The Protein Responsible for the Repeating Structure of Cytoplasmic Poly(A)-Ribonucleoprotein

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ABSTRACT A 75,000-dalton protein has been purified approximately 1,000-fold from rat liver, based on its capacity to organize poly(A) in a 27-residue repeating structure. This protein may be identified with the major polypeptide component of cytoplasmic poly(A)-ribonucleoprotein (RNP) previously described. The poly(A)-organizing activity of the protein is detected only in cytoplasmic fractions. Upon nuclease digestion of the 75,000-dalton protein-poly(A) complex, monomers, and higher multimers of RNP subunits can be resolved in a sucrose gradient. The sedimentation rate of the monomer is compatible with a composition of one 75,000-dalton protein molecule and one 27-residue segment of poly(A).

Most previous studies of cytoplasmic poly(A)-ribonucleoprotein (RNP) have been concerned with its protein composition. The RNP is generally purified from cell extracts by digestion with pancreatic and T1 RNases to degrade all but the poly(A), followed by fractionation in a sucrose gradient (3, 11, 20) or on poly(U)-Sepharose or oligo(dT)-cellulose (8, 10, 13). The purified RNP invariably contains a major protein, most often reported as ~78,000 daltons, and usually contains minor proteins as well. Evidence for the association of the 78,000-dalton protein with poly(A) in vivo has come from ultraviolet light-induced crosslinking of the protein to poly(A) in intact cells (7).

Structural studies of cytoplasmic poly(A)-RNP (1) have revealed a repeating pattern of organization with a periodicity of about 27 residues of poly(A). The repeating structure can be reconstituted from purified poly(A) and factors in a cell extract. The question naturally arises of whether these factors correspond with the poly(A)-RNP proteins mentioned above. The objectives of the work reported here were to identify the factors and determine their role in poly(A)-RNP structure. We took the approach of fractionating an extract, based on reconstitution of the 27-residue repeating structure as an assay. The results were of interest in regard to the structure of nuclear as well as cytoplasmic RNPs. They helped reconcile the seemingly paradoxical findings that nuclear and cytoplasmic poly(A)-RNPs contain similar proteins (5, 10, 21), but the 27-residue repeating structure occurs only in the cytoplasm (1).

MATERIALS AND METHODS

Purification of Poly(A)-organizing Protein: Rat livers (100 g) were homogenized in 50 ml of 0.34 M sucrose–2 mM EDTA–0.2 mM PMSF–buffer A (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM 2-mercaptoethanol, 15 mM Tris-HCl, pH 7.4) with four strokes of a motor-driven teflon-glass homogenizer. The homogenate was layered over 1.2 M sucrose in the same buffer (~30 ml over 12 ml) and centrifuged at 10,000 *g* for 20 min. The upper layer was transferred to fresh 1.2 M sucrose and the centrifugation repeated. The upper layer was treated with RNase A (0.5 μg/ml, Worthington Biochemical Co., Freehold, NJ) and RNase T1 (1,000 U/ml, Calbiochem Corp.) for 1 h at 4°C and centrifuged in a Beckman SW 41 rotor (Beckman Instruments, Spinco Div., Palo Alto, CA) at 40,000 rpm for 90 min. To the supernatant was added 0.32 vol of saturated ammonium sulfate in 0.34 M sucrose–2 mM EDTA–buffer A with stirring in ice, and a precipitate was removed by centrifugation at 10,000 *g* for 10 min. To the supernatant was added 0.2 vol of saturated ammonium sulfate and the precipitate collected as before. The precipitate was dissolved in 8 ml of buffer A, layered on four 5–28.8% isokinetic sucrose gradients (4) containing 1 M NaCl–buffer A, and centrifuged in a Beckman SW 27 rotor at 26,000 rpm for 50 h. Fractions (1.2 ml) were collected, 0.02 ml-aliquots assayed for poly(A)-organizing activity, the active fractions (typically 7–13 from the bottom of the gradient) pooled, micrococcal nuclease (50 U/ml, Worthington Biochemical Co.) was added, the mixture dialyzed against 1 mM $CaCl_2$–0.2 mM PMSF–buffer A for 16 h at 4°C, and a small precipitate removed by centrifugation at 10,000 *g* for 20 min. The supernatant was applied at 20 ml/h and room temperature to a poly(A)-Sepharose 4B column (1 × 1 cm, Pharmacia Fine Chemicals, Piscataway, NJ) which had been washed as described by the manufacturer and equilibrated with 10 mM EDTA–0.2 mM PMSF–0.1 mg/ml yeast tRNA–buffer A. The column was washed with 10 ml of the same buffer, followed by 3 ml of the same buffer supplemented with the following (or as indicated in the figures): 1 M NaCl, and 1.1, 1.2, and 1.3 M guanidine HCl. Eluates were dialyzed against buffer A, and 0.02-ml aliquots were assayed for poly(A)-organizing activity. Typically, ~0.15 mg of pure protein, determined as described (15), was eluted with 1.2 and 1.3 M guanidine HCl.

