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The polyribosomal mRNA–protein complex is a dynamic structure

[poly(A)/photocrosslinking]

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ABSTRACT **The metabolism of mRNA–protein complexes present in polyribosomes of mouse L cells was investigated with the aid of ultraviolet light-induced crosslinking of proteins to RNA. It was shown that a set of at least seven proteins which are synthesized and become associated with mRNA under conditions permitting mRNA synthesis also are synthesized and become associated with mRNA when mRNA synthesis is inhibited with a high dose of actinomycin D. This set includes a protein of M_r 78,000 which can be crosslinked to poly(A). These findings imply that mRNA-associated proteins exchange in the cytoplasm with a pool of free proteins. Taken together with results from other laboratories, they suggest a role for mRNA-associated proteins in translation. The labeling kinetics of mRNA-associated proteins were also investigated and found to be consistent with cytoplasmic addition to mRNA and a half-life of more than 2 hr. Additional implications of these findings are discussed.**

It is well established that mRNA molecules released from eukaryotic polyribosomes by treatment with EDTA or with high salt concentration and puromycin are associated with proteins other than ribosomal structural proteins (reviewed in refs. 1 and 2). Two of these proteins have M_r of 78,000 and 52,000; the larger protein appears to be associated with poly(A) (1, 2). There are probably other mRNA-associated proteins as well, although debate continues as to their exact numbers and sizes. Despite more than a decade of investigation, the functional significance of these proteins is unknown, and very little is known about their metabolic properties. However, it has been shown that there is a pool of soluble cytoplasmic proteins which contains species similar to mRNP proteins with respect to size, charge, and amino acid composition (3–5). These soluble proteins bind to RNA, and they can be isolated from postribosomal supernatants by affinity chromatography on columns of RNA (3–8). Furthermore, some studies have suggested that the soluble RNA binding proteins can substitute for elongation and initiation factors in translation (4, 7, 8).

The existence of this pool suggests the possibility of protein exchange between it and the mRNP proteins. The occurrence of such an exchange would imply that the latter proteins also have some role in translation. In this paper it is shown that the mRNP proteins are synthesized and become associated with mRNA in the absence of mRNA synthesis. This finding constitutes direct evidence for an exchange in the cytoplasm between the proteins associated with mRNA and a pool of free proteins. It is also shown that the labeling kinetics of the mRNA-associated proteins are consistent with cytoplasmic addition to mRNA.

The proteins associated with mRNA were investigated with the aid of ultraviolet light-induced crosslinking of proteins to RNA. There is considerable evidence (discussed in ref. 9) that irradiation by 254-nm light can join proteins covalently to nucleic acids in a manner that is highly specific with respect to the amino acid and nucleotide sequences involved in crosslinking. Therefore, crosslinking can be used to circumvent problems that have previously hindered research on mRNA–protein interactions and cast doubt on the validity of some of the conclusions of this research. These problems are nonspecific sticking of proteins to RNA and artifactual losses of true mRNA-associated proteins. The strategy with crosslinking is to isolate polyribosomes under conditions that minimize dissociation of proteins from RNA and, after irradiation, to isolate and work with crosslinked mRNA–protein complexes under conditions that prevent nonspecific sticking of proteins to RNA.

In a previous study in this laboratory (9), several proteins crosslinked to mRNA by irradiating polyribosomes were identified. Furthermore, results to date support the concept that specific proteins are crosslinked to specific regions of mRNA molecules. First, the same proteins were crosslinked to mRNA, whether intact polyribosomes or EDTA-released mRNA–protein complexes were labeled (9). Second, only one protein having a molecular weight of 78,000 (p78A) was crosslinked to poly(A), although six proteins were crosslinked to mRNA sequences other than poly(A) (9). One of these had a molecular weight of 78,000. However, further work has shown that this protein (p78X) differs from p78A in its partial peptide map. Therefore, p78A and p78X are probably products of different genes that bind to different sites on mRNA molecules (10).

In the study described here, the proteins crosslinked to mRNA in the presence and absence of mRNA synthesis, as well as the labeling kinetics of these proteins, are investigated by use of crosslinking methods.

