a substantial number of ribosomal proteins are dissociated from both the small and large ribosomal subunits (2). The existence of only two proteins in the mRNA–protein complex and their relatively strong interaction with the mRNA in the complex suggest that the association is specific and is not fortuitous like that known to occur at low salt concentrations for several RNAs in certain eukaryotic cell homogenates (3).

This paper reports the presence of a protein of molecular weight 78,000 in mRNAs released from polysomes prepared from liver (rat) and L cells (mouse), and, in addition, shows that this protein is bound to the poly(A) region of mRNA.

METHODS

L cells were labeled with [³H]adenosine or [³H]uridine or ³²PO₄ as follows: To 1 liter of L cells growing in suspension, beginning with an initial density of 7.0×10^5 cells per ml, 1 mCi of either [³H]adenosine or [³H]uridine was added. 2.5 hr later, 5 μg/ml of cycloheximide was added to the cultures to inhibit the subsequent readout of mRNA, and 30 min later the cells were harvested. Conditions for ³²PO₄ labeling were similar except that cells were labeled for 3 hr with 5 mCi of ³²PO₄ in an otherwise phosphate-free medium. The cells were harvested, transferred to 1 liter of fresh, phosphate-containing medium, and allowed to grow for an additional 3 hr before addition of cycloheximide and the final harvest.

Free polysomes were prepared from rat liver (4) and from rabbit reticulocytes as described (1). Free polysomes from L cells were obtained by the procedure used for reticulocyte polysomes. Procedures for washing polysomes (2) in high-salt

medium [500 mM KCl–50 mM Tris·HCl (pH 7.5)–5 mM MgCl₂], for their dissociation by puromycin–KCl treatment (1), and for the subsequent fractionation of the products of dissociation on continuous sucrose density gradients have been described (1, 2, 4, 5). Particles or components present in the fractions were recovered by either centrifugation at 4° for 20 hr at 40,000 rpm in a Spinco no. 40 rotor [large and small ribosomal subunit and messenger ribonucleoprotein (mRNP)] or precipitation with 2 volumes of ethanol (24 hr at −20°), followed by low-speed centrifugation.

RNase Treatment. Polysomes (20–40 A_{260} units) were suspended in 0.45 ml of H₂O; 50 μl of a compensating salt-buffer was added to the suspension to give a final concentration of 100 mM KCl (or NaCl)–50 mM Tris·HCl (pH 7.5)–2 mM MgCl₂. In addition, certain samples received 5 μl each of pancreatic RNase (400 μg/ml) and T1 RNase (1000 units/ml). The mixtures were incubated for 30 min at 37° and then layered on 12.5 ml of 5–20% sucrose gradients in 100 mM KCl (or NaCl)–50 mM Tris·HCl (pH 7.5)–2 mM MgCl₂. The gradients were centrifuged at 20° for 12.5 hr at 39,000 rpm (190,000 × g_{av}) in an SB-283 rotor of an IEC centrifuge.

For radioactivity assays of gradient fractions, 100-μl aliquots were pipetted on 3 MM Whatman filter paper disks, which were subsequently kept overnight at 0° in 10% trichloroacetic acid, washed once with cold 5% trichloroacetic acid, once with ethanol–ether (50:50) and ether, and finally counted in toluene–Liquifluor.

Electrophoresis of proteins in 10% acrylamide–sodium dodecyl sulfate (SDS) gels in a vertical electrophoresis cell was done as described (5). Base analysis of RNA samples was performed by thin-layer chromatography (6).

Source of Materials. [8-³H]Adenosine (18.3 Ci/mmol) and [5-³H]uridine (20 Ci/mmol) were from Schwarz/Mann, Orangeburg, N.Y.; H₃³²PO₄, carrier-free, from New England Nuclear, Boston, Mass.; bovine-pancreatic RNase, 5× crystallized, 65 Kunitz units/mg, and T1 RNase from Calbiochem, Los Angeles, Calif.