Assay of Poly(A)-organizing Activity: A mixture of 3×10^4 cpm (~0.3 μg) of ^{32}P-poly(A), prepared as described (1), with the protein fraction of interest was incubated in 0.2 ml of buffer A containing 0.1 mg/ml yeast tRNA for 30 min at 37°C. Then the mixture was made 1 mM in $CaCl_2$ and digested with micrococcal nuclease (1 U; Worthington Biochemical Co.) for 20 min at 37°C. Digestion was terminated by the addition of EDTA to 5 mM, and RNA fragments were phenol extracted, ethanol precipitated, and subjected to electrophoresis in a 7 M urea-containing 10% polyacrylamide gel (16). Autoradiograms

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of gels are shown.

Yeast tRNA was included in the buffers for chromatography of poly(A)-organizing activity and in the mixture for assay of the activity to prevent interference by nonspecific RNA-binding proteins, polycations, etc. There is no requirement for tRNA, however, for demonstrating the organization of poly(A)-RNP in a repeating structure, nor is tRNA present in isolated poly(A)-RNP (1).

RESULTS

Purification of Poly(A)-organizing Protein

The repeating structure of poly(A)-RNP is revealed by brief digestion with an enzyme that cleaves poly(A), such as T2 RNase or micrococcal nuclease, followed by extraction of the poly(A) fragments and gel electrophoresis. A pattern of bands that are multiples of about 27 residues is obtained (1). Addition of purified, ^{32}P-labeled poly(A) to a rat liver extract, followed by nuclease digestion, gel electrophoresis, and autoradiography, results in a similar pattern of bands (1). Evidently, the ^{32}P-poly(A) is organized in a repeating structure by an activity in the extract. The procedure that gives the pattern of bands in an autoradiogram serves as an assay of the activity, allowing its purification from the extract.

Poly(A)-organizing activity was purified in four steps: (*a*) treatment of a rat liver extract with pancreatic and T1 RNases to degrade all but the poly(A), followed by centrifugation at high speed to give a postribosomal supernatant; (*b*) precipitation with ammonium sulfate at 25% of saturation; (*c*) sedimentation in a sucrose gradient containing 1 M NaCl; and (*d*) treatment with micrococcal nuclease to degrade endogenous poly(A), followed by chromatography on poly(A)-Sepharose. All of the poly(A)-organizing activity precipitated with ammonium sulfate and sedimented at 12S in the sucrose gradient. The activity was unaffected by micrococcal nuclease digestion. It remained bound to poly(A)-Sepharose in high salt and was eluted with guanidine HCl (Fig. 1). Virtually all the activity was eluted with 1.2 and 1.3 M guanidine HCl (no further activity was recovered upon elution with 7 M guanidine HCl). The active fractions were analyzed by SDS gel electrophoresis, which revealed a single band of protein of about 75,000 daltons (Fig. 2).

The assay of poly(A)-organizing activity by observation of a band pattern in an autoradiogram is qualitative, but the relative activities of the various fractions could be estimated from the amounts of protein needed to obtain comparable patterns. On this basis, the four steps of purification gave an over-all enrichment of about 1,000-fold in 25% yield.