MATERIALS AND METHODS

Cell Culture, Labeling, and Fractionation. Mouse L cells were propagated in suspension cultures. In experiments to determine the effects of inhibiting mRNA synthesis on the synthesis of mRNA-associated proteins, 800-ml suspension cultures were grown overnight in medium containing L-methionine at 1.9 μg/ml, which is one-eighth the usual concentration. The medium also contained nondialyzed 4% calf serum and 4% fetal calf serum. When the cel' had reached a density of 3.0 to 3.5 $\times 10^5$ cells per ml, one-half of the culture was treated with actinomycin D at 0.08 μg/ml for 30 min to inhibit ribosome synthesis, and the other half was treated with the drug at 2 μg/ml for 30 min to inhibit all RNA synthesis. Each sample was then concentrated to 50 ml in the same medium and labeled for 70 min with [^{35}S]methionine (10–20 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) and [^{3}H]adenosine (10 μCi/ml) (New England Nuclear). In labeling kinetics experiments, cells were grown in medium containing L-methionine at 7.6 μg/ml, treated with actinomycin D at 0.08 μg/ml for 30 min, concentrated 8-fold, and labeled with [^{35}S]methionine only at 28.6 μCi/ml. At intervals, samples of cells were harvested for isolation of polyribosomes.

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In all experiments, the cells were lysed and polyribosomes were isolated as described (11). Briefly, cells were lysed gently in isotonic KCl buffer containing 0.1% Triton X-100, and polyribosomes were isolated from 16,000 × g supernatants by centrifuging through 15–45% (wt/wt) sucrose gradients in 0.01 M NaCl/0.01 M Tris·HCl, pH 7.6/2 mM $MgSO_4$. The polyribosomes were further purified and concentrated by pelleting through 2 M sucrose in the same buffer.

Irradiation of Polyribosomes and Isolation of Crosslinked mRNA–Protein Complexes. In most experiments, pelleted polyribosomes were dissolved in 0.01 M NaCl/0.01 M Tris·HCl, pH 7.6/2 mM $MgSO_4$/10% (vol/vol) glycerol at $A_{260} \approx 1.5$, and 1-ml aliquots were irradiated on ice in polyethylene vial caps (outer diameter, 22 mm) with a 15-W germicidal lamp having more than 90% of its emitted energy at 254 nm (Sylvania G15T8). The radiation intensity was adjusted to 2000 $\mu W/cm^2$ with the aid of an Ultraviolet Products (San Gabriel, CA) J225 meter, and a total incident dose of 2×10^5 ergs/mm^2 was delivered. The irradiated polyribosomes were precipitated from ethanol. In one experiment, polyribosomes (≈18 ml) were irradiated to 10^5 ergs/mm^2 in a 10-cm Petri dish prior to pelleting through 2 M sucrose, and the ethanol precipitation step was omitted. For chromatography on oligo(dT)-cellulose, samples were dissolved in 0.5% $NaDodSO_4$/0.01 M Tris·HCl, pH 7.6/1 mM EDTA/1% 2-mercaptoethanol which was then made 0.5 M in NaCl, heated to 60°C for 3 min, and applied to a column containing oligo(dT)-cellulose T_3 (Collaborative Research, Waltham, MA) at 0.1 g per ≤10 A_{260} units of polyribosomes. The column was washed with the above buffer containing 0.5 M NaCl, and then bound material was eluted with the same buffer lacking NaCl. The eluate was again made 0.5 M in NaCl, heated to 60°C, and recycled through the column. In the second cycle, 95% of the ^3H cpm and 70–80% of the ^{35}S cpm bound in the first cycle of chromatography were rebound.

For isolation of the poly(A)–protein complex, the crosslinked poly(A)-containing mRNA–protein complexes isolated by chromatography on oligo(dT)-cellulose were precipitated from ethanol, dissolved in 0.5 ml of 0.5% sodium lauroyl sarcosinate/0.01 M Tris·HCl, pH 7.6/1 mM EDTA/0.5 M NaCl/1% 2-mercaptoethanol, digested with 5 μg of RNase A and 50 units of RNase T1 (Worthington) at 37°C for 30 min, heated to 60°C for 3 min, and applied to an oligo(dT)-cellulose column. The column was washed and eluted as above.

Polyacrylamide Gel Electrophoresis. Polyribosomes and samples from oligo(dT)-cellulose columns were precipitated from ethanol. The dried precipitates were dissolved in 0.15 ml of 0.5% sodium lauroyl sarcosinate/0.0625 M Tris·HCl, pH 6.8/10% glycerol/1% 2-mercaptoethanol. The samples were digested with 12.5 units of RNase T_2 (Sigma, type VI) for 1 hr at 37°C, then 0.1 vol of 20% $NaDodSO_4$/0.01% bromophenol blue was added; the samples were boiled for 2 min and then applied to a 10% polyacrylamide slab gel (10 × 0.15 cm). The gels were prepared and run as described by Laemmli (12). ^{14}C-Labeled marker proteins having molecular weights of 92,000, 68,000, 45,000, 30,000, and 12,500 were included in all gels. After electrophoresis the gels were examined by fluorography (13).