RESULTS

In a previous study (2) in which two proteins were found associated with globin mRNA in mRNP particles released by puromycin–KCl treatment of rabbit-reticulocyte polysomes, it was noted that the same proteins were present, although in much lower amount, in gradient fractions sedimenting slightly slower or faster than the globin mRNP. The finding suggested that other minor mRNA species expected to occur in reticulocyte polysomes, and to be either

---

Abbreviations: SDS, sodium dodecyl sulfate; mRNP, messenger ribonucleoprotein.

smaller or larger than the dominant globin mRNA, were associated with identical proteins. By extension, this assumption implies that the two proteins may be associated with a large variety of different mRNAs in any particular cell type. To test this assumption for a cell with a highly heterogeneous mRNA population and without a dominant mRNA species, rat-liver polysomes were dissociated by the puromycin–KCl procedure; the released mRNP was analyzed by electrophoresis in acrylamide–SDS gels. Fig. 1 (slot *H*) shows that liver mRNP also contains two major bands. Moreover, the large protein (hereafter referred to as P78) was identical in mobility, and presumably molecular weight, to the protein in mRNP of rabbit reticulocytes (Fig. 1, slot *G*); the other protein (to be referred to as P52) was slightly smaller than its counterpart in globin mRNP. The occurrence of proteins with apparently identical molecular weights in mRNAs of two different cell types (one of them expected to have a highly heterogeneous mRNA population) obtained from two different animal species suggested that P78 is bound to a region or sequence common to many mRNAs. Such common sequences may be located in the nontranslated parts of the mRNA strands, near their 3′ or 5′ ends. Were this the case, one would expect to find these sequences not occupied by ribosomes in a polysome, and, therefore, unprotected and likely to be accessible to nucleolytic attack, which should release the proteins (and perhaps the associated sequences) from polysomes. This assumption was indeed borne out by the results obtained in the following experiment. Reticulocyte polysomes were digested with RNase and subsequently fractionated on a sucrose gradient in a centrifugal field in which components with sedimentation coefficients larger than 16 S sedimented to the bottom of the tube. Acrylamide–SDS gel electrophoresis of fractions collected from this gradient (defined as indicated in Fig. 6) showed, besides a great number of proteins [similar to those that can be removed from polysomes by a salt wash (see Fig. 4)], the two prominent proteins associated with mRNA, P78 and P52 (Fig. 1). P78 sedimented predominantly in fraction *3*, but was also present in fractions *2* and *4*, thus covering the range of estimated sedimentation coefficients, from 4 to 14 S; P52 appeared to sediment faster, mainly in fractions *4* and *5*, with sedimentation coefficients from 10–16 S (Fig. 1, *right*, slots *1–5*). Thus, both P78 and P52 sedimented considerably faster than expected from their molecular weights of 78,000 and 52,000, respectively. The finding suggested that they were still attached to segments of mRNA, even after RNase treatment. When isolated globin mRNP was analyzed in the same manner as the reticulocyte polysomes (Fig. 1, *left*, slots *1–5*), somewhat different results were obtained; P78 showed similar sedimentation rates, being present mainly in fraction *3*, while P52 now sedimented mainly in the top fraction, corresponding to a range of estimated sedimentation coefficients of from 0 to 4 S. The results suggested that, among the products of RNase digestion of isolated mRNP, P78 remained associated with a fragment of mRNA, whereas P52 had lost its corresponding fragment. The loss might be explained by extensive nucleolysis at the high concentrations of RNase used relative to the small amount of RNA present in the isolated mRNP. The much higher ratio of RNA to RNase when polysomes were used as a starting material might explain the protection of the P52-associated fragment of mRNA.

FIG. 1. Electropherogram in a 10% acrylamide–SDS gel of globin- and liver-mRNP and of gradient fractions obtained from reticulocyte polysomes and globin mRNP after RNase treatment. Slot *H*, liver mRNP; slot *G*, globin mRNP, each about 0.5 A_{260} units, obtained by centrifugation of the appropriate sucrose gradient fractions after puromycin–KCl disassembly of polysomes. Slots *1–5*, ethanol precipitates of gradient fractions (collected as indicated in Fig. 6) of reticulocyte polysomes (about 40 A_{260}; *right*) and of globin mRNP (1.0 A_{260}; *left*) after treatment with RNase. Downward- and upward-pointing *arrows* indicate bands of molecular weight of 78,000 (P78) and 52,000 (P52), respectively. Because of the large number of bands, the identity of P78 and P52 in the slots containing products of RNase digestion of polysomes with those of isolated globin mRNP in slot *G* is not certain, but highly probable (see Fig. 4).

