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Biochemistry

A major species of mammalian messenger RNA lacking a polyadenylate segment

[oligo(dT)-cellulose/18S mRNA/cell-free translation/45,000 dalton polypeptide]

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ABSTRACT **Translation of total polysomal RNA from sarcoma 180 ascites cells in a wheat germ cell-free system produces two major polypeptides, A and B, with molecular weights of 50,000 and 45,000, respectively. Fractionation on Millipore filters or on oligo(dT)-cellulose leads to retention of the mRNA specific for protein A in the poly(A)-containing fraction and to accumulation of the B mRNA in the unadsorbed poly(A)-deficient fraction. The mRNA for B sediments at approximately 18 S; it is released as a 50S ribonucleoprotein upon EDTA treatment of polysomes. Its translation is particularly sensitive to an inhibitor present in the polysomal RNA. The poly(A)-deficient mRNA for the 45,000 dalton polypeptide is also present in mouse myeloma MPC-11 cells, where it seems to be localized in membrane-bound polysomes.**

The great majority of eukaryotic messenger RNA species that have been isolated and characterized to date have been found to contain a poly(A) sequence at the 3′ terminus. The one well-documented exception is the group of RNAs that code for histones (1, 2). The reovirus-specific mRNAs have also been reported to lack poly(A) (3). It is possible that a large population of mRNA components without poly(A) exists in mammalian cells. This is suggested by the occurrence in polysomes of heterodisperse RNA, labeled in the absence of rRNA synthesis, that fails to bind to poly(A)-specific adsorbents (4, 5). Hybridization experiments have indicated that this fraction in HeLa cells represents a population of molecules distinct from that of poly(A)-containing species (5). Similar results have been obtained with sea urchin RNA (6). It remains to be demonstrated, however, that this heterogeneous polysomal RNA fraction is in fact mRNA. Conclusive evidence would require the identification of specific polypeptides coded for by this material.

Studies on the role of the poly(A) sequence have been inconclusive. RNAs lacking poly(A), such as the histone mRNAs, appear to be about as stable as the poly(A)-containing RNAs (7). Furthermore, they do not show unusual requirements for translation *in vitro* (8). It may be of significance, however, that the histone RNA species are subject to a specific inactivation mechanism linked to the cessation of DNA replication (9).

We report here on the identification of a major mammalian mRNA component that lacks a poly(A) sequence. This material sediments at about 18 S and codes for a 45,000 dalton polypeptide. Its behavior in wheat germ extracts suggests that its efficiency of translation is lower than that of poly(A)-containing mRNA components.

MATERIALS AND METHODS

Cells and Tumors. Mouse sarcoma 180 ascites (S-180) cells were maintained by weekly transfers into the peritoneal cavity of albino mice. They were harvested 6–8 days after inoculation and washed as described (10). Mouse myeloma tumors were obtained by subcutaneous inoculation of Balb/c mice with 3×10^6 MPC-11 cells grown in Dulbecco's modified Eagle's medium supplemented with 15% decomplemented horse serum and antibiotics (11).

Isolation of Polysomes and Polysomal RNA. The S-180 cells were incubated for 1 hr in Krebs' medium (without antibiotics) and then disrupted by hypotonic swelling followed by Triton X-100 lysis (12). Polysomes were obtained from the 17,000 × *g* supernatant by precipitation with 30 mM $MgCl_2$ (12). Minced myeloma tumors were suspended in a volume of buffer A (100 mM KCl–5 mM $MgCl_2$–50 mM Tris·HCl, pH 7.6–0.8 M sucrose) equal to 2.5 times their weight and disrupted by gentle homogenization in a motor-driven Teflon-glass homogenizer (Thomas Co., Philadelphia). The homogenate was centrifuged at 17,000 × *g* for 10 min, and the supernatant (cytoplasm) was removed without disturbing the fluffy material above the nuclear pellet. A membrane-bound polysome pellet was obtained from the cytoplasm by centrifugation through a 2-ml layer of buffer A for 20 min at 60,000 × *g*. It was resuspended in a small volume of high speed supernatant from a rat liver extract [which contains a ribonuclease inhibitor (13)], and an equal volume of 10 mM KCl–1 mM $MgCl_2$–10 mM Tris·HCl, pH 7.6. Polysomes, released from the membranes by addition of Triton X-100 (1% final concentration), were precipitated with $MgCl_2$.