RESULTS

To investigate the synthesis of mRNP proteins in the presence and absence of mRNA synthesis, mouse L cells were treated with actinomycin D at 0.08 μg/ml to inhibit ribosome synthesis or at 2 μg/ml to inhibit all RNA synthesis (14, 15). Cells treated with the low dose of actinomycin D were used as the control, rather than untreated cells, in order to prevent entrance of labeled rRNA and ribosomal proteins into polyribosomes. Labeling of rRNA and ribosomal proteins would complicate analysis of the effects of a high dose of actinomycin D on labeling of mRNA and mRNA-associated proteins. After a 30-min preincubation, which is sufficiently long to terminate the flow of mRNA from the nucleus to the cytoplasm in high-actinomycin-treated cells (16, 17), they were doubly labeled with [^{35}S]methionine (for protein) and [^3H]adenosine (for RNA). Table 1 summarizes the incorporation data for three separate experiments. The high dose of actinomycin D inhibited [^3H]adenosine incorporation into polyribosomes by 79–94% relative to the low dose. However, there was no inhibition of [^{35}S]methionine incorporation. In fact, in two of the three experiments there may have been a slight stimulation of incorporation.

When the extent of crosslinking of [^{35}S]methionine-labeled proteins to mRNA was analyzed by chromatography on oligo(dT)-cellulose in the presence of $NaDodSO_4$ (9), no difference was found between the two actinomycin treatments; 3.2–5.5% of the labeled protein (as cpm) was crosslinked to mRNA. Similarly, there was no difference between high and low doses with respect to the extent of labeled protein crosslinking to poly(A) (0.27–0.55% of the total ^{35}S cpm in polyribosomes). The crosslinked poly(A)–protein complex was isolated by chromatography on oligo(dT)-cellulose after digestion of poly(A)$^+$mRNA–protein complexes with RNases A and T1 in high-salt medium.

In order to investigate the possible effects of inhibiting mRNA synthesis on the synthesis of individual polyribosomal and mRNA-associated proteins, they were analyzed in $NaDodSO_4$/polyacrylamide gels. Fig. 1 shows the total polyribosomal proteins labeled in low- and high-dose actinomycin D-treated cells (lanes *A* and *B*). All of the proteins detectable in the low-dose-treated cells were also detectable in the high-dose-treated cells. Fig. 1 also shows the proteins crosslinked to mRNA in high- and low-dose-treated cells. The proteins with molecular weights 98,000, 78,000, 75,000, 68,000, 62,000, and 52,000 previously identified as the major proteins crosslinked to mRNA sequences other than poly(A) were labeled in both cases. Similarly, the 78,000 protein crosslinked to poly(A) was labeled in both low- and high-dose-treated cells. As noted above, the 78,000 proteins crosslinked to poly(A) and to se-

Table 1. Effects of inhibiting mRNA synthesis on synthesis of mRNA-associated proteins

Effect	Exp.	Low actinomycin D ^{35}S	Low actinomycin D ^3H	High actinomycin D ^{35}S	High actinomycin D ^3H
Incorporation into total polyribosomes, cpm/A_{260}	1	529,896	56,165	513,885	3,540
	2	509,158	62,423	520,914	13,448
	3	342,686	57,175	380,903	3,740
% inhibition of incorporation*	1	0	0	3.0	93.6
	2	0	0	−2.3	78.5
	3	0	0	−11.0	93.5
% of cpm bound to oligo(dT)	1	5.5	60	4.4	ND
	2	4.4	46	5.0	ND
	3	3.2	57	3.4	ND
% of cpm crosslinked to poly(A)	1	ND	—	ND	—
	2	0.55	—	0.57	—
	3	0.36	—	0.27	—

Actinomycin doses: low, 0.08 μg/ml; high, 2 μg/ml. ND, not done.
* Incorporation was set at 100% in the case of low-actinomycin-treated cells.