Taking these results into account, the following experiments were designed to characterize the presumptive mRNA fragment associated with P78. Since efficient labeling of mRNA is difficult to achieve in whole animals, tissue culture cells were chosen on the assumption that their mRNAs should also be associated with the two proteins. L cells were incubated for 3 hr with [³H]adenosine. Their free polysomes were isolated and disassembled by the puromycin–KCl procedure. The sedimentation profile and the distribution of acid-insoluble radioactivity (Fig. 2) show a broad peak of high specific activity with a sedimentation rate of about 27 S that, by analogy with the reticulocyte system (1), can be assumed to be mRNP. Acrylamide–SDS gel electrophoresis of fractions obtained from this gradient (Fig. 3) indeed show the two characteristic proteins of estimated molecular weights 78,000 and 52,000 (slot *m*, *arrows*), primarily in the fraction in which mRNP sedimented. However, substantial amounts of these two proteins also sedimented in the small-subunit region (S^0). This result is probably due to incomplete dissociation of mRNP from the small ribosomal subunit. Further evidence in support of this interpretation can be detected in the radioactivity profile in Fig. 2; the shoulder of high specific radioactivity on the faster-sedimentating side of the small-subunit peak probably represents a mRNP–small subunit complex. A small amount of P78 is apparently present
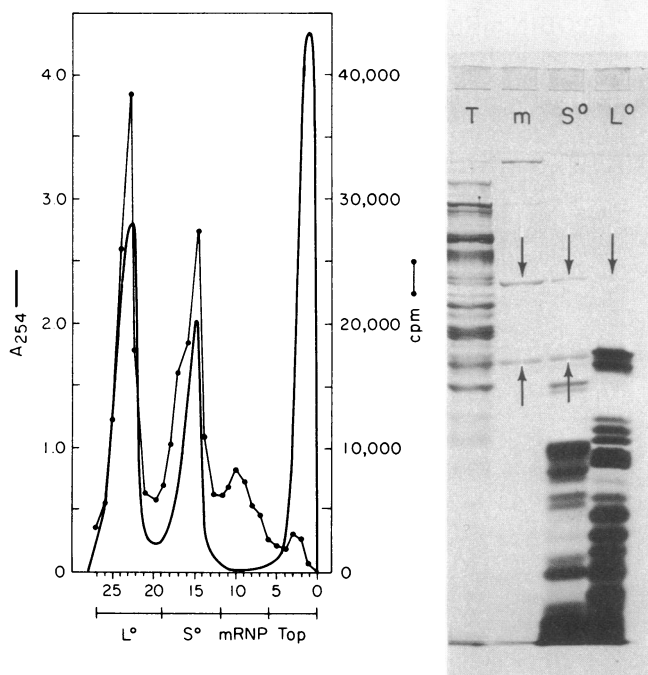
FIG. 2. (*left*) Sedimentation profile and distribution of acid-insoluble radioactivity among the dissociation products of polysomes from L cells labeled for 3 hr with [³H]adenosine. Polysomes (15 A_{260} units) were suspended in 0.5 ml of 500 mM KCl, 50 mM Tris·HCl (pH 7.5), 2 mM MgCl₂, 1 mM dithiothreitol, and 1 mM puromycin (2). After incubation for 10 min at 37°, the mixture was layered on 12.5 ml of a 5–20% sucrose gradient in 500 mM KCl–50 mM Tris·HCl (pH 7.5)–5 mM MgCl₂. The gradients were centrifuged in an SB 283 rotor of an IEC centrifuge for 2.8 hr at 39,000 rpm; A_{254} was recorded by an ISCO UV analyzer, and 27 0.5-ml fractions were collected with an ISCO fractionator. 100 μl from each 0.5-ml fraction was spotted on 3 MM Whatman filter paper disks for determination of acid–insoluble radioactivity (●——●). The remaining 0.4 ml was recombined into 4 successive fractions [*top*, *mRNP*, small ribosomal subunit (*S⁰*) and large ribosomal subunit (*L⁰*). After ethanol precipitation, these fractions were subjected to electrophoretic analysis on an acrylamide-SDS gel (see Fig. 3).

