Proc. Nat. Acad. Sci. USA
Vol. 71, No. 7, pp. 2632–2636, July 1974

Nuclear Ribonucleoprotein Complexes Containing Polyadenylate from Mouse Ascites Cells

(nuclear proteins/heterogeneous nuclear RNA/Millipore filter binding)

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Communicated by Hewson Swift, April 22, 1974

ABSTRACT **Nuclear poly(A)-containing RNA of mouse ascites cells can be extracted in the form of 15–17S ribonucleoprotein complexes under conditions in which the bulk of the heterogeneous nuclear RNA is released as 30S complexes. The poly(A)-containing fraction of nuclear extracts has been resolved into two distinct components, 15 and 17 S; neither contains the two polypeptides of 30S ribonucleoprotein. The 17S particle contains approximately six polypeptide species of molecular masses 17,000–30,000 daltons. The 15S complex has four distinct polypeptides of higher molecular mass, including a prominent 80,000-dalton species.**

In higher eukaryotes the rapidly labeled nuclear RNA, characterized as DNA-like by its base composition and ease of hybridization, is transcribed as large sequences sedimenting heterogeneously in sucrose gradients (heterogeneous nuclear RNA). The bulk of these RNA sequences is rapidly degraded in the nucleus (1–6). Only a relatively small fraction of the rapidly labeled RNA is conserved, reaching the cytoplasm as mRNA. Considerable evidence suggests that mRNA is, in fact, transcribed as part of a much larger nuclear precursor which is cleaved to yield the true messenger (7–9). Some heterogeneous nuclear RNA and most mRNA molecules contain polynucleotide sequences rich in adenylic acid [poly(A)] at the 3′-OH termini (10–14). Poly(A) appears to be a characteristic sequence in all mRNA with the exception of histone mRNA (15, 16). The addition of poly(A) is most likely a post-transcriptional nuclear event (12, 17), occurring before or during the nucleolytic processing of the putative precursors into mRNA.

The DNA-like RNA of higher eukaryotes is found both in nuclear and cytoplasmic extracts in a form complexed with specific proteins (18). Ribonucleoprotein (RNP) particles containing heterogeneous nuclear RNA sedimenting homogeneously at about 30 S have been extracted from the isolated nuclei of many higher vertebrates (19–21). The protein composition of these 30 S RNP complexes is relatively simple, consisting of multiple copies of only a very few specific polypeptides (21, 22). The RNA from 30S complexes contains sequences that hybridize to the moderately repetitive DNA, and includes sequences not present in the cytoplasm (23, 24). Insofar as DNA-like RNA that is extracted from 30S complexes sediments at only 4–8 S (19, 25), it must be concluded that 30S complexes derive from much larger nuclear ribonucleoprotein complexes.

Our interest in the possible role of the 30S RNP complexes in the transport of mRNA from the nucleus to the cytoplasm has prompted us to look for poly(A)-containing RNA species in nuclear extracts that contain 30S RNP complexes. Preliminary reports have indicated that the 30S complex contains little if any poly(A), but that poly(A) sequences are found in a 15–20S fraction (21, 26). Experiments reported here confirm the preliminary results, and indicate that a RNP complex of about 15–17 S is associated with the bulk of poly(A)-containing RNA in our nuclear extracts. These RNP particles contain a more complex protein moiety than is associated with 30S RNP.

Abbreviation: RNP, ribonucleoprotein; $NaDodSO_4$, sodium dodecyl sulfate.

MATERIALS AND METHODS

Enzymes, Radioisotopes, Nucleic Acids, and Reagents. Bovine pancreas RNase (Preparation XIIA) and glycogen (Type III from rabbit liver) were obtained from Sigma; T1 RNase (B grade) was from Calbiochem. DNase I (DPFF) was purchased from Worthington. [*2,8*-^{3}H]Adenosine (30–60 Ci/mmol) was obtained from New England Nuclear Corp., and synthetic [^{3}H]poly(A) was from Miles. The [^{3}H]uridine pulse-labeled, whole-cell RNA and unlabeled cytoplasmic RNA from the Taper ascites cells were prepared as in Martin and McCarthy (24). Earle's balanced salt solution and minimum essential medium containing glutamine were from Gibco.

