JOURNAL OF BACTERIOLOGY, July 1977, p. 240-246
Vol. 131, No. 1
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# Messenger Ribonucleoprotein Complexes Isolated by Oligodeoxythymidylate-Cellulose Chromatography from *Neurospora crassa* Polysomes

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Received for publication 9 February 1977

Messenger ribonucleoprotein (mRNP) complexes were isolated from ethylenediaminetetraacetic acid-dissociated polysomes of *Neurospora crassa*. Approximately 15% of the [$^3$H]uridine incorporated into polysomal ribonucleic acid (RNA) during a 15-min pulse was eluted from oligodeoxythymidylate-cellulose as an mRNP complex. The isolated mRNP complexes exhibited sedimentation coefficients ranging from 15*S* to greater than 60*S*. RNA isolated from these mRNP complexes sedimented in sucrose gradients between 4*S* and 40*S*, with broad peaks at 15*S* and 24*S*. The buoyant density of mRNP complexes eluted with 25% formamide was 1.42 to 1.44 g/cm$^3$, whereas for mRNP complexes eluted with 50% formamide it was 1.48 to 1.50 g/cm$^3$. Six polypeptides, with molecular weights of 14,000, 19,000, 24,000, 31,000, 44,000, and 66,000, were associated with mRNP complexes eluted with 25% formamide. The mRNP complexes eluted with 50% formamide had one associated polypeptide, of molecular weight 27,000.

An ever-increasing volume of information is accumulating which indicates that messenger ribonucleic acid (mRNA) is always associated with proteins in the form of messenger ribonucleoprotein (mRNP) complexes (7). The association of specific proteins with mRNA begins in the nucleus during transcription (11) and continues during the transport of mRNA to the ribosomes and subsequent recruitment into polysomes (16, 19, 22). In fact, specific proteins associate with mRNA during translation since mRNA is released as an mRNP complex from ethylenediaminetetraacetic acid (EDTA)- or puromycin-dissociated polysomes (3, 8, 10).

The size of the mRNA-associated proteins varies from 23,000 daltons in rat brain (13) to 160,000 daltons in rat liver (17). Several recent reports indicate that two proteins with molecular weights between 49,000 and 50,000 and between 68,000 and 78,000 predominate (2, 3, 6).

The exact function of the proteins in the mRNP complex is unknown. They may function in providing protection against ribonuclease attack (24, 25), in facilitating mRNA transport from nucleus to cytoplasm (20, 21), or as a key element in translational control mechanisms (22).

The present study was initiated to determine the characteristics of mRNP complexes released from EDTA-dissociated polysomes of *Neurospora crassa*. This information will form the basis for further studies concerning the regulation of protein synthesis during the initial stages of spore germination.

## MATERIALS AND METHODS

**Polysome isolation.** *N. crassa* wild-type strain 74-OR23-1A was used in all experiments. Conditions of harvest and culture were those outlined previously (15). Conidia were germinated for 3 h in Vogel minimal medium at 32°C. During the last 15 min the conidia were labeled with [$^3$H]uridine ([5-$^3$H]uridine; specific activity, 20 Ci/mmol) to a final concentration of 1 $\mu$Ci/ml. A post-mitochondrial supernatant was prepared and layered onto a sucrose step gradient consisting of 3 ml of 2 M sucrose, 1 ml of 1.5 M sucrose, and 1 ml of 0.5 M sucrose (0.03 M triethanolamine [TEA] [pH 7.5], 0.01 M KCl, 0.01 M $MgCl_2$). The polysomes were pelleted by centrifugation for 3 h at 45,000 rpm in a type 65 rotor (Beckman). The supernatant was discarded, and the pellet was resuspended in 2 to 4 ml of dissociation buffer (0.05 M TEA [pH 7.5], 0.01 M EDTA).

**Isolation of mRNP complexes by oligo(dT)-cellulose chromatography.** The NaCl concentration of the dissociated polysomes was adjusted to 0.25 M, and the mixture was chromatographed on oligodeoxythymidylate [oligo(dT)]-cellulose (Collaborative Research, Inc., Waltham, Mass.) according to the procedures of Lindberg and Sundquist (10). The column was initially washed with 20 ml of elution buffer (0.05 M TEA [pH 7.5], 0.25 M NaCl), followed by successive 10-ml washes of elution buffer containing 25% formamide and 50% formamide (purified according to the procedure of Tibbets et al. [26]). One-milliliter fractions were collected, and the radioactivity was determined by counting a 50-$\mu$l por-

tion. Peak fractions eluted with formamide were pooled and dialyzed against buffer (0.03 M TEA [pH 7.5], 0.01 M KCl, 0.01 M $MgCl_2$) to remove formamide and reduce the salt concentration.

