PHOSPHORYLATION OF MESSENGER RNA-BOUND PROTEINS IN HeLa CELLS

Susan Auerbach* and Thoru Pederson

Worcester Foundation for Experimental Biology
Shrewsbury, Massachusetts 01545

Received January 6,1975

SUMMARY: A limited ribonuclease digestion (0.5 µg/ml RNase A, 20 min., 4°C) of salt-washed HeLa cell polyribosomes can be used to release the messenger RNA as ribonucleoprotein. The messenger-bound proteins are different from ribosomal structural proteins, as judged by their respective electrophoretic migrations in polyacrylamide gels containing sodium dodecyl sulfate or in urea-containing gels at pH 4.5. Studies with ^{32}P-labeled cells reveal that some of the messenger RNA-associated proteins are phosphorylated *in vivo*.

Recent studies have demonstrated that a very similar set of proteins are bound to messenger RNA in a variety of eukaryotic cells, inlcuding rabbit reticulocytes (1), mouse L-cells (2), rat liver tissue (2), chick cerebral cells (3), and HeLa and KB cells (4,5). This fact suggests that these protein-mRNA interactions are physiological, and that the proteins have some function in the life history of messenger RNA. In this report, we describe a new method for purifying mRNA-associated proteins which is based on the ability of pancreatic ribonuclease, at low concentrations, to selectively attack mRNA in polyribosomes (6). Using this method to isolate mRNA-associated proteins from cells grown in the presence of ^{32}P, we find that these proteins are phosphorylated *in vivo*.

MATERIALS AND METHODS

Cell culture and labeling: HeLa S_3 cells were grown in suspension culture as detailed previously (7). Messenger RNA was preferentially labeled with ^{3}H-uridine as described previously (8). In other cases, the cells were resuspended in phosphate-free medium at a concentration of 3 x 10^5 cells/ml and were labeled with ^{32}P

*present address: 1570 Cambridge Street, Cambridge, Mass. 02139.

(2.0 μCi/ml) for 24 hours at 37°C. Cell growth was monitored and found to be unaffected by this labeling protocol (doubling time = 21 hours).

Preparation of mRNA-associated proteins and ribosomal proteins: The preparation of mRNA-associated proteins is based upon a limited digestion of salt-washed polyribosomes with ribonuclease A. Cells were homogenized in RS buffer* as detailed previously (8). The post-mitochondrial supernatant was centrifuged at 100,000 x g for 40 min over 20% sucrose in RS buffer. The pellet of polyribosomes was resuspended in 0.5M KCl, 5mM $MgCl_2$, 0.05M Tris-HCl, pH 7.4 and centrifuged again at 100,000 x g for 12 hrs through 20% sucrose in RS buffer, yielding a pellet of "salt-washed polyribosomes." The polyribosomes were resuspended into 0.025M KCl, 1mM $MgCl_2$, 0.05M Tris-HCl, pH 7.4 and dissociated by incubation with 1mM puromycin for 30 min at 37°C, then chilled. The sample was incubated with ribonuclease A at 0.5 μg/ml for 20 min at 4°C. Ice-cold salt solutions were then added to final concentrations of 0.5M KCl and 5mM $MgCl_2$. The sample was layered over 2 ml of 15% sucrose in RS buffer and centrifuged for 2 hrs at 56,000 rpm in the SW56 rotor at 4°C. The top 2 ml was removed, 2 vol of 95% ethanol was added, and the solution kept at -20°C overnight, yielding a precipitate of mRNA-bound proteins. The pellet of ribosomes obtained from the 56,000 rpm centrifugation was used to prepare ribosomal proteins.

LiCl precipitation was employed to remove most of the remaining RNA from its associated proteins in both samples, i.e. messenger ribonucleoprotein and ribosomes. The samples were dissolved in 0.01M sodium acetate, pH 4.5, and made 4.4M in urea and 2.2M in LiCl and then left at 4°C for 16 hours. RNA was removed by centrifugation at 1000 x g for 20 min. For gel-electrophoresis, the supernatant proteins were dialysed against either 0.01M sodium acetate, pH 4.5, 6M urea, 0.1% 2-mercaptoethanol, or 0.1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, 0.01M sodium phosphate buffer pH 7.0.

Electrophoresis and autoradiography: mRNA-associated proteins and ribosomal proteins were electrophoresed in 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (9) or 6M urea (10). Gels were stained with Coomassie brilliant blue (11) or 0.5% Amido black in 7% acetic acid. The gels were then placed in 7% CCl_3COOH at 90°C for 30 min in order to hydrolyze completely any traces of RNA remaining with the proteins (see Results). Gels were placed in 10% acetic acid overnight with stirring to remove nucleic acid hydrolysis products. The gels were sliced longitudinally, dried for autoradiography (12), and placed on single-sided X-ray film (Kodak SB54) and exposed for 2-3 weeks.