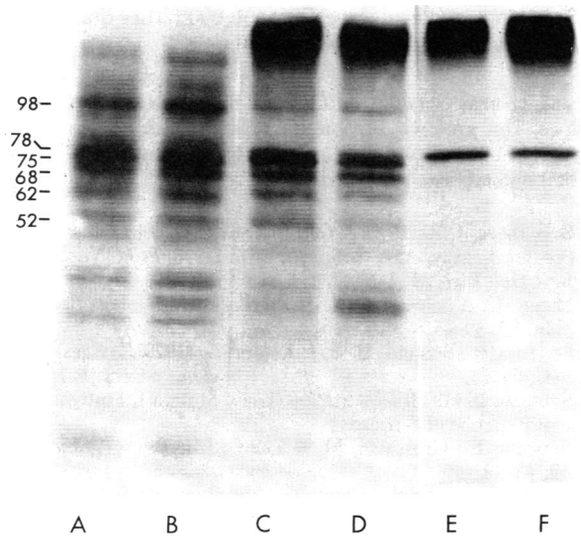

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis of polyribosomal proteins, proteins crosslinked to mRNA sequences other than poly(A), and proteins crosslinked to poly(A) in the presence or absence of mRNA synthesis. Samples of polyribosomes or samples eluted from oligo(dT)-cellulose were analyzed electrophoretically. The numbered lines indicate the apparent molecular weights ($\times 10^{-3}$) of the proteins. Lanes: A and B, total polyribosomal proteins, 20,000 cpm per gel, 4-day exposure; C and D, proteins crosslinked to mRNA sequences other than poly(A), 20,000 cpm per gel, 4-day exposure; E and F, proteins crosslinked to poly(A), 6000 cpm (lane E) and 8000 cpm (lane F) per gel, 14-day exposure. Samples in lanes A, C, and E were from low-dose-treated cells. Samples in lanes B, D, and F were from high-dose treated cells.

quences other than poly(A) are probably products of different genes. In high-dose-treated cells there was a band at about 35,000, visible both in the proteins crosslinked to mRNA and in the total polyribosomal proteins, which was relatively faint in low-dose-treated cells. Although the significance of this result is unknown, it was reproducibly obtained. The radioactivity remaining at the origin of the gel in some lanes of Fig. 1 probably resulted from protein–protein crosslinking (9).

It has been reported that the synthesis of the 78,000 poly(A)-associated protein is inhibited in the absence of mRNA synthesis (18). When the effect of a high dose of actinomycin D on the synthesis of the 78,000 protein crosslinked to poly(A) was analyzed quantitatively by densitometry of autoradiograms, a slight reduction in its synthesis was found. However, this reduction was never more than a factor of 2 in experiments in which mRNA synthesis was inhibited by a factor of 20 (for example, experiments 1 and 3 in Table 1). Therefore, it seems clear that this protein, as well as the other proteins crosslinked to mRNA, is synthesized and becomes associated with mRNA in the absence of mRNA synthesis.

The labeling kinetics of the polyribosomal proteins crosslinked to mRNA were analyzed to obtain information on their time of addition to mRNA and on their metabolic stability. For this purpose the cells were exposed continuously to [^{35}S]methionine in the presence of actinomycin D at 0.08 μg/ml in medium containing half the amount of L-methionine in complete growth medium. Incorporation of [^{35}S]methionine into trichloroacetic acid-insoluble form in whole cells was linear for 2 hr under these conditions (not shown). Fig. 2 shows [^{35}S]methionine incorporation into total polyribosomal proteins and proteins crosslinked to mRNA. In both cases, incorporation was linear for the duration of the experiment. In the case of proteins crosslinked to mRNA the labeling curve extrapolates to the origin. This is the result expected if addition of labeled