FIG. 3. (*right*) Electropherogram in a 10% polyacrylamide-SDS gel of the gradient fractions collected in the experiment described in Fig. 2.

even within the large-subunit region, where it might be accounted for by a single mRNP with two small subunits still attached to it. Such complexes may, in fact, explain the usually observed small contamination of large subunits by small subunits after puromycin–KCl dissociation of polysomes from other sources (4).

The two proteins associated with mRNA were also detected among the products of RNase treatment of L-cell polysomes. Fig. 4 shows the results of an experiment similar to that described in Fig. 1, except that the polysomes were washed in a high salt medium before treatment with RNase and fractionation on sucrose gradients. The absorbance profile after RNase treatment showed a large peak near the top of the gradient (see Fig. 6), resulting from partial fragmentation of ribosomal RNA; this peak was considerably smaller when polysomes were treated with RNase at 0°. Acrylamide–SDS gel electrophoresis of the various fractions throughout the gradient showed the elimination of many proteins that were seen in Fig. 1 (slots *1–5*, *right*) and that

TABLE 1. *Base composition of the products of RNase digestion of ³²PO₄-labeled L-cell polysomes. Nucleotide distribution among gradient fractions*

	Fraction							
	1		2		3		4	
	cpm	%	cpm	%	cpm	%	cpm	%
AMP	3750	22	405	14	1060	78	195	36
CMP	4210	24	862	29	88	7	91	17
GMP	6770	39	1140	39	118	9	148	27
UMP	2640	15	513	18	87	6	108	20

Treatment with RNase was as described in *Methods*. Fractions *1–5* were collected and numbered as indicated in Fig. 6. Fraction 5 was not analyzed.

were previously shown to be removed by a salt wash (2). As a result, P78 and P52 now stood out as the major proteins that were removed from polysomes by RNase treatment. P78 has identical mobilities, whether derived from reticulocyte, liver, or L-cell polysomes. Furthermore, it was released from polysomes, even when the RNase treatment was performed at 0°. As in reticulocyte polysomes not washed with salt (Fig. 1), P78 was again present in fractions *2*, *3*, and *4*, with some differences among the various polysomes; for instance, the bulk of P78 from liver polysomes appeared to sediment somewhat faster than that from L-cell or reticulocyte polysomes, being concentrated in fraction *4*, but also present in fraction *5*. P52 is present in fractions *2*, *3*, *4*, and *5* after RNase digestion at 37°, but is missing from fraction *2* and more concentrated in fractions *4* and *5* after RNase digestion at 0°, a finding suggesting again that the fragment of RNA attached to P52 is not as consistently RNase-resistant as that associated with P78. The removal of P78 after RNase digestion was complete (Fig. 4 slots P⁻ and P⁺). Complete removal of P52 could not be ascertained by gel analysis of the pellets since its mobility appears to coincide with that of a ribosomal protein of the large subunit.