RESULTS AND DISCUSSION

Unlike 20S globin mRNP, mRNP particles from HeLa cells are heterogeneous in their sedimentation behavior (20 to 70S) and therefore overlap with both the 60S and 40S ribosomal subunits

*Abbreviations: RS buffer = 0.01M NaCl, 1.5mM $MgCl_2$, 0.01M Tris-HCl, pH 7.2.

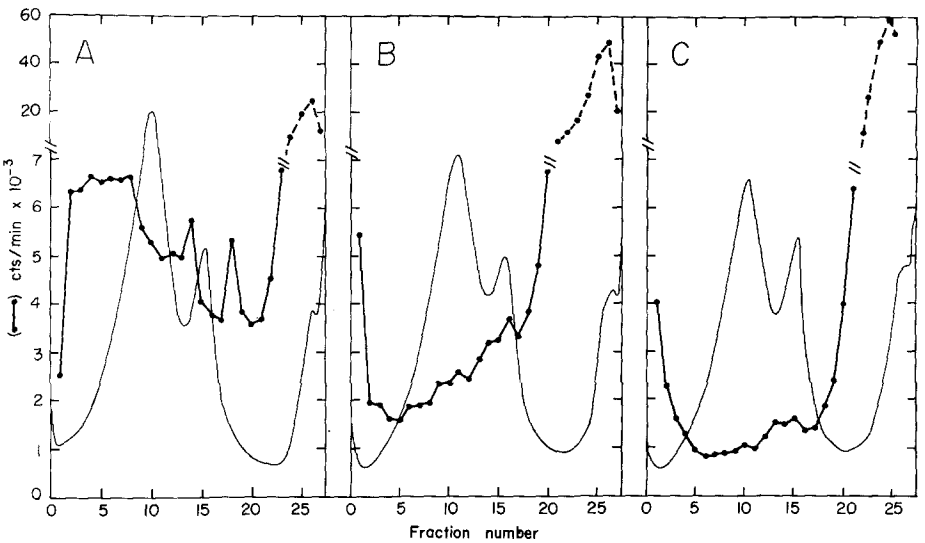
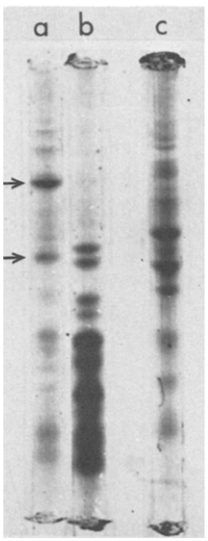


Figure 1. Sucrose gradient sedimentation of HeLa cell ribosomal subunits and mRNP particles. 6 x 10^8 cells were incubated with ^{3}H-uridine under conditions permitting selective labeling of messenger RNA. 0.5M KCl-washed polyribosomes were resuspended in 0.025M KCl, 1mM $MgCl_2$, 0.05M Tris-HCl pH 7.4, 1mM dithiothreitol (DTT), made 1mM in puromycin, incubated at 37°C for 30 min and chilled to 0°C. Pancreatic ribonuclease, 0.5 µg/ml, was added to portions for (A) 0 min., (B) 2 min., or (C) 20 min. Salts were added to final concentrations 0.5M KCl and 5mM $MgCl_2$ and the samples were layered on 5-20% sucrose gradients (0.5M KCl, 5mM $MgCl_2$, 0.05M Tris-HCl, pH 7.4). Centrifugation was at 41,000 rpm in the Beckman Spinco SW41 rotor for 3 hours (4°C). The gradients were collected into 0.5 ml fractions while continuously monitoring A_{260}. Fractions were precipitated with 10% trichloroacetic acid (TCA), collected on glass fiber filters, and the radioactivity measured by liquid scintillation counting. Direction of sedimentation is from right to left. (——), A_{260}; (●——●), cts/min.

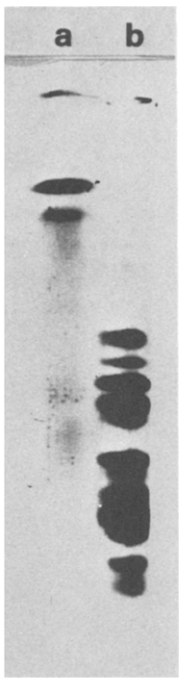
during gradient centrifugation of EDTA- or puromycin-dissassembled polyribosomes (5). In the present report we have isolated messenger RNA-associated proteins by exploiting the differential sensitivity of ribosomes and mRNP to low concentrations of pancreatic ribonuclease, as was first demonstrated by Warner, Knopf and Rich (6). Salt-washed polyribosomes were dissociated into ribosomal subunits and mRNP with puromycin and high salt (13). Aliquots were then incubated for varying lengths of time with 0.5 µg/ml pancreatic ribonuclease at 4°C and examined by velocity