Structural Role of Poly(A)-organizing Protein

When the RNP reconstituted from ^{32}P-poly(A) and purified poly(A)-organizing protein was digested with micrococcal nuclease and fractionated in a sucrose gradient, a series of peaks of radioactivity was obtained (Fig. 3 *B*). The pattern of intensity of the peaks corresponded with that of the bands resulting from deproteinization of the total digest and analysis in a gel (Fig. 3 *A*). Moreover, RNA from the first peak in the gradient (the first peak following the one at the top of the gradient, which contained oligonucleotides) was found, on extraction and gel electrophoresis, to correspond with the first band in the gel, while RNA from the second peak corresponded with

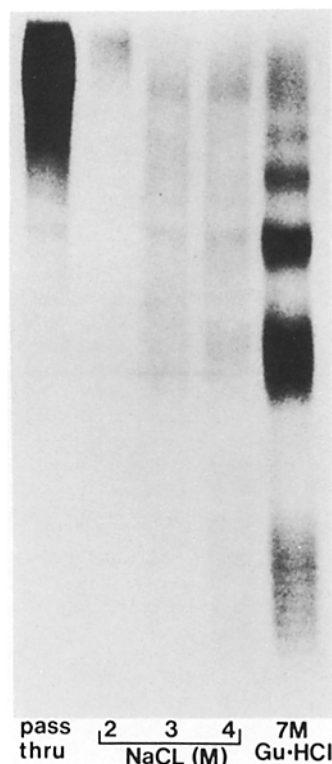

FIGURE 1 Chromatography of poly(A)-organizing activity on poly(A)-Sepharose. The column was eluted with the concentrations of NaCl or guanidine HCl indicated and eluates were assayed, all as described.

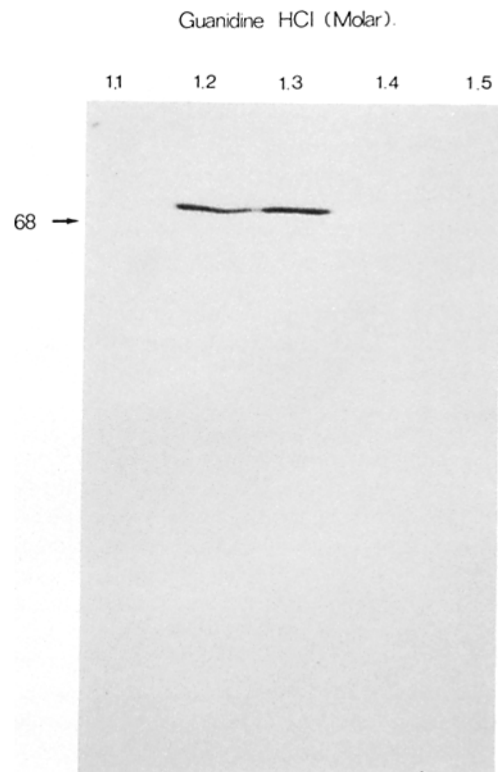

FIGURE 2 Proteins eluted from poly(A)-Sepharose. The column was eluted as described with the concentrations of guanidine HCl indicated. Eluates were dialyzed against water, lyophilized, and analyzed by electrophoresis in an SDS-12% polyacrylamide gel (16). A photograph of the Coomassie Blue-stained gel is shown. The position of BSA (68,000 daltons) run in another lane of the same gel is indicated on the left.