Isolation of Nuclei and Preparation of Nuclear Extracts. The nature and propagation of the Taper hepatoma ascites cell line have been described (24). All cell fractionation procedures were done at 0–4°. The isolation of nuclei and extraction with buffered salt solution were based on the method of Samarina *et al.* (19, 27) and have been described (21, 24). After brief washes in 0.1 M NaCl–10 mM Tris·HCl–1 mM $MgCl_2$ (pH 7) and the same buffer at pH 9, the isolated nuclei were shaken in a small volume of the buffer (pH 9) for about 4 hr to extract RNP complexes. The nuclei were then pelleted at low speed, and the nuclear extract (2.5 ml from 30–40 ml of ascites fluid) was layered onto a 35-ml 15–30% (w/v) sucrose gradient in NaCl–Tris·HCl–$MgCl_2$ (pH 8) and was centrifuged in a Beckman SW 27 rotor at 25,000 rpm for 15 hr at 4°. The gradient was then passed through an ISCO model D Gradient Fractionator with UA-2 Ultraviolet Analyzer. Fig. 1 illustrates a typical absorbance profile obtained from 30 to 40 ml of ascites fluid.

Cell-Labeling Procedures. For *in vivo* experiments, the radioisotope was injected into the peritoneal cavity of several tumor-bearing mice, and samples of ascites fluid were withdrawn at various times. Initial experiments indicated that an 80-min labeling period was convenient for studies using [^{3}H]-adenosine. For the *in vitro* experiment, ascites fluid was removed from tumor-bearing mice, and the cells were pelleted by low-speed centrifugation. The cells were washed twice

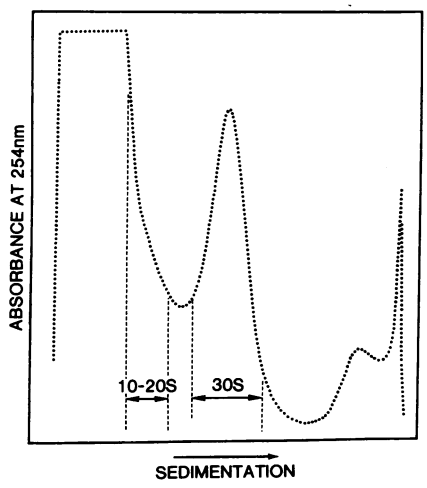

FIG. 1. Absorbance profile of a nuclear extract after centrifugation on a sucrose density gradient. About 2.5 ml of a nuclear extract (prepared as in *Materials and Methods*) was layered onto a 35-ml 15–30% (w/v) sucrose gradient in 0.1 M NaCl–10 mM Tris·HCl–1 mM $MgCl_2$, pH 8, and centrifuged in a Beckman SW 27 rotor at 24,000 rpm for 15 hr at 4°. The gradient was analyzed as in *Materials and Methods;* full scale was about 1 A_{254} unit. In routine preparations with unlabeled cells, 10–20S and 30S fractions (as indicated) were collected and stored at −20°.

with Earle's solution, and finally suspended in minimum essential medium in a volume about five times the ascites fluid volume. The cell suspension was shaken gently in a 37° water bath for 80 min after addition of [^{3}H]adenosine.

RNA Extraction and Nuclease Digestions. The phenol–sodium dodecyl sulfate procedure of Shearer and McCarthy (6) was used, but phenol was replaced with a phenol–chloroform (1:1) mixture (28). In addition, glycogen (50 μg/ml) was added as a carrier in the ethanol precipitations. Samples for DNase digestion were incubated at 37° for 40 min in 10 mM $MgCl_2$, 10 mM Tris·HCl (pH 7.8) containing 50 μg/ml of DNase I. RNase digestions were in 0.2 M NaCl, 10 mM Tris·HCl (pH 7.4) containing 2 μg/ml of bovine pancreas RNase and 8 units/ml of T1 RNase at 37° for 40 min. [These conditions, based on the methods of Philipson *et al.* (29), were slightly modified in some experiments.]