sedimentation on sucrose gradients. Fig. 1A shows the sedimentation distribution of the 40S and 60S ribosomal subunits (A_{260}) and mRNP particles (^3H radioactivity) from puromycin-dissembled polyribosomes not treated with RNase. After two min. of enzyme treatment (Fig. 1B) the ^3H radioactivity from mRNP is displaced to lower S values, and after 20 min. of RNase treatment (Fig. 1C) most of the mRNA is degraded to material smaller than 20S. However, the ribosomal subunits (A_{260}) continue to display the same sedimentation coefficients as in the control sample. The slight decrease in the 60S:40S A_{260} ratio after RNase treatment is due to the co-sedimentation of 40S dimers with 60S subunits in the untreated sample, and their appearance at 40S after enzyme treatment (data not shown).

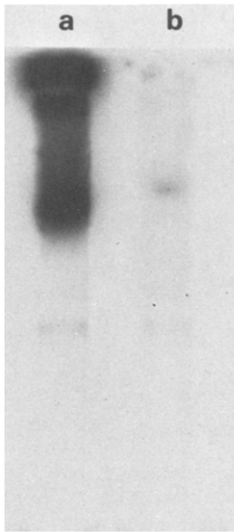
Electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate resolved the mRNP proteins into two prominent bands having estimated molecular weights of 73,000 and 50,000 (arrows, Fig. 2a), and a number of minor bands. The molecular weight values of 73,000 and 50,000 are the same as those obtained for proteins from HeLa mRNP isolated by the alternative method of oligo(dT)-cellulose chromatography (4). The mRNP preparation contained only a very small amount of ribosomal structural proteins, which are 10,000-55,000 mol wt. (Fig. 2b). The mRNP proteins were distinct from those which are removed from the polyribosomes during the high salt wash (compare Fig. 2a with 2c). In addition, the electrophoretic distribution of the mRNP proteins differed from that of the proteins which are complexed with HeLa heterogeneous nuclear RNA (4,8). In urea-polyacrylamide gels run at pH 4.5, the mRNP proteins electrophoresed as two sharp bands (Fig. 3a) while the more basic ribosomal structural proteins displayed a faster cathodal migration (Fig. 3b).



2



3



4

Figure 2. Electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. Protein samples were dialyzed against 0.01M sodium phosphate buffer pH 7.0, 0.1% sodium dodecyl sulfate, 0.1% mercaptoethanol. Samples containing 20-40 µg of protein were electrophoresed as detailed previously (11). Migration is from top to bottom. (A) mRNA-associated proteins; (B) ribosomal proteins; (C) proteins released from polyribosomes by 0.5M KCl.

Figure 3. Electrophoresis of mRNA-associated proteins and ribosomal proteins in urea-polyacrylamide gels at pH 4.5. a: 14 µg of mRNA-associated protein. b: 60 µg of ribosomal protein. Direction of electrophoresis is from top to bottom (cathodal).

Figure 4. Effect of trichloroacetic acid hydrolysis at 90°C on ^{32}P-labeling or ribosomal proteins in polyacrylamide gels. Ribosomal proteins were prepared from ^{32}P-labeled cells as detailed in Materials and Methods, and electrophoresed as in Figure 3. One gel was incubated in 7% (w/v) trichloroacetic acid at 90°C for 30 min prior to autoradiography while a replicate gel was incubated similarly but at 20°C. a: autoradiogram of gel incubated at 20°C. b: autoradiogram of gel incubated at 90°C.

Before analyzing ^{32}P labeled mRNA-proteins by autoradiography of polyacrylamide gels, it was necessary to eliminate any ^{32}P radioactivity in RNA. For example, in ribosomes, the hot CCl_3COOH-precipitable ^{32}P (i.e. in phosphoprotein) is only 0.1% of the total

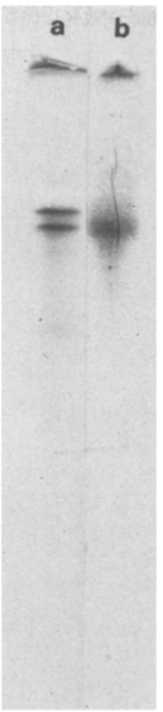


Figure 5. Phosphorylation of mRNA-associated proteins. 3.5 L of HeLa cells at 3 x 10^5/ml were labeled for 24 hours with 2.0 µCi of ^{32}P per ml. mRNA-associated proteins were prepared from salt-washed polyribosomes and electrophoresed as described in Materials and Methods. After trichloroacetic acid hydrolysis at 90°C (see Fig. 4) the gels were sliced longitudinally (12) and autoradiographed with Kodak SB54 X-ray film (exposure 23 days).
a: photograph of stained gel (amido black) after trichloroacetic acid hydrolysis. b: autoradiogram of gel illustrated in a.