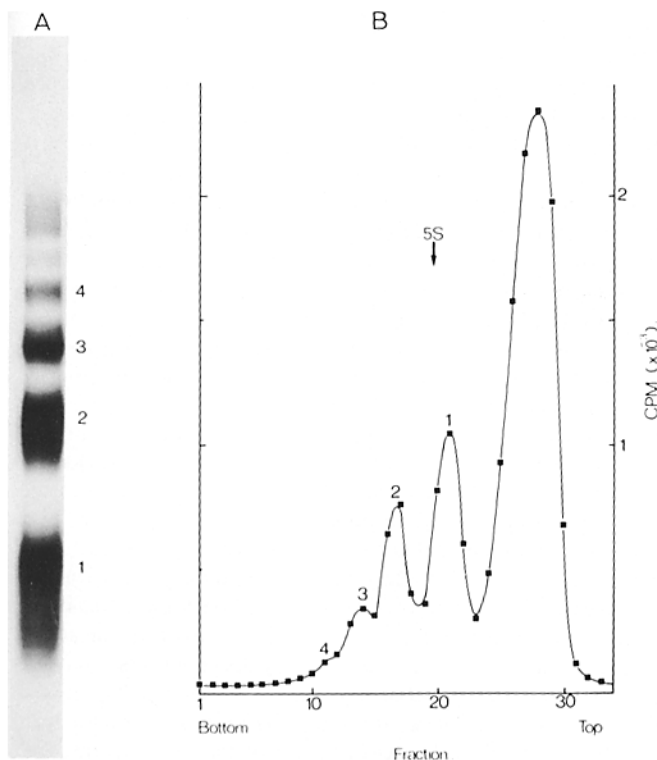

FIGURE 3 Subunit structure of poly(A)-RNP. Purified poly(A)-organizing protein was mixed with ^{32}P-poly(A), the mixture was digested with micrococcal nuclease, and digestion was terminated by the addition of EDTA, all as described. RNA was extracted from half of the mixture and analyzed by electrophoresis and autoradiography (*A*) as described. The other half of the mixture (*B*) was layered on a 5-28.8% isokinetic sucrose gradient (4) containing 10 μg/ml yeast tRNA-2 mM EDTA-buffer A and centrifuged in a Beckman SW 41 rotor at 40,000 rpm for 20 h. An arrow indicates the position of 200 residue poly(A) which sedimented at about 5S in a parallel gradient.

the second band, and so forth. Apparently, monomers, dimers, and higher multimers of RNP subunits are resolved in the gradient. These must represent nucleoprotein complexes and not naked RNA segments, since the monomer sediments at about 4S, far faster than expected for naked 27-residue poly(A). The sedimentation rate is comparable to that of a globular protein of 60,000–80,000 daltons (cf. hemoglobin, 66,000 daltons, 4.3S). The monomer must contain nearly this weight of protein, because 27 residues of poly(A) contribute only 9,000 daltons. On reconstitution with this weight of total poly(A)-organizing protein for every 27 residues of poly(A), half or more of the poly(A) was converted to 4S monomer and higher multimers (Fig. 3). It follows that at least half of the poly(A)-organizing protein was involved in the formation of repeating structure. Because the 75,000-dalton protein was the only component of the poly(A)-organizing protein preparation present in this amount (Fig. 2), we conclude that it is responsible for the repeating structure. The simplest possibility is that a unit of the structure contains a single 75,000-dalton protein molecule complexed with a 27-residue segment of poly(A). A stoichiometry of two or more 75,000-dalton protein molecules for every 27 residues of poly(A) is not excluded, but a rather asymmetric shape of these molecules would be required to explain why the sedimentation coefficient of the monomer is only 4S.

Subcellular Distribution of Poly(A)-organizing Protein

Previously, we found that nuclease digestion of poly(A) in nuclei gives no pattern of bands in a gel but only a smear (1). The 27-residue repeating structure of poly(A)-RNP is apparently restricted to the cytoplasm. We now report two additional lines of evidence consistent with this conclusion. First, an extract of cultured cells was separated into nuclear and cytoplasmic fractions, and poly(A)-organizing activity was assayed as described above. Activity was found only in the cytoplasmic fraction (Fig. 4). The second line of evidence comes from sedimentation analysis. Nuclear and cytoplasmic poly(A)-RNP were analyzed in sucrose gradients containing 1 M NaCl (Fig. 5). The sedimentation of the cytoplasmic RNP was unaffected by the high salt concentration, as expected from the stability of the poly(A)-organizing protein–poly(A) complex in these conditions (Fig. 1). The nuclear RNP, on the other hand, appeared to dissociate in 1 M NaCl, the poly(A) component sedimenting at the same rate as free poly(A). Thus the protein component of nuclear poly(A)-RNP differs from the poly(A)-organizing protein found in the cytoplasm.