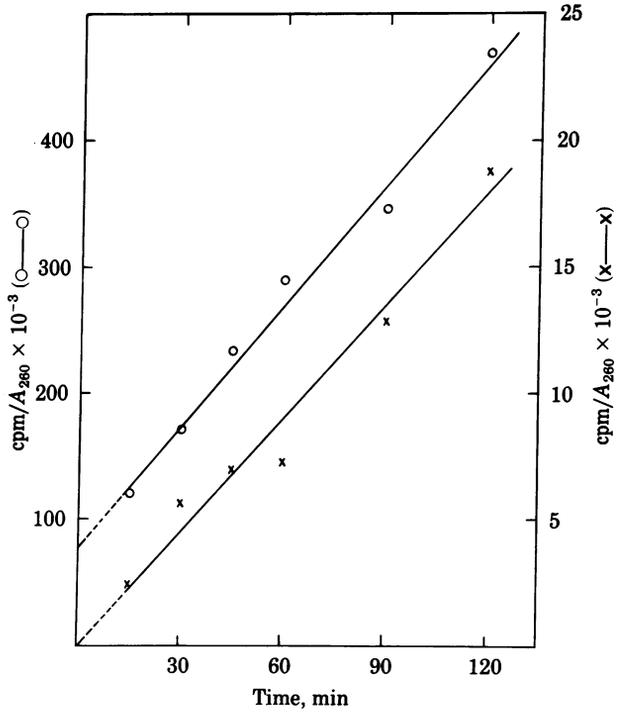

FIG. 2. Labeling kinetics of mRNA-associated proteins and total polyribosomal proteins. Cells were labeled with [^{35}S]methionine in medium containing actinomycin D at 0.08 μg/ml. At intervals, samples of cells were harvested for isolation of polyribosomes and measurement of the total trichloroacetic acid-insoluble radioactivity. In the experiment shown, polyribosomes were made 5 mM in EDTA prior to irradiation. However, similar results were obtained in another experiment in which intact polyribosomes were irradiated (not shown). The solid straight lines are fitted by a least-squares procedure; the broken lines are extrapolations of the fitted lines. ×, Proteins crosslinked to poly(A)-containing mRNA; ○, total polyribosomal proteins.

proteins to mRNA occurs in the cytoplasm. If addition of the proteins to mRNA occurred at the time of transcription, or early in posttranscriptional processing, then there would be a lag of 20–30 min in the appearance of labeled proteins in the cytoplasm, similar to the lag in the appearance of labeled mRNA (17, 19, 20). The finding that incorporation into these proteins was linear for 2 hr indicates that their average half-life must be greater than 2 hr. In the case of total polyribosomal proteins, the labeling curve extrapolates to a positive value of cpm incorporated at $t = 0$. This indicates the presence of a rapidly labeling component, presumably nascent peptides, that is maximally labeled in about 2 min (21). A rapidly labeling component was not observed in the case of proteins crosslinked to mRNA. Therefore, nascent peptides were not a detectable fraction of the proteins crosslinked to mRNA.

DISCUSSION

Evidence has been presented that the proteins associated with mRNA in L-cell polyribosomes are synthesized and become associated with mRNA in the absence of mRNA synthesis. Therefore, the proteins must associate in the cytoplasm with previously existing mRNA. Also, the newly synthesized labeled proteins must displace previously existing unlabeled ones from the mRNA. In other words, there is an exchange between proteins bound to mRNA and free proteins, and the mRNA–protein complex is a dynamic structure rather than a static one. This behavior contrasts with the behavior of ribosomal structural proteins which, for the most part, become associated with preri-

bosomal RNA in the nucleolus and remain an integral part of the ribosome throughout its functional lifetime (15, 22). The observed turnover of the mRNP proteins is consistent with the idea that they function in translation because it is already known that the soluble proteins with which the mRNP proteins apparently exchange can substitute for initiation and elongation factors in translation (4, 7, 8).

The labeling kinetics of the mRNP proteins are also consistent with cytoplasmic addition to mRNA. They do not by themselves demonstrate an exchange between proteins bound to mRNA and free proteins. However, they partially corroborate the high-dose actinomycin experiments without having to inhibit mRNA synthesis. The labeling kinetics also suggest that the mRNP proteins are fairly stable, having an average half-life of greater than 2 hr. This half-life, which is long compared with the protein synthesis time of about 2 min, suggests that each protein molecule is used in multiple rounds of translation if these proteins do indeed function in translation.

The present results also show that the mRNP proteins are not derived from hnRNP. This conclusion is in agreement with various observations that hnRNP proteins are both more complex than and qualitatively different from mRNP proteins (23, 24). The finding in the present work that the 78,000 protein associated with the poly(A) segment of mRNA is synthesized and becomes associated with mRNA in the absence of mRNA synthesis indicates that this protein also cannot be entirely derived from hnRNP, although it has been reported that a protein of this size is associated with the poly(A) of hnRNA (25). In agreement with a previous report (18) there was some reduction in the synthesis of the 78,000 cytoplasmic poly(A)-associated protein in the absence of mRNA synthesis. However, this reduction was at most a factor of 2, whereas mRNA synthesis was reduced by a factor of 20. This finding argues that the synthesis of this protein is not tightly coupled to mRNA synthesis. Baer and Kornberg (26) recently reported that the cytoplasmic poly(A)–protein complex has a structure that is fundamentally different from that of the nuclear complex. Their results also suggest that the cytoplasmic poly(A)-associated protein is not derived from hnRNP. In a study, by means of ultraviolet light-induced crosslinking, of the proteins associated with the poly(A) of hnRNP, a single protein having a molecular weight of 60,000 rather than 78,000 has been detected (10).

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