Millipore Assay of Protein-Associated RNA and Poly(A)-Containing RNA. The Millipore filter-binding assay of Lee *et al.* (14) was used to determine the amount of poly(A)-containing RNA in a given sample. Small volumes of either a [^{3}H]poly(A) solution or a [^{3}H]uridine pulse-labeled, whole-cell RNA were mixed with 1.0 ml of KCl buffer (10 mM Tris·HCl, pH 7.6; various KCl concentrations; 1 mM $MgCl_2$). The mixture was immediately passed through HAWP Millipore filters (0.45-μm size) which were then washed with about 30 ml of the buffer, dried, and counted in toluene–Permafluor scintillation fluid. In some cases 0.2 ml of an unlabeled 30S RNP fraction from preparative sucrose gradients was mixed with the buffer and RNA before the sample was filtered. The binding of pulse-labeled RNA to 30S RNP has been described (21, 30). The input radioactivity (greater than 20,000 cpm in all cases) was assayed by counting in scintillation fluid containing 1 volume of Triton X-100: 2 volumes of toluene–Permafluor. The maximum percentages of input radioactivity recovered on Millipore filters were: [^{3}H]poly(A) with 30S particles, 96% at 100 mM KCl; [^{3}H]poly(A) without 30S particles, 95% at 700 mM KCl; [^{3}H]uridine-labeled RNA with 30S particles, 19% at 50 mM KCl. The binding of [^{3}H]-uridine-labeled RNA without 30S particles was negligible.

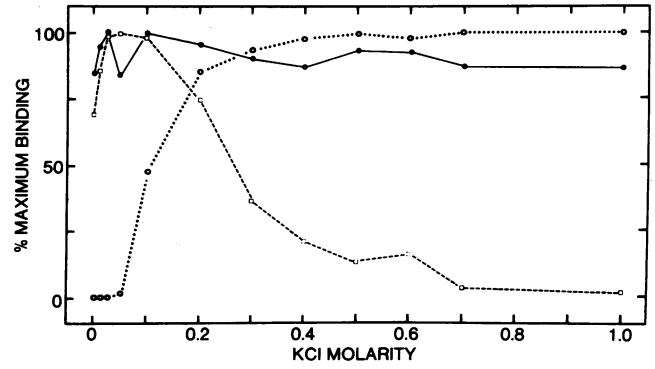

FIG. 2. Binding of radioactive RNA to Millipore filters as a function of KCl concentration. Small volumes of either [^{3}H]-poly(A) solution or [^{3}H]uridine pulse-labeled, whole-cell RNA (with or without unlabeled 30S material) were mixed with KCl buffer and passed through Millipore filters (see *Materials and Methods*). [^{3}H]Poly(A) with 30S particles (●——●); [^{3}H]poly(A) without 30S particles (O····O); [^{3}H]uridine RNA with 30S particles (□– – –□).

Poly(A) without exogenous 30S particles bound maximally at about 700 mM KCl but the binding was very low at concentrations below 50 mM, whereas in the presence of particles the binding was quite high across a large range of KCl concentrations (Fig. 2). The uridine-labeled RNA alone did not bind significantly, but added 30S RNP did cause binding at low KCl concentrations to about 20% of the input radioactivity in this experiment; the binding was reduced by increasing KCl concentrations even in the presence of the 30S fraction. On the basis of this experiment, 20 mM KCl and 700 mM KCl were chosen as the salt concentrations for this assay; at 20 mM KCl, protein-associated RNA was bound to the Millipore filters, whereas at 700 mM KCl, only poly(A)-containing RNA was bound.

While this simple assay enabled the rapid detection of protein-associated RNA and poly(A)-containing RNA in nuclear extracts, the localization of poly(A)-containing RNA was verified by Millipore filter binding at 700 mM KCl with RNA purified with phenol–chloroform and the nuclease digestion techniques described above.

Polyacrylamide Slab-Gel Electrophoresis. Protein samples were electrophoresed on 10% acrylamide slab gels containing $NaDodSO_4$ (21, 31), then fixed and stained with Coomassie brilliant blue. Densitometric tracings of the gel were made on a Joyce-Lobel Microdensitometer.

RESULTS

Our first experiment was designed to assay for the possible presence of poly(A)-containing RNA in 30S RNP complexes, and more generally, for its distribution in the nuclear extract (Fig. 3). Two major peaks of protein-associated, filter-binding radioactivity are resolved at low salt: one at about 15–20 S, and the other at 30 S (absorbance profile not shown).