DISCUSSION

The 75,000-dalton protein purified here on the basis of its poly(A)-organizing activity appears to be identical with the

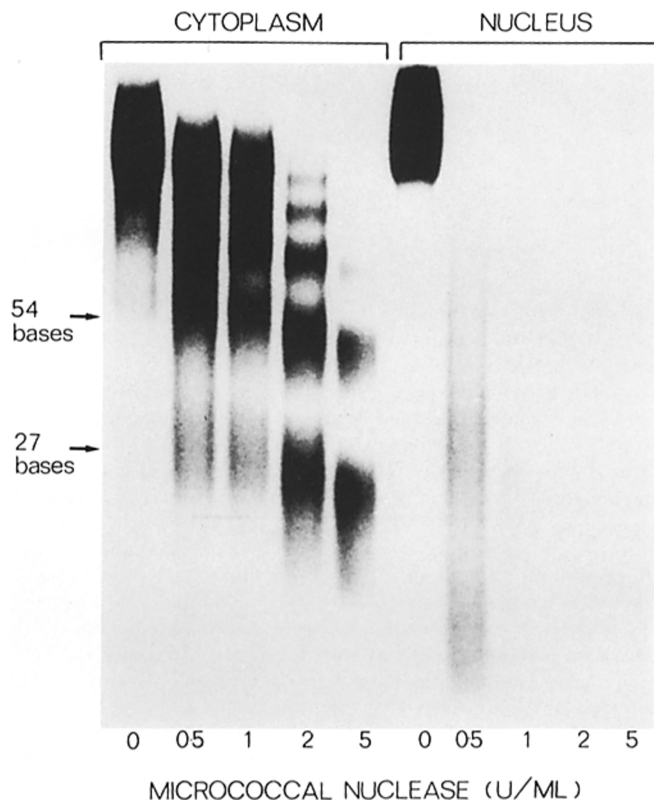

FIGURE 4 Poly(A)-organizing activity in nuclei and cytoplasm from mouse erythroleukemic cells. Nuclear and cytoplasmic fractions were prepared by lysis of cells in hypotonic medium and centrifugation as described (1), except that the cells were unlabelled. Each fraction was adjusted to 0.2 mM EDTA and 0.1% Triton X-100, passed through a 22-gauge needle, and assayed for poly(A)-organizing activity by the addition of ^{32}P-poly(A) and digestion with micrococcal nuclease at the levels indicated.