For characterization of the presumptive mRNA fragment associated with P78, L cells were labeled with [³H]uridine or [³H]adenosine or with ³²PO₄. RNase digestion of the labeled polysomes and subsequent sucrose gradient analysis of their digestion products showed (Fig. 5) a prominent broad peak of radioactivity in the 11S region after labeling with [³H]adenosine—but not with [³H]uridine. Base composition analysis after ³²PO₄ labeling (Table 1) showed a peak of AMP in the identical position of the gradient. This peak was composed of about 80% AMP. Thus, a poly(A)-containing fragment of mRNA, which has been described as a common feature for many eukaryotic mRNAs (7–11), sedimented together with a large portion of P78, suggesting that the two are associated with one another. The association of P78 with the poly(A) region of mRNA could explain the abnormally high sedimentation rate of the complex since the poly(A)-rich segment obtained from deproteinized mRNA sediments at about 4 S (7–11). Dissociation of the poly(A)–P78 complex should therefore result in a lower sedimentation rate for both the poly(A) segment and the protein. Such a shift was obtained by treating the material released by RNase with SDS or by sedimenting it into a sucrose gradient containing 2.0 M LiCl. The radioactivity peak shifted after both SDS
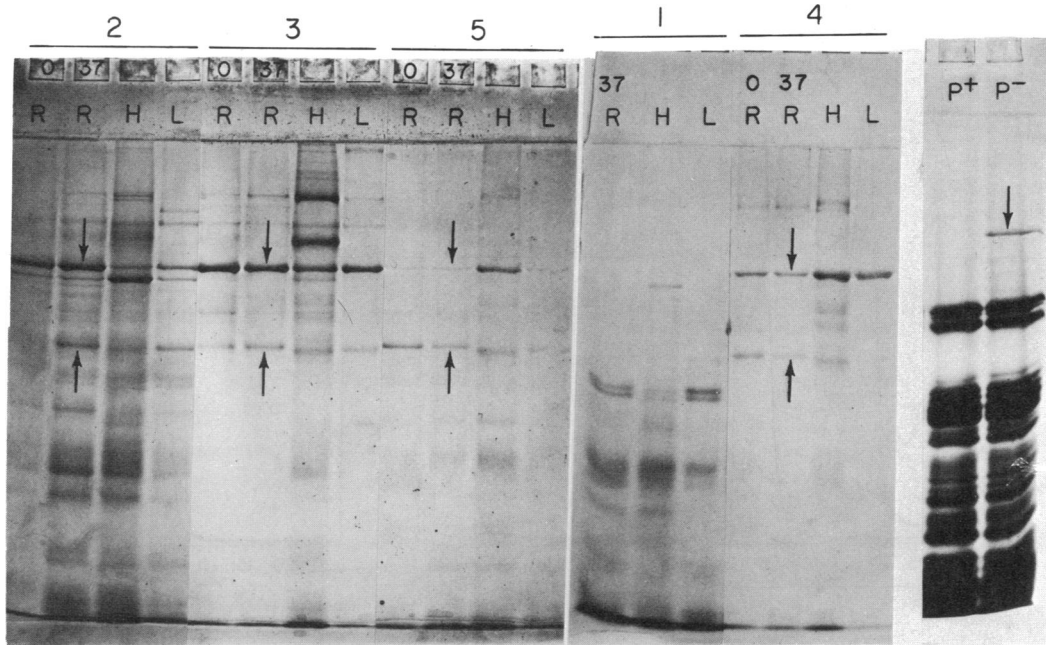
FIG. 4. Electropherogram in a 10% acrylamide–SDS gel of gradient fractions obtained from salt-washed polysomes of rabbit reticulocytes, rat liver, and L cells after RNase treatment. The salt-washed polysomes (35 A_{260} units) were treated with RNase at 37° (all) or at 0° (reticulocyte polysomes only). After sucrose gradient centrifugation five fractions were collected (see Fig. 6, *left*) and treated with ethanol. Slots *1–5*, gradient fractions *1–5*; *RO* and *R37*, temperature at which reticulocyte polysomes were incubated with RNase; *H*, hepatic; and *L*, L-cell polysomes. Slots *P⁻* and *P⁺* represent ¹/₄ of the pellets found after sucrose gradient centrifugation of L-cell polysomes without (*P⁻*) or with (*P⁺*) RNase treatment.

and LiCl treatment, from about 11 S to about 5 S (Fig. 6). Concomitant with the shift of the radioactivity peak, there was a shift of P78 from fractions *3* and *4* to fraction *1* (data not shown).