Under conditions where we would expect poly(A)-associated RNA to bind (700 mM KCl), we find a distinct change in the radioactivity profile. All fractions have lost some filter-binding ability, but the loss is most noticeable in the 30S

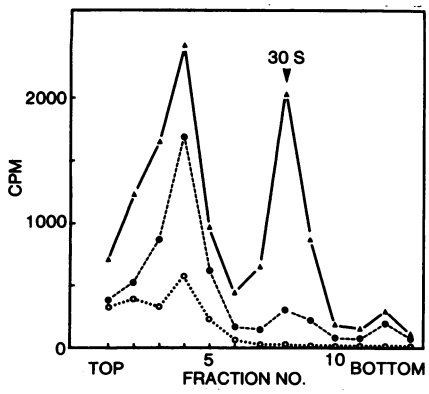

FIG. 3. Distribution of adenosine-labeled RNA in a nuclear extract. Three mice were each injected with 0.5 mCi of [2,8-^{3}H]-adenosine, and 2 ml of ascites fluid was removed from each mouse after 80 min. These samples were pooled and a nuclear extract was prepared and centrifuged as in *Materials and Methods;* 3-ml fractions were obtained from the sucrose gradient. 0.5 ml of each fraction was mixed with 20 mM KCl buffer (▲——▲) or 700 mM KCl buffer (●- - -●) before Millipore filtration. 0.5 ml of each fraction was digested with DNase and RNase, then diluted and filtered with 700 mM KCl buffer (O····O).

region. It is evident that the majority of any possible poly(A) must be located in the 15–20S fraction. In addition, no new peaks of radioactivity have been resolved, suggesting that the poly(A) exists in a protein-associated complex. [Poly(A) not complexed with proteins binds at 700 mM KCl, but not at

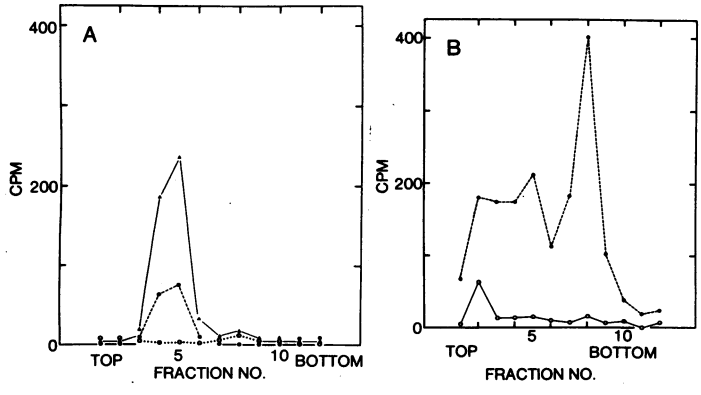

FIG. 4. Distribution of adenosine-labeled RNA in fractions, extracted with phenol–chloroform–NaDodSO$_4$, from a sucrose density gradient. Two tumor-bearing mice were each injected with about 400 μCi of [2,8-^{3}H]adenosine. After 80 min, the mice were killed and 13.7 ml of ascites fluid was recovered. A nuclear extract was prepared and centrifuged on a sucrose density gradient as in *Materials and Methods,* and twelve 3-ml fractions were obtained. RNA was extracted by the phenol–chloroform–NaDodSO$_4$ method and treated with DNase. The resulting purified RNA from each fraction was dissolved in 2 ml of water. (*A*) 0.2 ml of each sample was mixed with 20 mM KCl buffer (O——O) or 700 mM KCl buffer (▲- - -▲) before filtration. 0.2 ml of each sample was digested in RNase and then diluted and filtered with 700 mM KCl buffer (●···●). (*B*) Trichloroacetic acid-precipitable RNA after incubation in 0.3 M NaOH (O——O) or pH 7 Tris buffer (●- - -●) for 60 min at 37°. Samples were precipitated with 5% cold trichloroacetic acid and bovine-serum albumin carrier, and passed through Whatman GF/C filters, which were washed with about 30 ml of cold 5% trichloroacetic acid, dried, and counted in toluene–Permafluor scintillation fluid.