^{32}P (in RNA). LiCl precipitation removes only 95-98% of the RNA. Therefore even the 2% of RNA remaining after LiCl precipitation would still be a substantial contaminant with respect to ^{32}P. For this reason, a 90°C CCl_3COOH hydrolysis step was introduced after electrophoresis and before autoradiography. The results of the hot CCl_3COOH treatment are shown in Fig. 4a and Fig. 4b for ribosomal proteins. Additional controls (not shown) established that this procedure selectively and quantitatively removes nucleic acid from the gels.

When cells were grown for one generation in the presence of

^{32}P, the more cathodal of the two mRNA-associated protein bands was found to contain hot CCl_3COOH-resistant radioactivity (Fig. 5). This labeling was abolished by treatment of the proteins with trypsin or alkaline phosphatase prior to electrophoresis. In sodium dodecyl sulfate-polyacrylamide gels (not shown) the phosphorylated proteins were resolved into a 50,000 mol wt. component and a small number of minor bands.

It is of interest to consider the cellular abundance of the two mRNA-associated proteins (73,000 and 50,000 mol wt.). Since the 73,000 molecular weight species is bound to poly(A) (Kish and Pederson, unpublished data) and since about 70% of HeLa mRNA contains poly(A) (14), the Coomassie-blue dye intensity of this protein following gel electrophoresis establishes what one can expect to see for other proteins of undetermined abundance. (This assumes equivalent dye binding for all proteins, which is not always the case, and also assumes a constant protein:polyribonucleotide packing ratio in all RNP). It follows that the 50,000 mol wt. dalton species (Fig. 2a) must also be associated with a highly abundant mRNA sequence, since its dye intensity after electrophoresis is close to that of the 73,000 mol wt. component when densitometric scans are corrected for molecular weight (i.e. the 50,000 mol wt. protein binds 50/73 as much dye per mole as the 73,000 mol wt. component). The 50,000 mol wt. component therefore is probably complexed with a nucleotide sequence that is common to the majority of the cell's messenger RNA molecules.

Two previous reports have suggested the presence of phosphorylated protein in messenger RNP, but neither of these studies employed salt-washed polyribosomes (15,16). The present results demonstrate that one of the two major proteins (50,000 mol. wt.), or groups of proteins, that remains bound to HeLa cell messenger

RNA in 0.5M KCl is phosphorylated. Further studies are needed to determine whether mRNA-associated proteins play a regulatory role in protein synthesis or mRNA storage, and whether their phosphorylation is an aspect of such a role.

Acknowledgment: Supported by research grants from the National Institute of General Medical Sciences (GM 21595), the National Cancer Institute (CA 13400; CA 12708), the American Cancer Society (VC 199, IN 100), and a Scholar Award from the Leukemia Society of America (T.P.).

REFERENCES

1. Blobel, G. (1972) Biochem. Biophys. Res. Commun. 47, 88-95.
2. Blobel, G. (1973) Proc. Nat. Acad. Sci. U.S.A. 70, 924-928.
3. Bryan, R.N., and Hayashi, M. (1973) Nature New Biol. 244, 271-274.
4. Kumar, A., and Pederson, T. (1975) J. Mol. Biol., in press.
5. Lindberg, U., and Sundquist, B. (1974) J. Mol. Biol. 86, 451-468.
6. Warner, J.R., Knopf, P., and Rich, A. (1963) Proc. Nat. Acad. Sci. U.S.A. 49, 122-129.
7. Pederson, T. (1972) Proc. Nat. Acad. Sci. U.S.A. 69, 2224-2228.
8. Pederson, T. (1974) J. Mol. Biol. 83, 163-183.
9. Bhorjee, J.S., and Pederson, T. (1972) Proc. Nat. Acad. Sci. U.S.A. 69, 3345-3349.
10. Leboy, P.S., Cox, E.C., and Flaks, J.G. (1964) Proc. Nat. Acad. Sci. U.S.A. 52, 1367-1374.
11. Bhorjee, J.S., and Pederson, T. (1973) Biochem. 12, 2766-2773.
12. Fairbanks, G., Levinthal, C., and Reeder, R.H. (1965) Biochem. Biophys. Res. Commun. 20, 393-399.
13. Blobel, G., and Sabatini, D. (1971) Proc. Nat. Acad. Sci. U.S.A 68, 390-394.
14. Milcarek, C., Price, R., and Penman, S. (1974) Cell 3, 1-10.
15. Egly, J.M., Johnson, B.C., Stricker, C., Mandel, P. and Kempf, J. (1972) FEBS Letters 22, 181-184.
16. Morel, C., Gander, E.S., Herzberg, M., Dubochet, J., and Scherrer, K. (1973) Europ. J. Biochem. 36, 455-464.