RNA prepared from cytoplasm or from polysomes by direct phenol extraction in the presence of Tris·HCl, pH 9 (14) was precipitated with ethanol overnight, and washed with a 95% ethanol–0.1 M NaCl (2 vol:1 vol) mixture. Ethanol was removed by an ether wash.

Cell-Free Polypeptide Synthesis. Reaction mixtures (50 μl total volume) contained 15 μl of wheat germ lysate (15), 26 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) (pH 7.2), 12 μM spermidine, 3 mM magnesium acetate, 2 mM dithiothreitol, 1.8 mM 2-mercaptoethanol, 1 mM ATP, 0.2 mM GTP, 8 mM creatine phosphate, 160 mM potassium acetate, 50 μg/ml of creatine phosphokinase, 30 μM each of the common amino acids (except methionine), 5 μCi of [^{35}S]methionine (100–300 Ci/mmol, New England Nuclear), and indicated amounts of exogenous RNA. After incubation at 22° for 2 hr, pancreatic ribonuclease A was added to a final concentration of 200 μg/ml; reaction mixtures were digested at 37° for 30 min. Samples were removed for measurements of acid-insoluble radioactivity.

Polyacrylamide Gel Electrophoresis. Proteins were precipitated with 10% trichloroacetic acid containing 3% casamino acids. The precipitates were washed with 0.1% and 0.01% trichloroacetic acid, dissolved in 50 mM Tris·HCl, pH 9.0–2% sodium dodecyl sulfate ($NaDodSO_4$), and heated in boiling

Abbreviations: S-180, sarcoma 180 ascites cells; $NaDodSO_4$, sodium dodecyl sulfate.

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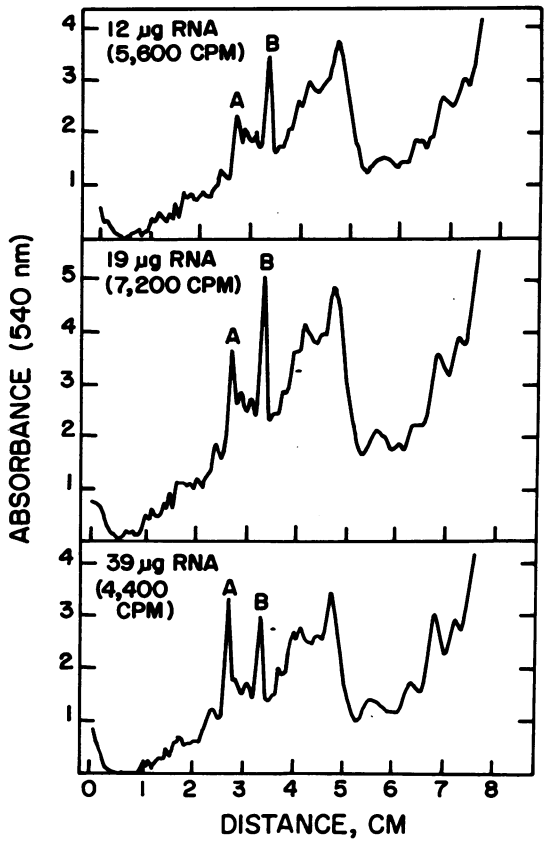

FIG. 1. Electrophoretic analysis of *in vitro* translation products of S-180 polysomal RNA at different RNA concentrations. Polypeptides produced in a wheat germ, cell-free, protein-synthesizing system were subjected to electrophoresis on polyacrylamide–Na-DodSO$_4$ slab gels. Autoradiograms of dried gels were scanned as described in *Materials and Methods*. The values in parentheses indicate acid-insoluble radioactivity incorporated in a 4-μl sample of the incubation mixture. Endogenous incorporation was 1150 cpm.

water for 1 min. The proteins were treated with 2-mercaptoethanol at a final concentration of 75 mM for 1 hr at 37° to reduce disulfide bridges, and alkylated with iodoacetamide (0.1 M) for 30 min at 37°. They were subjected to electrophoresis on either 12% or 13% polyacrylamide-NaDodSO$_4$ slab gels (1/32 inch or 0.08 cm thick) with a 5% stacking region according to the procedure of Studier (16). Combs which provide 1/4 inch (0.63 cm) wells were used. Electrophoresis was for 16 hr at 27 V. The gels were stained with Coomassie blue, destained, and dried onto paper. Autoradiograms produced by exposure of Kodak No-screen x-ray film to the gels were scanned (λ = 540 nm) with a Beckman model DU spectrophotometer adapted with a Gilford gel scanner.