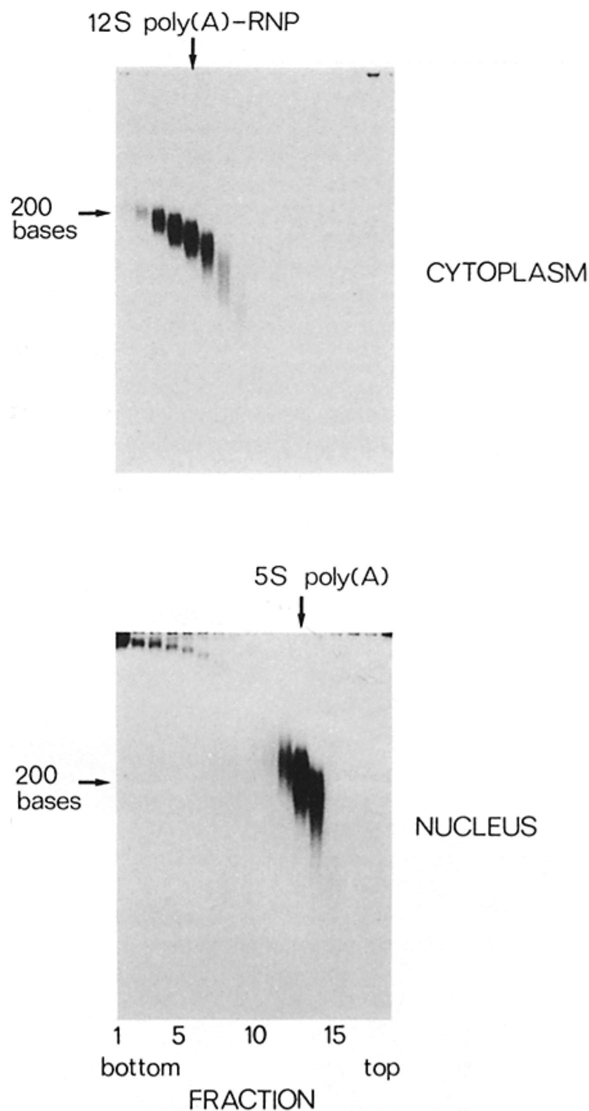

FIGURE 5 Sedimentation of nuclear and cytoplasmic poly(A)-RNPs in 1 M NaCl. Nuclei and cytoplasm were prepared from mouse erythroleukemic cells grown for 4 h in the presence of [^{32}P]orthophosphate as described (1), digested with RNase A (2 μg/ml, Worthington Biochemical Co.) and RNase T1 (550 U/ml, Calbiochem Corp.) for 20 min at 37°, and centrifuged at 10,000 *g* for 1 min. The supernatants were layered on 5–28.8% isokinetic sucrose gradients (4) containing 1 M NaCl-0.2 mM PMSF-buffer A and centrifuged in a Beckman SW 41 rotor at 40,000 rpm for 20 h. Fractions were collected, phenol extracted, ethanol precipitated, and subjected to electrophoresis in 7 M urea-containing 10% polyacrylamide gels (16). Autoradiograms of the gels are shown. The position of a peak of free poly(A) was determined by deproteinization and analysis in a parallel gradient. The radioactive material at the top of the gel from fractions at the bottom of the gradient run on nuclear poly(A)-RNP was DNA.

major polypeptide component of cytoplasmic poly(A)-RNP previously described (2, 3, 6–10, 13, 17, 20, 21). These proteins are similar in the following respects: (*a*) molecular weight, variously reported for the major poly(A)-RNP protein as 74,000–81,000 daltons; (*b*) high affinity for poly(A), especially the lack of dissociation from poly(A) at high ionic strength (Fig. 1 and refs. 2, 6, 18, 21); (*c*) occurrence in cytoplasmic fractions in a form capable of binding to free poly(A), detected in the case of the 75,000-dalton protein by the formation of the 27-residue repeating structure, and shown in the case of the major poly(A)-RNP protein by adsorption to poly(A)-Sepharose (6, 21).

The state of the cytoplasmic 75,000-dalton protein that interacts with free poly(A) remains to be established. During purification, cytoplasmic poly(A)-organizing activity sedimented at 12S in a sucrose gradient, as does native poly(A)-RNP (1, 2, 11), suggesting that the activity resides in poly(A)-RNP. One explanation for this would be that the 75,000-dalton protein is capable of exchange between poly(A)-RNP and the free poly(A) used to assay the activity. Consistent with this, nuclease digestion of endogenous poly(A) did not enhance the poly(A)-organizing activity, as would have occurred if the 75,000-dalton protein, incapable of exchange, were liberated by digestion. An alternative explanation of all these findings is that the 75,000-dalton protein is present in the cytoplasm in a 12S form distinct from poly(A)-RNP. An additional, minor protein could be involved, analogous to the nucleosome assembly protein from *Xenopus* oocytes, which binds histones in an 8S form and mediates their interaction with DNA (14).

Proteins of about 75,000 daltons have been reported as major components of both nuclear and cytoplasmic poly(A)-RNPs (5, 10, 21), while the 27-residue repeating structure is restricted to the cytoplasm (1). The problem of how to reconcile these findings is made even more difficult by the conclusion reached here that the 75,000-dalton protein is responsible for the repeating structure. The solution appears to be that the nuclear and cytoplasmic poly(A)-associated proteins are different. Ultraviolet light-induced cross-linking of proteins to RNA in intact cells reveals a 60,000-dalton protein associated with the poly(A) of hnRNA, and a 78,000-dalton protein associated with the poly(A) of mRNA (22). Sedimentation analysis of nuclear and cytoplasmic poly(A)-RNPs in the presence of 1 M NaCl (Fig. 5) has provided further evidence for a difference in the protein components of these RNPs and for a corresponding difference in structure. The functional significance of these differences is not known. It has been suggested that the 75,000-dalton protein is involved in the transport of mRNA from nucleus to cytoplasm (9, 19, 20), which may occur through an active role in the transport process or through simple binding to the RNA in the cytoplasm.