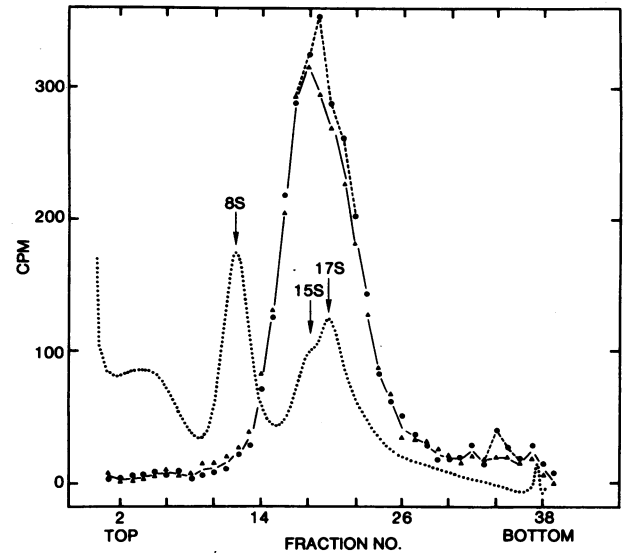

FIG. 5. Location of poly(A)-containing RNP complexes in the 10–20S fraction of nuclear extracts. Twenty ml of ascites fluid was labeled *in vitro* with [2,8-^{3}H]adenosine for 80 min, and the 10–20S region (about 5 ml) of this nuclear extract was identified by low- and high-salt filter-binding analysis, as in Fig. 3. This material was mixed with 100 ml of unlabeled 10–20S material from routine preparations (see *Materials and Methods*), and the mixture was then centrifuged at 39,000 rpm, 4°, for 18 hr, in a Beckman type 40 rotor. The pellets were resuspended in 2.5 ml 0.1 M NaCl–10 mM Tris·HCl–1 mM $MgCl_2$, pH 7, and large particulate matter was removed by a 10-min centrifugation at 10,000 rpm in a Sorvall SS-34 rotor. The supernatant was layered onto a 35-ml 15–30% (w/v) sucrose gradient in the buffer at pH 8, and centrifuged at 27,000 rpm, 4°, in a Beckman SW 27 rotor for 23 hr. Cytoplasmic RNA was centrifuged on another 35-ml 15–30% (w/v) sucrose gradient in 10 mM Tris·HCl (pH 7) in the same rotor to provide an absorbance marker at 18 S. The 10–20S gradient was fractionated into 1.0-ml samples. A_{254} (*dotted line*); 0.5 ml of each sample mixed with 20 mM KCl buffer (▲——▲) or 700 mM KCl guffer (●- - -●) and filtered.

20 mM KCl.] After incubation of the fractions with DNase and RNases, which will digest nucleic acids other than poly(A), the 30S region and the bottom of the gradient contain negligible filter-binding radioactivity at 700 mM KCl, but the 15–20S fraction retains a significant amount of filter-binding radioactivity.

To eliminate possible artifacts due to the presence of proteins not removed at 700 mM KCl, we have also examined the filter-binding properties of deproteinized RNA prepared from sucrose gradient fractions (Fig. 4*A*). The filter-binding of purified RNA at 20 mM KCl is extremely low across the entire gradient. The filter-binding at 700 mM KCl shows only one significant peak of radioactivity, at about 15–20 S. The RNase-resistant filter-binding again indicates a single peak of radioactivity at about 15–20 S, with essentially background filter-binding in other regions of the gradient. To more rigorously exclude the possibility that the filter-bound radioactivity is in DNA rather than poly(A), we have determined the trichloroacetic acid-precipitable radioactivity before and after alkaline hydrolysis in 0.3 M NaOH (Fig. 4*B*). Fractions 3–12 have essentially background radioactivity after hydrolysis; this observation suggests that the filter-binding peak at 700 mM KCl in Fig. 4*A* is indeed RNA. A possible DNA contaminant may give rise to the peak of alkali-resistant radioactivity in fraction 2.

In order to further characterize the poly(A)-containing fractions, adenosine-labeled 15–20S material was sedimented with unlabeled 10–20S material from routine preparations, resuspended, and centrifuged on a sucrose gradient; another tube containing ribosomal RNA provided an 18S marker (Fig. 5). The previously unresolved 10–20S region of Fig. 1 is now shown to contain a number of particulate components. We repeatedly observe two major particle fractions (8 S and 17 S) in addition to the soluble material at the top of the gradient; the 17S peak has always been observed to have a 15S shoulder associated with it, and we have occasionally been able to resolve this as a separate component (see below). The filter-binding radioactivity is localized in the 15–17S region; all other fractions have essentially background radioactivity. In the 15–17S region most of the radioactivity binding at 20 mM KCl also binds at 700 mM KCl, suggesting that most of the RNA molecules contain poly(A) sequences.

Our previous studies had characterized the protein composition of 30S RNP, and we have now sought to determine the nature of the proteins associated with the poly(A)-containing RNA in nuclear extracts. In preparing fractions for analysis of the proteins we have been able to resolve the 8S, 15S, and 17S particles (Fig. 6).