**Sucrose density gradient analysis of mRNP complexes.** The 25 and 50% formamide eluates were pooled, dialyzed, layered over 1 ml of 2 M sucrose, and centrifuged for 20 h at 35,000 rpm at 5°C in a type 65 rotor. The supernatant above the cushion was discarded, and the cushion was diluted with 5 ml of buffer (0.01 M TEA [pH 7.5], 0.25 M NaCl, 0.01 M EDTA). Two milliliters of this material was layered on a 36-ml 15 to 30% (wt/wt) sucrose gradient (0.01 M TEA [pH 7.5], 0.25 M NaCl, 0.01 M EDTA) and centrifuged at 25,000 rpm for 15 h at 5°C in the SW27 rotor. Gradients were monitored continuously at 254 nm, and 1.5-ml fractions were collected. Bovine serum albumin was added to each fraction to a final concentration of 100 μg/ml, and trichloroacetic acid was added to a final concentration of 10%. After 1 to 2 h at 5°C, precipitates were collected on GF/C filters, which were counted in an Omnifluor scintillation cocktail.

**Sucrose density gradient analysis of RNA from mRNP complexes.** The 25 and 50% formamide eluates were pooled and dialyzed, and the mRNP complexes were precipitated by adding 100% ethanol to a final concentration of 70%. The precipitate was pelleted by centrifugation and resuspended in buffer (0.01 M TEA [pH 7.5], 0.001 M EDTA, 0.01 M LiCl, 0.5% sodium dodecyl sulfate [SDS]). An equal volume of chloroform-phenol-isoamyl alcohol (48:48:4) saturated with buffer (0.01 M sodium acetate [pH 6.0], 0.10 M LiCl, 0.001 M EDTA) was added. The mixture was vortexed for 10 min at room temperature and then centrifuged for 15 min at 12,000 rpm. The aqueous phase was decanted and extracted a second time. After the second extraction, no interphase was observed. The aqueous phase was adjusted to 0.3 M LiCl, and the RNA was precipitated with ethanol. The RNA was precipitated overnight at −20°C, resuspended in buffer, and reprecipitated. A portion of the purified RNA was layered on a 5 to 20% (wt/wt) sucrose-SDS gradient (0.01 M TEA [pH 7.5], 0.01 M LiCl, 0.001 M EDTA, 0.5% SDS) and centrifuged at 25,000 rpm for 16 h at 20°C in the SW41 rotor. Gradients were analyzed as described previously.

The polyadenylic acid [poly(A)] content of the high-salt wash and formamide-eluted RNA was assayed by the method of Sheldon et al. (23) as described previously (14).

**Isopycnic centrifugation of mRNP complexes.** Dialyzed mRNP fractions were fixed with formaldehyde (6% final concentration) neutralized with 1 M $NaHCO_3$ just before use. Portions of 1 ml were adjusted to a density of 1.3 g/cm³ and layered on top of a preformed step gradient consisting of 1-ml blocks of CsCl solutions at densities of 1.4 to 1.7 g/cm³. Gradients were centrifuged for 24 h at 45,000 rpm at 20°C in the SW65 rotor. Eight-drop fractions were collected, and the density of every fifth fraction was determined by refractometry. To each fraction, 0.2 ml of distilled water and 2 ml of Aquasol were added. Samples were analyzed in a Packard Tri-Carb liquid scintillation counter.

**SDS-polyacrylamide gel electrophoresis.** The 25 and 50% mRNP eluates were separately precipitated with trichloroacetic acid (10% final concentration). The precipitates were pelleted and washed successively with 100% ethanol, ethanol-ether (1:1), and ether. The air-dried precipitates were resuspended in buffer (0.1 M $PO_4$ [pH 7.1], 1% SDS, 1% 2-mercaptoethanol) and boiled for 1 to 2 min. Samples were applied to a 6% polyacrylamide slab gel (12 by 15 cm) and electrophoresed for 4 h at 15°C and 200-mA constant current. Bovine serum albumin, alcohol dehydrogenase, and cytochrome *c* were electrophoresed in parallel. Molecular weights were calculated by the method of Weber et al. (27).