RESULTS

Translation of messenger RNAs from mammalian tumor cells

Translation of total polysomal RNA extracted from S-180 cells in a wheat germ cell-free system led to the production of several major polypeptides, as well as a heterogeneous mixture of poorly resolved components. Fig. 1 shows densitometry tracings of [^{35}S]methionine-labeled polypeptides subjected to slab gel electrophoresis followed by autoradiography. Two polypeptides, A and B, having molecular weights of 50,000 and 45,000, respectively, were particularly prominent. Polypeptides of identical mobilities were found among the translation products

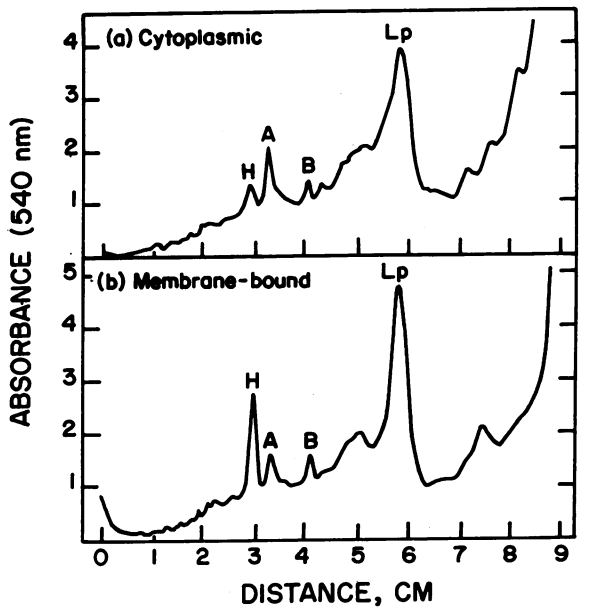

FIG. 2. Electrophoretic analysis of translation products of 16 μg of either myeloma cytoplasmic or membrane-bound polysomal RNA. Products were analyzed as in Fig. 1. H, immunoglobulin heavy chain; Lp, precursor of immunoglobulin light chain.

of mouse myeloma MPC-11 cytoplasmic RNA (Fig. 2a). Translation of the myeloma RNA also led to synthesis of the immunoglobulin heavy chain and light chain precursor (refs. 17 and 18; Sonenshein and Brawerman, submitted for publication).

When the RNA from myeloma membrane-bound polysomes was used, immunoglobulin synthesis was enhanced substantially (Fig. 2b). Polypeptide B was also present among the translation products of the membrane-bound polysomal RNA, while polypeptide A appeared to be reduced. Separate experiments with the RNA of free polysomes showed a considerable enrichment in activity for the synthesis of polypeptide A in this fraction, without concomitant enrichment in activity for polypeptide B (Sonenshein and Brawerman, submitted for publication).

Adsorption of the poly(A)-containing RNA from either S-180 or myeloma cells to Millipore filters (14) led to separation of the components responsible for the synthesis of A and B. The mRNA specific for A was retained on the filters, while the activity for B was recovered in the nonadsorbed fraction (data not shown). Under these conditions 98% of the poly(A) in the preparations adsorbed to the filters, as measured by annealing with radioactive poly(U) (19). When polysomal RNA from S-180 cells was subjected to extensive adsorption to oligo-(dT)-cellulose until all material that reacted with poly(U) was removed, the resulting unadsorbed fraction was considerably enriched in activity for B and showed no mRNA activity for A (Fig. 3). The material adsorbed on oligo(dT)-cellulose was enriched in mRNA for A, but still contained activity for B. The latter was considerably reduced after a second adsorption (Fig. 3). Since a significant amount of rRNA tended to be adsorbed to oligo(dT)-cellulose, the binding of at least some of the B band RNA may be due to nonspecific adsorption. It is possible, however, that a portion of this mRNA contains a poly(A) segment.