It is apparent from densitometric tracings of the $NaDodSO_4$ gels that distinct proteins are associated with each of the three fractions recovered by recentrifugation of the crude 10–20S fraction (Fig. 7). Polypeptides similar in size to the two proteins of 30S RNP are recovered, together with numerous high molecular mass species in the 8S fraction; however, they are absent from both the 15 and 17S fractions. At least six polypeptides in the molecular mass range 17,000–30,000 daltons are recovered in both the 15 and 17S fractions. The 15S fraction, however, contains four to six additional protein components of molecular mass greater than 55,000 daltons not found in the 17S region. The major band of these high molecular mass proteins associated with the 15S fraction has a $NaDodSO_4$ mobility corresponding to a molecular mass of about 80,000 daltons. Since the 15S and 17S particles have not been completely separated, the proportion of low molecular mass polypeptides in the 15S complex remains uncertain.

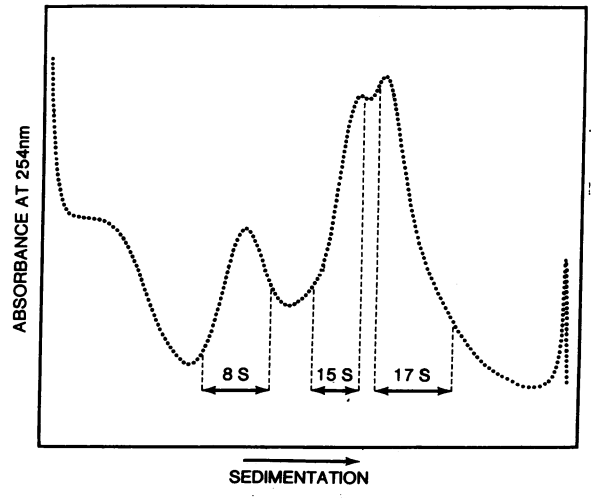

FIG. 6. Separation of the components of the 10–20S material on a sucrose gradient. Pooled fractions (100 ml) from the 10–20S region of gradients used in the initial isolation of RNP complexes (Fig. 1) were centrifuged 22 hr at 37,000 rpm (40 rotor). Pellets were resuspended in 0.1 M NaCl–10 mM Tris·HCl–1 mM $MgCl_2$, pH 8 (as in Fig. 5), and centrifuged on a 15–30% sucrose gradient in the same buffer, pH 8 (27,000 rpm for 24 hr in the SW 27 rotor). Fractions indicated by the *horizontal bars* were again pelleted (49,000 rpm, 17 hr in the SW 50.1 rotor), and the pellets were dissociated in $NaDodSO_4$ buffer.

DISCUSSION

The heterogeneous nuclear RNA of many higher vertebrates can be extracted in the form of 30S RNP particles, which are presumed to represent subunits of larger nuclear complexes (20, 21). The protein composition of the 30S RNP is simple. We find only two distinct polypeptide species of molecular mass 37,500 and 40,000 daltons in purified particles; these values are slightly higher than we had previously reported (21). The RNA of the 30S RNP is both rapidly synthesized and degraded, and in addition contains sequences that are not found in the cytoplasm. Such evidence as exists at present suggests that 30S RNP are not associated with mRNA on polyribosomes (32). In view of these observations, it is significant that the poly(A)-containing RNA of the nucleus is extracted in the form of a complex of distinctly different properties from the 30S RNP. It is unlikely, therefore, that the 30S RNP components are directly involved in the transport of mRNA from nucleus to cytoplasm. While it is certainly possible that the 15–17S poly(A)-containing RNP may be derived from large nuclear complexes that also contain the 30S RNP subunits (26), the results reported here imply that a distinct set of proteins becomes associated with RNA sequences which will be conserved for transport to the cytoplasm as mRNA. Our present studies, which indicate that the poly(A) sequence contained in 15–17S nuclear RNP is similar in size and base composition (Kinniburgh, Quinlan, and Martin, unpublished results) to that contained in cytoplasmic mRNA, support but of course do not prove this hypothesis. Since the intact cytoplasmic poly(A)-containing RNA molecules are larger than the RNA species isolated from