## RESULTS

**Isolation of mRNP complexes.** The procedure of Lindberg and Sundquist (10) was used to isolate mRNP complexes from EDTA-dissociated polysomes of *N. crassa*. A typical elution profile is shown in Fig. 1. Approximately 85% of the [³H]uridine incorporated into polysomal RNA during a 15-min pulse did not bind to oligo(dT)-cellulose under the salt conditions used (high-salt wash fraction). The remaining 15% was sequentially eluted with high-salt buffers containing 25 and 50% formamide. Approximately 40% of the bound material could be eluted with buffer containing 25% formamide, whereas 55% was eluted with buffer containing 50% formamide. A small amount of bound material was eluted with 90% formamide and another fraction that could only be released by alkaline digestion. The nature of these latter two eluates was not investigated further, and in all experiments reported here elution was carried out with 25 and 50% formamide solutions.

**Sucrose density gradient analysis of mRNP complexes.** To determine the size of the mRNP complexes, portions of 25 and 50% formamide eluates were pooled and analyzed on 15 to 30% sucrose-SDS gradients. The distribution of labeled mRNP complexes is shown in Fig. 2a. Labeled mRNP complexes were found heterogeneously distributed in the gradient, exhibiting sedimentation coefficients from approximately 15*S* to greater than 60*S*. In contrast, the sedimentation profile of the high-salt wash material is shown in Fig. 2b. The major fraction of radioactivity cosedimented with the 40*S* and 60*S* ribosomal subunits, although a significant fraction sedimented between the top of the gradient and the small ribosomal subunit. Based upon these sedimentation characteristics, the high-salt wash fraction contained the ribosomal subunits generated by EDTA dissociation of

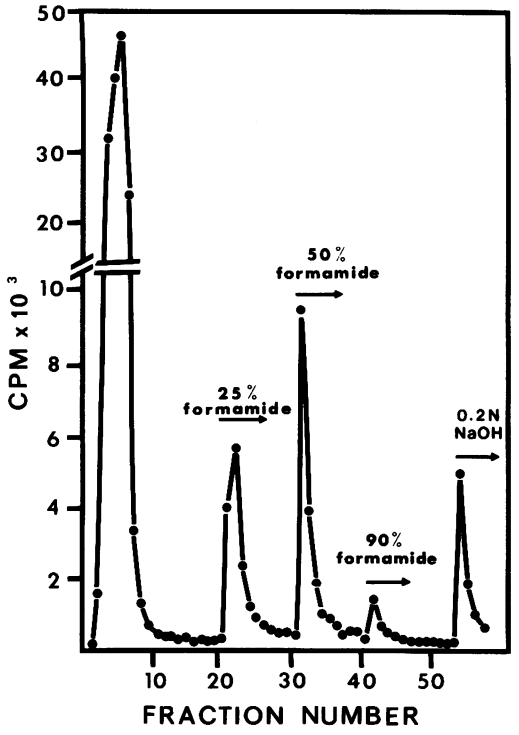


FIG. 1. *Fractionation of EDTA-dissociated polysomes on oligo(dT)-cellulose. Polysomes were isolated from 3-h germinated conidia and dissociated with EDTA. Dissociated polysomes were chromatographed on oligo(dT)-cellulose (T2). Elution was affected by successive washes of elution buffer (25 ml), elution buffer containing 25% formamide (10 ml), elution buffer containing 50% formamide (10 ml), elution buffer containing 90% formamide (10 ml), and finally 0.2 N NaOH (10 ml). One-milliliter fractions were collected, and 50 μl of each fraction was counted in Aquasol.*

polysomes and some nonribosomal complexes. The latter probably represented mRNP complexes that failed to bind to oligo(dT)-cellulose under the conditions used.