DISCUSSION

The results reported in this paper show that the tight, i.e., relatively salt-resistant, association of two distinct proteins to mRNA is not restricted to globin mRNA, but is a more general feature of many eukaryotic mRNAs. The striking similarity of the molecular weights of these two proteins associated with mRNAs in polysomes of cells as different as rabbit reticulocytes, rat hepatocytes, and L cells (of which at least the last two must have a highly heterogeneous mRNA population) suggested that these proteins are bound to regions or sequences common to all mRNAs, most likely not translated, and therefore located at the mRNA termini. In support for this interpretation, it was found that nucleolysis of polysomes, even under mild conditions (incubation at 0°), led to a release of fragments of mRNA separated from ribosomes but still associated with their distinct proteins. In polysomes the mRNA termini are expected to be uncovered and, hence, sufficiently exposed to nucleolytic attack, in contrast to most of the translated sequences, which are protected by ribosomes.

The different sedimentation rates of the two protein-polynucleotide complexes obtained by RNase digestion suggested that they were derived from different regions of the mRNAs. One of the two proteins was shown to be attached to an adenylate-rich region of mRNA, which is a common feature of many eukaryotic mRNAs (7–11) and which is located at the 3' terminus (12).

The location of the other protein of molecular weight 52,000 (P52) needs further investigation. The results obtained in the present study suggest that its associated mRNA
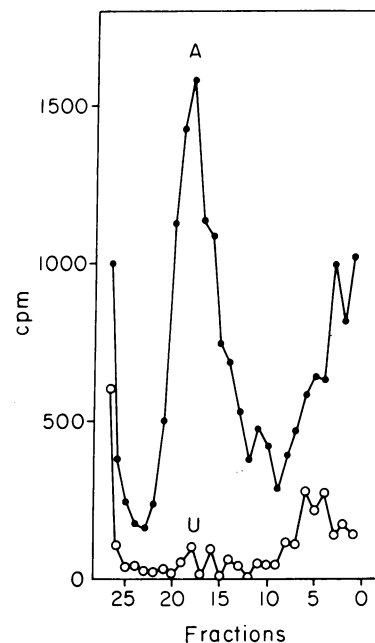

FIG. 5. Distribution of acid-insoluble radioactivity in sucrose gradients after incubation with RNase of polysomes from L cells labeled with [³H]adenosine or [³H]uridine. 12.5 A_{260} units containing 1.3 × 10⁶ cpm, or 14.5 A_{260} units containing 3.4 × 10⁶ cpm of L-cell polysomes labeled with [³H]uridine (O——O) or [³H]adenosine (●——●) were subjected to RNase treatment. For further details, see *Methods*.
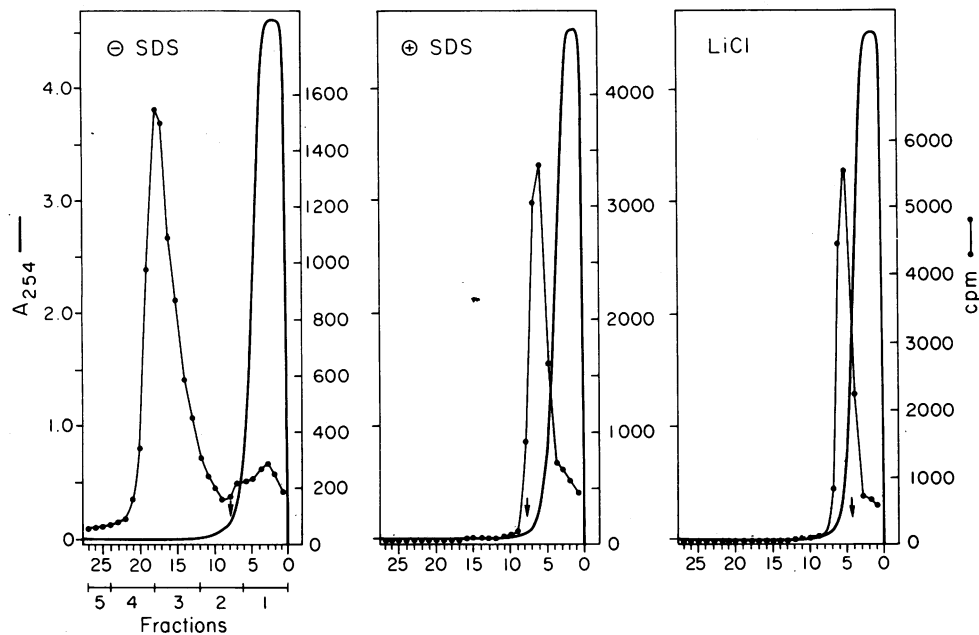
FIG. 6.   Sedimentation profile and distribution of acid-insoluble radioactivity in sucrose gradients of [³H]adenosine-labeled L-cell poly-somes after RNase incubation and subsequent treatment with dissociating agents. Incubation with RNase was extended to 1 hr and performed in NaCl rather than in KCl. During this incubation period, the ribosome solution (45.5 A_{260} units and 6.9 × 10⁶ cpm in 1.5 ml) became very turbid. Centrifugation of this solution for 10 min at 2000 x g resulted in a pellet containing the bulk of the ribosomal proteins (data not shown). The resulting supernatant was divided into three aliquots. The first aliquot (*left*) was layered directly on a gradient containing NaCl instead of KCl; the second aliquot (*middle*) was first treated with 10% SDS in 0.1 M Tris·HCl (pH 7.5) to give a final concentration of 1% SDS before being layered on an identical sucrose gradient; the third aliquot (*right*) was layered on a gradient containing 2.0 M LiCl and no MgCl₂. For other detals see *Methods*. Arrow indicates position of 5S RNA centrifuged under identical conditions.