The myeloma RNAs for A and B behaved in the same fashion as the corresponding components from the sarcoma cells. The unadsorbed fraction from Millipore binding of RNA extracted from the membrane-bound myeloma polysomes had activity

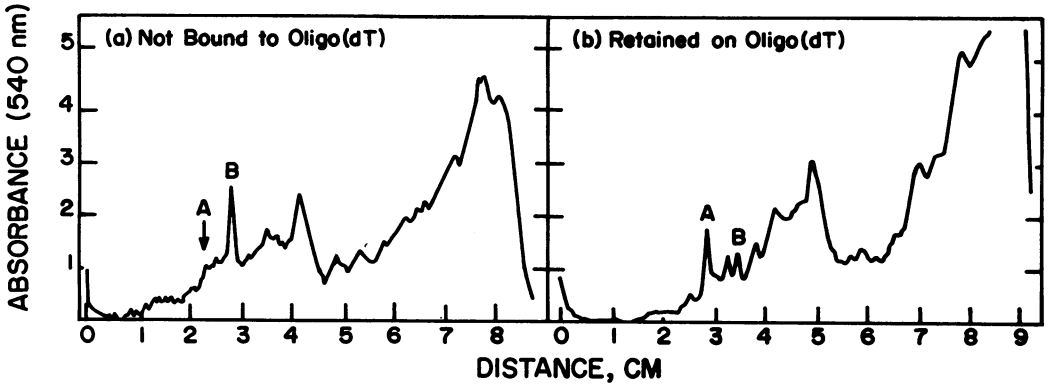

FIG. 3. Electrophoretic analysis of products of translation of S-180 polysomal RNA chromatographed on oligo(dT)-cellulose. S-180 polysomal RNA was mixed with oligo(dT)-cellulose at room temperature in the presence of binding buffer (500 mM NaCl–5 mM $MgCl_2$–50 mM Tris·HCl, pH 7.6–0.1% $NaDodSO_4$) and washed extensively with the same buffer. (a) The unadsorbed RNA (95% of the total) was precipitated and washed with ethanol; a 24-μg sample was used to direct the cell-free system. (b) The adsorbed RNA was eluted and passed a second time through oligo(dT)-cellulose in the binding buffer containing 0.5% $NaDodSO_4$. The amount of RNA adsorbed during the second passage represented about 1% of the original. A 5-μg sample of the RNA retained in the second passage was used to direct translation *in vitro*.

for B while that from free polysomes did not (data not shown). Thus, it appears that the mRNA for polypeptide B is a species without a poly(A) sequence; it may be associated with membrane-bound polysomes, at least in myeloma cells. The RNA for polypeptide A, on the other hand, is a poly(A)-containing species associated with free polysomes.