**Characterization of RNA from mRNP complexes.** Pulse-labeled poly(A)-containing RNA isolated from *Neurospora* polysomes exhibited the heterogeneous profile seen in Fig. 3a. The profile of RNA extracted from mRNP complexes eluted by formamide is shown in Fig. 3b. The two profiles are quite similar, although the peak of radioactivity at approximately 24*S* associated with the mRNP RNA is not seen in the profile of poly(A)-containing mRNA isolated from polysomes. Although not coincident with the 25*S* ribosomal marker, the radioactivity sedimenting at 24*S* may indicate contamination of the mRNP fraction with large ribosomal subunits. The RNA of the high-salt wash fraction was also analyzed (Fig. 3c). In this fraction, the dominant species of labeled RNA were the 25*S* and 17*S* ribosomal RNAs. Some labeled material did sediment between 4*S* and 17*S* and may reflect mRNA molecules that did not bind to the column. RNA from the high-salt fraction and from the formamide eluates was assayed for poly(A) content by binding to polyuridylic acid [poly(U)] filters (Table 1). Less than 1% of the radioactivity in the high-salt wash fraction bound to poly(U) filters, whereas approximately 20% of the radioactivity in the formamide eluates did.

**Isopycnic centrifugation of mRNP complexes.** Since mRNP complexes were differentially eluted by increasing formamide concentrations, the 25 and 50% formamide eluates were separately analyzed by CsCl gradient centrifugation in order to compare the two mRNP populations. The mRNP complexes eluted by 25% formamide banded at a peak density of 1.42

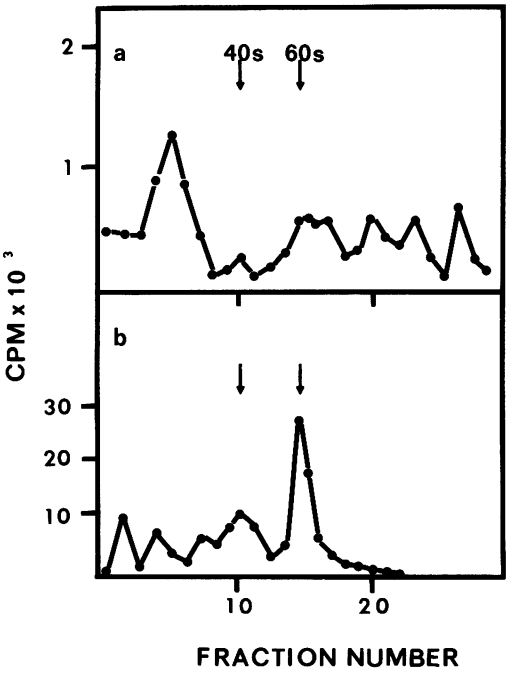


FIG. 2. *Size distribution of RNP complexes isolated from oligo(dT)-cellulose. Fractions 22 to 25 and 31 to 34 were pooled, dialyzed, layered over 1 ml of 2 M sucrose, and centrifuged for 20 h at 35,000 rpm at 5°C in a type 65 rotor. The supernatant above the cushion was discarded and the cushion was diluted with 5 ml of buffer. Two milliliters was layered on a 36-ml 15 to 30% (wt/wt) sucrose gradient and centrifuged at 25,000 rpm for 15 h at 5°C in an SW27 rotor. Fractions of 1.5 ml were collected and precipitated with trichloroacetic acid (a). Fractions 3 to 8 were treated in an identical manner (b).*

to 1.44 g/cm³ (Fig. 4a). Using the formula of Perry and Kelley (19), a density of 1.42 to 1.44 g/cm³ corresponds to an RNP complex composed of approximately 20% RNA and 80% protein, calculated with the values of 1.87 and 1.35 g/cm³ for the densities of RNA and protein, respectively (Irwin et al. [8]). The mRNP complexes eluted with 50% formamide banded at a peak density of 1.48 to 1.50 g/cm³, suggesting a complex composed of 35% RNA and 65% protein (Fig. 4b). Although these particle compositions are only approximations (12), they do indicate that the two formamide eluates contain mRNP complexes that differ in the RNA-protein ratios. Furthermore, the mRNP complexes eluted with formamide were distinct from ribosomes (subunits), which banded at a peak density of 1.58 g/cm³ (data not shown), indicating a composition of 50% RNA and 50% protein.

**Polypeptides associated with high-salt wash and formamide eluates.** The polypeptides associated with the formamide-eluted mRNP complexes and the high-salt wash fraction were characterized by SDS-polyacrylamide gel electrophoresis, and densitometric scans of the gels are shown in Fig. 5. The 25% formamide fraction yielded a gel profile containing three major polypeptides, with apparent molecular weights of 19,000, 24,000, and 31,000, and three minor components, with molecular weights of 14,000, 44,000, and 66,000 (Fig. 5a). The 50% formamide fraction contained only one detectable polypeptide, with an apparent molecular weight of 27,000 (Fig. 5b). In contrast, the profile of polypeptides present in the high-salt wash (Fig. 5c) is complex and typical of gel profiles obtained using purified ribosomes (data not shown). A comparison of the three profiles indicates that only the 24,000-dalton polypeptide of the 25% formamide fraction co-migrates with a ribosomal protein.