fragment is either larger or more compact than that of the 3'-terminal poly(A)-P78 complex. Compactness due to considerable secondary structure would also explain the relative RNase resistance of the fragment. In fact, a large amount of base pairing was proposed for the 130 untranslated nucleotides at the 5' end of MS2 bacteriophage RNA (13). If untranslated sequences, capable of extensive base-pairing, would also occur at the 5' termini of eukaryotic mRNAs, then that segment might represent a reasonable association site for P52.

The observed variability from 50–250 nucleotides in the length of the poly(A) terminus of mRNA (7–11) is probably reflected in the wide range of sedimentation rates (4–14 S) of the poly(A)-P78 complex observed here, provided one P78 of constant molecular weight is associated with one fragment of poly(A) of variable length.

After completion of the experiments reported in this paper, evidence indicating that the poly(A) segment of mRNA is associated with a particle in mouse sarcoma 180 was published by Kwan and Brawerman (15). It is likely that that particle is the protein P78 described here.

1. Blobel, G. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 832–835.
2. Blobel, G. (1972) *Biochem. Biophys. Res. Commun.* **47**, 88–95.
3. Baltimore, D. & Huang, A. S. (1970) *J. Mol. Biol.* **47**, 263–273.
4. Blobel, G. & Sabatini, D. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 390–394.
5. Blobel, G. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1881–1885.
6. Greenman, D. L., Huang, R. C., Smith, M. & Furrow, L. M. (1969) *Anal. Biochem.* **31**, 348–357.
7. Lim, L. & Canellakis, E. S. (1970) *Nature* **227**, 710–712.
8. Kates, J. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35**, 743–752.
9. Edmonds. M., Vaughan, M. H. & Nakazato. H. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1336–1340.
10. Darnell, J. E., Wall R. & Tushinski, R. J. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1321–1325.
11. Lee, S. Y., Mendecki, J. & Brawerman, G. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1331–1335.
12. Sheldon, R., Kates, J., Kelley, D. E. & Perry, R. P. (1972) *Biochemistry* **11**, 3829–3834.
13. De Wachter, R., Merregaert, J., Vandenberghe, A., Contreras, R. & Fiers, W. (1971) *Eur. J. Biochem.* **22**, 400–414.
14. Adesnik, M. & Darnell, J. E. (1972) *J. Mol. Biol.* **67**, 397–406.
15. Kwan, S. W. & Brawerman, G. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3247–3250.