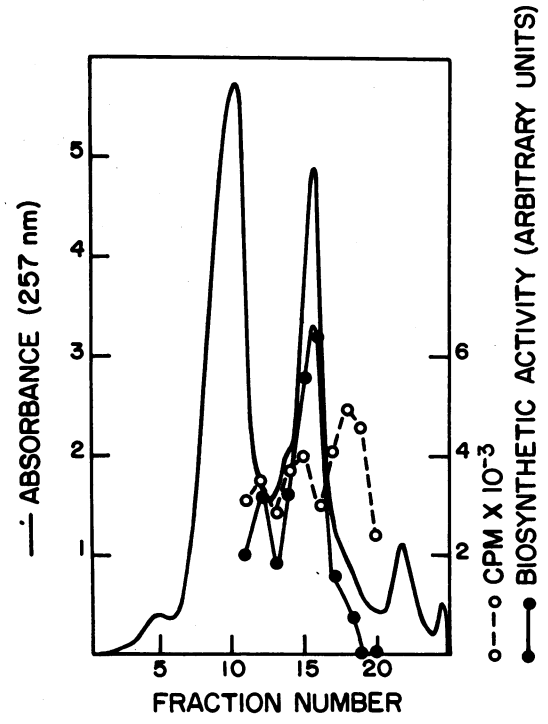

FIG. 4. Determination of the size of the mRNA coding for the B band polypeptide. S-180 polysomal RNA lacking poly(A), prepared as described in the legend of Fig. 3, was sedimented through gradients of 5–30% sucrose (10 mM KCl–20 mM Tris·HCl, pH 7.6) for 5 hr at 41,000 rpm in a Spinco SW41 rotor. The gradients were monitored for absorbance at 257 nm. The collected fractions were precipitated with ethanol and samples used to direct translation *in vitro*. Fraction 1 represents the bottom-most part of the tube. Autoradiograms of the electrophoretic analysis of the products were scanned. The peak height of the B band obtained with the RNA of each fraction was determined and used as a measure of its biosynthetic activity for B. Endogenous incorporation was 1500 cpm.

Sedimentation characteristics of the poly(A)-deficient mRNA

The sarcoma RNA remaining after removal of poly(A)-containing components by adsorption to oligo(dT)-cellulose was subjected to zone centrifugation. Fractions were assayed for messenger activity, and the products of cell-free translation were analyzed by gel electrophoresis in order to localize the component specific for polypeptide B. Fig. 4 shows that this mRNA sedimented primarily with the 18S rRNA. Polypeptide B was present as a major translation product of fraction 16 RNA, and was nearly completely missing from the products of fraction 18 RNA (Fig. 5). The latter contained a higher proportion of small polypeptides. A similar experiment carried out with unfractionated S-180 polysomal RNA showed that the mRNA for A sediments slightly more slowly than that for the B (data not shown).

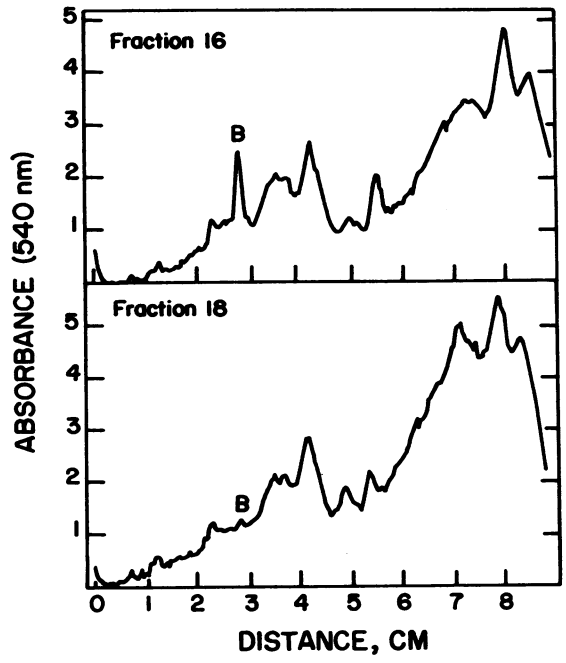

FIG. 5. Electrophoretic analysis of the translation products of RNA from fractions 16 and 18 of the gradient presented in Fig. 4.