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REFERENCES

1. Baer, B. W., and R. D. Kornberg. 1980. Repeating structure of cytoplasmic poly(A)-ribonucleoprotein. *Proc. Natl. Acad. Sci. USA.* 77:1890–1892.
2. Barrieux, A., H. A. Ingraham, S. Nystul, and M. G. Rosenfeld. 1976. Characterization of the association of specific proteins with messenger ribonucleic acid. *Biochemistry.* 15:3523–3528.
3. Blobel, G. 1973. A protein of molecular weight 78,000 bound to the polyadenylate region of eukaryotic messenger RNAs. *Proc. Natl. Acad. Sci. USA.* 70:924–928.
4. Finch, J. T., M. Noll, and R. D. Kornberg. 1975. Electron microscopy of defined lengths of chromatin. *Proc. Natl. Acad. Sci. USA.* 72:3320–3322.
5. Firtel, R. A., and T. Pederson. 1975. Ribonucleoprotein particles containing heterogeneous nuclear RNA in the cellular slime mold *Dictyostelium discoideum. Proc. Natl. Acad. Sci. USA.* 72:301–305.
6. Fukami, H., and H. A. Itano. 1976. In vitro translation of globin: effect of proteins purified by affinity chromatography on polyadenylate-sepharose. *Biochemistry.* 15:3529–3535.
7. Greenberg, J. R. 1980. Proteins crosslinked to messenger RNA by irradiating polyribosomes with ultraviolet light. *Nucleic Acids Res.* 8:5685–5701.
8. Jeffrey, W. R. 1977. Characterization of polypeptides associated with messenger RNA and its polyadenylate segment in Ehrlich ascites messenger ribonucleoprotein. *J. Biol. Chem.* 252:3525–2532.
9. Kish, V. M., and T. Pederson. 1975. Ribonucleoprotein organization of polyadenylate sequences in HeLa cell heterogeneous nuclear RNA. *J. Mol. Biol.* 95:227–238.
10. Kish, V. M., and T. Pederson. 1976. Poly(A)-rich ribonucleoprotein complexes from HeLa

cell messenger RNA. *J. Biol. Chem.* 251:5888–5894.
11. Kwan, S. W., and G. Brawerman. 1972. A particle associated with the polyadenylate segment in mammalian messenger RNA. *Proc. Natl. Acad. Sci. USA.* 69:3247–3250.
12. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
13. Lindberg, U., and B. Sundquist. 1974. Isolation of messenger ribonucleoproteins from mammalian cells. *J. Mol. Biol.* 86:451–468.
14. Laskey, R. A., B. M. Honda, A. D. Mills, and J. T. Finch. 1978. Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. *Nature (Lond.).* 275:416–420.
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. *J. Biol. Chem.* 193:265–275.
16. Maniatis, T., A. Jeffrey, and H. van deSande. 1975. Chain length determination of small double- and single-stranded DNA molecules by polyacrylamide gel electrophoresis. *Biochemistry.* 14:3787–3794.
17. Morel, C., B. Kayibonda, and K. Scherrer. 1971. Proteins associated with globin messenger RNA in avian erythroblasts: isolation and comparison with the proteins bound to nuclear messenger-like RNA. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 18:84–88.
18. Morel, C., E. S. Gander, M. Herzberg, J. Dubochet, and K. Scherrer. 1973. The duck-globin messenger-ribonucleoprotein complex. *Eur. J. Biochem.* 36:455–464.
19. Quinlan, T. J., Billings, B. P., and T. E. Martin. 1974. Nuclear ribonucleoprotein complexes containing polyadenylate from mouse ascites cells. *Proc. Natl. Acad. Sci. USA.* 71:2632–2634.
20. Schwartz, H., and J. E. Darnell. 1976. The association of protein with the polyadenylic acid of HeLa cell messenger RNA: evidence for a "Transport" role of a 75,000 molecular weight polypeptide. *J. Mol. Biol.* 104:833–851.
21. Schweiger, A., and G. Mazur. 1975. Rat liver soluble nuclear and cytoplasmic proteins with high affinity to polynucleotides. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 60:114–118.
22. Setyono, B., and J. R. Greenberg. Proteins associated with poly(A) and other regions of mRNA and hnRNA molecules as investigated by cross-linking. 1981. *Cell.* 24:775–783.