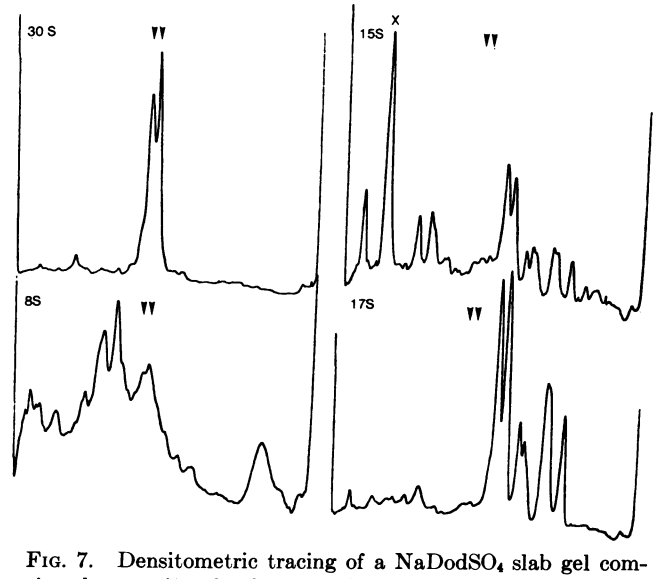

FIG. 7. Densitometric tracing of a $NaDodSO_4$ slab gel comparing the proteins of 30S nuclear RNP complexes with those of the 8S, 15S, and 17S fractions recovered from the sucrose gradient in Fig. 6. Electrophoresis was performed as in *Materials and Methods*. *Reference arrows* indicate the mobilities of the two polypeptides of 30S RNP. The major polypeptide band (*X*) of the 15S component has an apparent molecular mass of 80,000 daltons. Direction of migration is *left* to *right*.

15 to 17S complexes, we would have to assume that these RNP are subunits that contain at least the poly(A) sequence and perhaps the 3'-OH end of mRNA.

Given these considerations, it is relevant to mention the nature of proteins that have been reported to be associated with mRNA and poly(A) derived from cytoplasmic polyribosomes. Scherrer and coworkers have reported finding two main protein bands of molecular mass 49,000 and 73,000 daltons, and six minor bands of molecular mass greater than 60,000 daltons associated with duck globin mRNP prepared by EDTA treatment of polysomes (33). These authors argue that the proteins of globin mRNP are different from those associated with its nuclear precursor (33). In similar studies, Blobel has found proteins of molecular mass 78,000 and 52,000 daltons in rabbit globin mRNP prepared by puromycin–salt dissociation of reticulocyte polysomes (34). Proteins of identical mobilities are common to polysomal mRNP from mouse L cells and rat hepatocytes (35), which possess more diverse populations of mRNA than do reticulocytes. The 78,000-dalton protein appears to be tightly associated with the RNase-resistant polyadenylate-rich region of polysomal mRNP (35). Our discovery of a protein of similar molecular mass in a 15S nuclear poly(A)-containing ribonucleoprotein particle suggests that this protein is bound in the nucleus and remains bound during transport and translation of the mRNA. That the poly(A) sequence of mRNA exists in a conserved RNP complex is indicated by the release of a 12–15S poly(A)-containing particle by RNase treatment of mouse ascites polyribosomes (36). More rigorous comparisons of nuclear and cytoplasmic RNP proteins will be required to substantiate or refute this hypothesis.

There is at present no indication of the nature of the association between the RNA and the other proteins in nuclear 15–17S RNP complexes. Although more distinct species of polypeptides seem to be present in these complexes than in the 30S RNP, which are presumed to contain mainly heterogeneous nuclear RNA sequences, the number still seems to be small (six to ten), implying a general role for these proteins rather than a mRNA-specific function. Whether certain of the proteins are directly associated with true mRNA sequence in the complex while others are associated only with the poly(A), for example, the species of about 80,000 daltons, remains to be determined, as does the functional relationship, if any, between the 15S and 17S complexes.

Finally, some comment should be made on the 8S particles in our nuclear extracts. We do not have any evidence that this fraction contains any nucleic acid; however, it does appear to have a relatively complex polypeptide composition, which includes species that may be related to the 30S RNP proteins. The finding that the 8S complex contains rapidly phosphorylated proteins (Billings and Martin, unpublished results) warrants further studies on this fraction.

We acknowledge the excellent technical assistance of Mrs. Ljerka Urbas. This work was supported by the National Institutes of Health (Grant CA 12550). T.J.Q. is a trainee in the Department of Biophysics and Theoretical Biology, and is supported by USPHS Training Grant GM-780. P.B.B. is a trainee in the Department of Biology and is supported by USPHS Training Grant HD-00174.

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