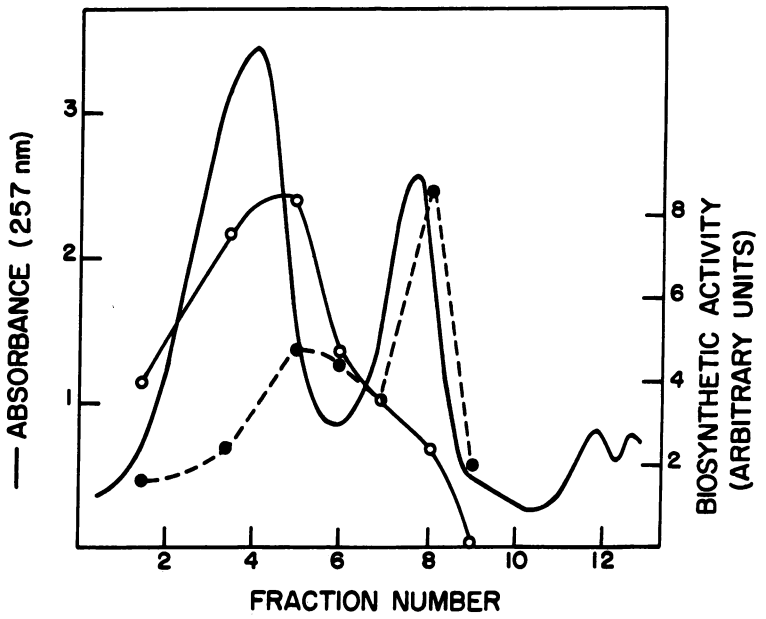

FIG. 6. Zone centrifugation of messenger ribonucleoprotein released by EDTA treatment of S-180 polysomes. S-180 polysomes, treated with 40 mM EDTA for 10 min at 4°, were layered on gradients of 10–30% sucrose (100 mM KCl–1 mM $MgCl_2$–50 mM Tris·HCl, pH 7.6). After centrifugation for 4.5 hr at 41,000 rpm in a Spinco SW41 rotor, fractions were collected and precipitated with ethanol as described in the legend of Fig. 4. RNA was extracted with phenol and used to direct a wheat germ cell-free system. The products were analyzed for A and B band synthesis as described in Fig. 4. (○) B band; (●) A band.

Sedimentation characteristics of the messenger ribonucleoprotein complexes

Purified sarcoma polysomes were dissociated by treatment with EDTA followed by zone sedimentation. Fractions of the sucrose gradient were deproteinized and the resultant RNA was translated in the wheat germ system. The activity for polypeptide B was found in a broad peak with a sedimentation coefficient slightly less than that of the large ribosomal subunit (Fig. 6). The activity for polypeptide A showed a biomodal distribution, with one peak between the two ribosomal subunits and another sedimenting as a 30S component. The more rapid sedimentation of the messenger ribonucleoprotein for B is of interest, in view of the similarity in sizes of the mRNAs for A and B. The distribution of polypeptides produced by fractions 5 and 8 is shown in Fig. 7. The relative distributions of A and B produced by these two fractions are very different. Other polypeptides also show substantial differences in distribution.

Differential translation of the mRNAs for polypeptides A and B

The amount of polypeptide synthesized in the wheat germ cell-free system increases with the amount of added polysomal RNA, but excessive amounts of this RNA lead to strong inhibition (Fig. 1). Not all polypeptides are affected to the same extent under the inhibitory conditions. Polypeptide A did not appear to be affected at all, while the amount of polypeptide B was reduced substantially. This led to a drastic change in the relative amounts of A and B produced *in vitro* (Fig. 1). A similar pattern of A and B synthesis as a function of RNA concentration was observed in myeloma RNA translation (Sonenshein and Brawerman, submitted for publication). Translation with relatively small quantities of polysomal RNA indicates that the amount of mRNA for B in S-180 cells far exceeds that for A.

The effect observed in Fig. 1 is due apparently to an inhibitor of translation present in S-180 polysomal RNA; inhibitor activity has been detected in other preparations as well. For example, an 18S RNA fraction rich in inhibitory activity has been isolated from the poly(A)-deficient fraction of rat liver polysomal RNA (which does not direct the translation of B polypeptide *in vitro*, data not shown). The effect of this inhibitor on the products of S-180 RNA *in vitro* is shown in Fig. 8. While synthesis of polypeptide A was not affected, that of polypeptide B was substantially reduced. This effect was observed consistently, and was also evident in mouse myeloma RNA translation (data not shown). Because of the presence of this selective inhibitor, it was important to use levels of RNA below the inhibitory range in the experiments designed to test the sedimentation properties of mRNA and messenger ribonucleoprotein for polypeptides A and B.

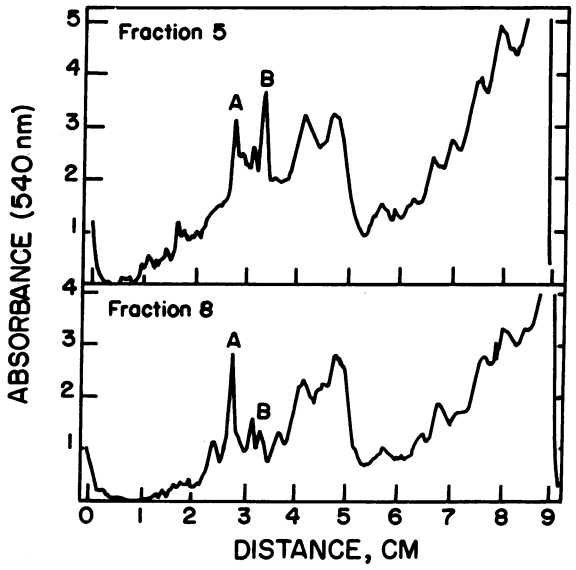

FIG. 7. Electrophoretic analysis of translation products of RNA from fractions 5 and 8 of the gradient presented in Fig. 6.