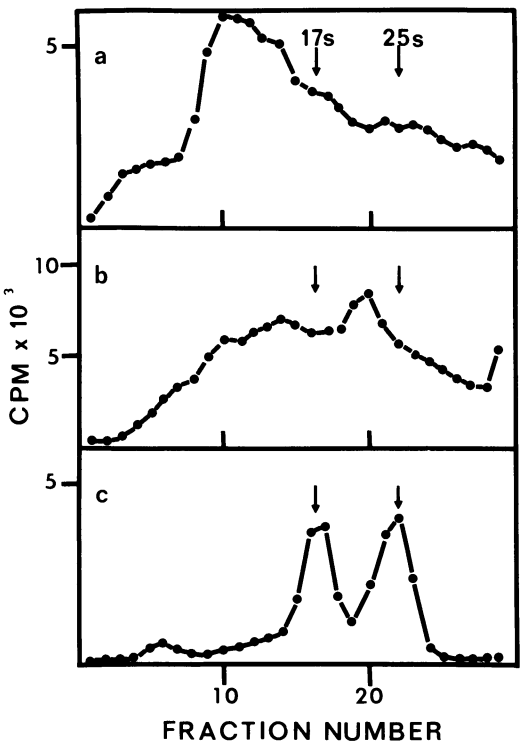


FIG. 3. *Size distribution of RNA isolated from RNP complexes. (a) Polysomes were collected from 10 to 40% (wt/wt) sucrose gradients, and the RNA was extracted with phenol-chloroform-isoamyl alcohol. Purified polysomal RNA was chromatographed on oligo(dT)-cellulose, and the poly(A)-containing RNA was layered on a 5 to 20% (wt/wt) sucrose gradient and centrifuged at 25,000 rpm for 16 h at 20°C in the SW41 rotor. (b) The 25 and 50% formamide eluates were pooled and dialyzed, and the mRNP complexes were precipitated with ethanol. The dried precipitate was resuspended in buffer and extracted with phenol-chloroform-isoamyl alcohol. The purified RNA was layered on a 5 to 20% (wt/wt) sucrose gradient and centrifuged as above. (c) The high-salt wash fraction was dialyzed and precipitated with ethanol. RNA was extracted and analyzed as described above.*

## DISCUSSION

The results presented constitute an initial characterization of mRNP complexes derived from EDTA-dissociated polysomes of the fungus *N. crassa*. The use of oligo(dT)-cellulose chromatography to isolate mRNP complexes from KB cells was introduced by Lindberg and Sundquist (10), and the procedure has now been used to isolate mRNP complexes from mouse kidney cells (8), HeLa cells (9), duck and rabbit erythrocytes (6), and silkmoth oocytes (18). Presumably the retention of mRNP complexes on oligo(dT)-cellulose involves A-T hybridization between the poly(A) region of the mRNA and the oligo(dT) chains covalently linked to the cellulose. However, the requirement of formamide to affect elution suggests that the protein moieties associated with the mRNA add stability to the hybridization process (10).

Although limited evidence is available, the differential elution of mRNP complexes with increasing concentrations of formamide seems, in part, to be related to the mRNP-associated proteins (8, 10). The 50% formamide-eluted mRNP complexes in *Neurospora* contain only one polypeptide species, yet they bind more tenaciously to the column than do the 25% formamide-eluted complexes, which contain six polypeptide species. This suggests that the