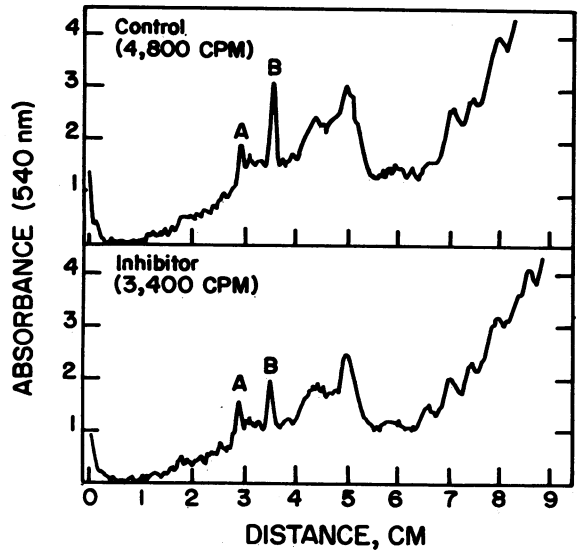

FIG. 8. Effect of rat liver inhibitor on translation of S-180 polysomal RNA. Rat liver 18S RNA was purified from poly(A)-deficient polysomal RNA by two cycles of centrifugation through sucrose gradients as described in the legend of Fig. 4. S-180 polysomal RNA (12 μg) was used to direct an *in vitro* system in the absence or presence of 8 μg of the rat liver 18S inhibitor fraction. Endogenous incorporation was 820 cpm.

DISCUSSION

The mRNA for polypeptide B described here represents an abundant species of large size that lacks a poly(A) sequence. The absence of this sequence is inferred from the fact that the RNA fails to bind to Millipore filters or to oligo(dT)-cellulose under conditions that remove most or all the material capable of annealing with poly(U) under our assay conditions. The presence of very short sequences not precipitable by trichloroacetic acid cannot be excluded, since the assay involves precipitation of the poly(U) segments protected from RNase by the poly(A) in the preparation. From the sedimentation coefficient of the RNA, a size of about 7×10^5 daltons can be tentatively deduced, bearing in mind the uncertainties of size determination by this technique. Since the size of the polypeptide is 45,000 daltons, the mRNA would seem to have an excess of about 900 nucleotides over what is needed for amino acid coding. Dissociation of the polysomes releases the mRNA as a rather large messenger ribonucleoprotein, as judged by its sedimentation rate. The apparent large excess of noncoding sequences in the RNA may provide many binding sites for proteins, thus accounting perhaps for the large size of the messenger ribonucleoprotein.

The availability of this species of poly(A)-deficient mRNA provides the opportunity for a preliminary evaluation of several possible poly(A) functions. This sequence does not seem to be essential for the occurrence of mRNA as nucleoprotein. The studies of translation *in vitro* indicate that the poly(A)-deficient mRNA component is particularly vulnerable to the effect of an inhibitor of translation present in polysomal RNA. It is also sensitive to the presence of small amounts of poly(A), an inhibitor of initiation of protein synthesis *in vitro* (Sonenshein and Brawerman, submitted for publication). Whether this is due to the lack of poly(A) or to some other feature of the mRNA is unknown.

The nature of polypeptide B remains to be determined. In preliminary experiments, cytoplasmic extracts of S-180 cells were found to contain large amounts of a polypeptide with the same mobility as the B band. This material could possibly represent actin, which is an abundant polypeptide present in a variety of vertebrate cells and which has a molecular weight of 45,000 (20–22). Further work will be required for the positive identification of our B component.

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