TABLE 1. *Poly(A) content of RNP complexes isolated by oligo(dT)-cellulose chromatography*[a]

| Sample | Vol (ml) | Filter | Treatment | cpm | % Binding | $\bar{X}$ |
|---|---|---|---|---|---|---|
| High-salt wash | 0.1 | GF/C | Spotted and dried | 152,801 | | |
| | 0.1 | GF/C + poly(U) | Spotted and dried | 145,631 | | |
| | 0.1 | GF/C + poly(U) | Poly(U) binding assay | 970 | 0.65 | |
| | 0.1 | GF/C + poly(U) | Poly(U) binding assay | 622 | 0.42 | 0.53 |
| | 0.2 | GF/C + poly(U) | Spotted and dried | 278,637 | | |
| | 0.2 | GF/C + poly(U) | Poly(U) binding assay | 1,645 | 0.59 | 0.54 |
| | 0.2 | GF/C + poly(U) | Poly(U) binding assay | 1,406 | 0.50 | |
| Formamide eluate | 0.2 | GF/C | Spotted and dried | 43,174 | | |
| | 0.2 | GF/C + poly(U) | Spotted and dried | 43,634 | | |
| | 0.2 | GF/C + poly(U) | Poly(U) binding assay | 10,830 | 24.95 | 19.68 |
| | 0.2 | GF/C + poly(U) | Poly(U) binding assay | 6,257 | 14.41 | |
| | 0.4 | GF/C + poly(U) | Spotted and dried | 82,832 | | |
| | 0.4 | GF/C + poly(U) | Poly(U) binding assay | 14,862 | 17.94 | 18.06 |
| | 0.4 | GF/C + poly(U) | Poly(U) binding assay | 15,070 | 18.19 | |

[a] Purified RNA was resuspended in buffer (0.03 M TEA [pH 7.5], 0.12 M NaCl). Portions of RNA were diluted to 5.0 ml with buffer (0.01 M TEA [pH 7.5], 0.12 M NaCl) and gravity-filtered through a GF/C filter containing bound poly(U) according to the procedure of Sheldon et al. (23) as previously described (15).

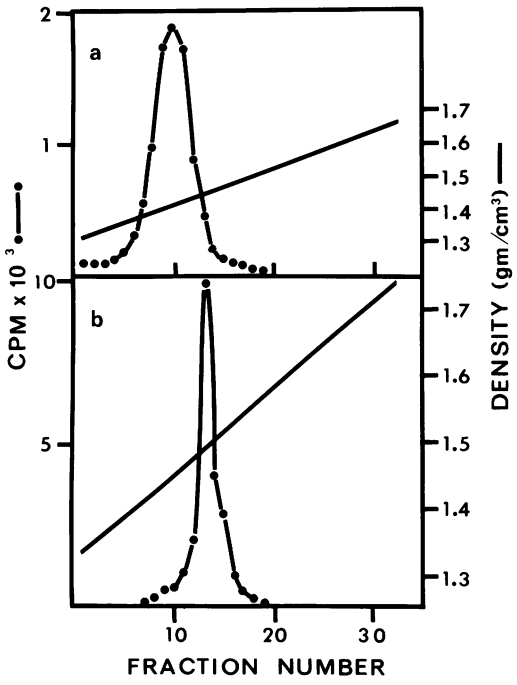


FIG. 4. *Bouyant densities of mRNP complexes eluted with formamide. (a) The 25% formamide eluate was dialyzed, and a portion was fixed with 6% formaldehyde. The density was adjusted to 1.3 g/cm³, and the sample was layered over a step gradient (density, 1.4 to 1.7 g/cm³). Gradients were centrifuged for 24 h at 45,000 rpm at 20°C in the SW65 rotor. Eight-drop fractions were collected and counted. The density of every fifth fraction was determined by refractometry. (b) The 50% formamide eluate was analyzed as described above.*

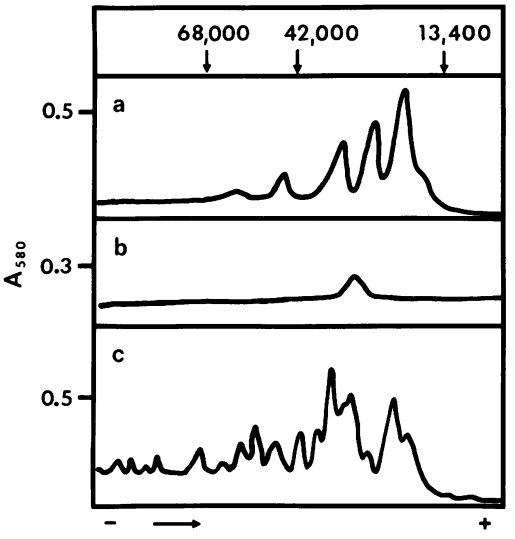


FIG. 5. *SDS-polyacrylamide gel electrophoresis of RNP proteins. Fractions to be analyzed were precipitated with trichloroacetic acid. Precipitates were washed with 100% ethanol, ethanol-ether, and ether. The air-dried precipitates were resuspended in buffer, and 100-μl portions were electrophoresed on a 6% polyacrylamide slab gel (12 by 15 by 3 mm; E-C Apparatus) for 4 h at 15°C and 200-mA constant current. (a) 25% formamide eluate; (b) 50% formamide eluate; (c) high-salt wash eluate.*

27,000-dalton polypeptide confers added stability to the hybridization of the mRNP and oligo(dT)-cellulose. The location of this polypeptide with respect to a specific region of the mRNA molecule is unknown. In rat liver and mouse L cells, Blobel (3) has reported that a

protein of molecular weight 78,000 is specifically associated with the poly(A) tract. Likewise, Irwin et al. (8) suggest that the 84,000-dalton polypeptide associated with the 50% formamide mRNP complex is associated with the poly(A) region. Experiments are currently in progress to determine whether the 27,000-dalton polypeptide in *Neurospora* is associated with the poly(A) tract and is therefore conparable to the 78,000- to 84,000-dalton polypeptides studied in other systems. An alternate explanation for the differential elution is that the poly(A) region of the 50% formamide-eluted mRNP complex is longer than the 25% formamide-eluted mRNP complexes, thus conferring greater hybrid stability. The size of the poly(A) region of the 25 and 50% formamide-eluted mRNP complexes has not yet been determined.

The specific location and function of the polypeptides associated with the 25% formamide-eluted mRNP complexes have not been determined in any system studied. A possible regulatory function for mRNP-associated proteins is suggested by the work of Lindberg and Sundquist (10). In normal KB cells, mRNP complexes contain four polypeptides, whereas mRNP complexes isolated from adenovirus-infected KB cells possess the same four polypeptides and one additional polypeptide. Although the evidence is far from compelling, the regulation of mRNA selection, utilization, and degradation by means of the specific addition, deletion, or modification of mRNP-associated proteins is an attractive hypothesis. This research constitutes a first step in determining whether mRNA-associated proteins are involved in the control of protein synthesis in fungal spores.

The major difference between the mRNP complexes isolated from *N. crassa* and similarly derived complexes from other organisms is the size of the mRNP-associated proteins. The *Neurospora* mRNP-associated proteins range in molecular weight from 17,000 to 66,000, whereas those in most other organisms studied range from 49,000 to 118,000 (2, 8, 10, 16). The reason for this size difference is not known, but two explanations are suggested. First, the observed difference is related to the phylogenetic position of *N. crassa*. In the other closely related organism studied, *Dictyostelium*, RNP complexes isolated from nuclei contain several polypeptides in the molecular-weight range of 13,000 to 40,000 as well as higher-molecular-weight species (5). More comparative data are needed to determine whether this size difference is of general occurrence among lower eukaryotes. Second, *Neurospora* mRNP-associated proteins are larger in the native state but are partially degraded during isolation of the mRNP complexes. It is difficult to preclude with certainty limited proteolysis, but the profile presented in Fig. 5 has been exactly reproduced in several different experiments, an unlikely result unless the proteolysis is exceedingly specific.

The results presented in this paper also provide some information regarding the specificity of RNA-protein interactions in *Neurospora*. Baltimore and Huang (1) showed that purified RNA could complex with proteins in a cytoplasmic extract and form particles with properties indicative of an RNP complex. However, these "artificial" RNP complexes were not formed at salt concentrations above 0.15 M. In those studies reporting the isolation and characterization of mRNP complexes, the salt concentration was maintained at some level above 0.15 M in order to prevent adventitious association of protein and RNA. In the present study, mRNP complexes were isolated in 0.25 M salt. However, during the disruption of the spores and the subsequent isolation of polysomes, the salt concentration was below 0.15 M. Parallel experiments in which the salt concentration was maintained at 0.5 M during the entire isolation procedure yielded results identical to those already reported. Thus, the proteins associated with mRNA are tightly bound and do not represent adventitious binding. The question of the association of specific proteins with mRNA can only be determined by additional experiments which: (i) localize specific proteins with respect to specific mRNA regions; (ii) determine the relationship between these proteins and mRNA under different metabolic conditions; and (iii) determine the effects of these proteins on mRNA translation in vitro. Such studies are currently in progress.

ACKNOWLEDGEMENT

This work was supported by a grant from the Research Corporation.

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