Proteins associated with poly(A)⁺RNA of cockerel liver: Effects of estradiol stimulation

(two-dimensional electrophoresis/silver staining/messenger ribonucleoprotein)

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ABSTRACT The protein population of poly(A)-containing messenger ribonucleoprotein (mRNP) from cockerel liver was analyzed before and after estradiol induction. Polysomal and free mRNP were isolated by thermal elution from oligo(dT)-cellulose. The proteins were separated by two-dimensional electrophoresis (of which the first dimension was a nonequilibrium pH gradient) and were visualized by silver staining. In order to determine similarities and differences between proteins of various mRNP fractions, trace amounts of ¹²⁵I-labeled protein from one fraction were coelectrophoresed with stainable amounts of protein from another fraction on the same gel. Of 27 proteins analyzed, 15 were common to polysomal and free mRNP, 7 were specific to polysomal mRNP, and 5 were specific to free mRNP. Moreover, estradiol strongly influenced 11 proteins. Six proteins changed either in relative intensity or in distribution between polysomal and free mRNP fractions. Three major proteins appeared in both fractions, and two additional proteins disappeared from the free mRNP fraction after estradiol treatment. The results suggest that the protein population in polysomal mRNP is quite complex and that the profound influence of estradiol on protein synthesis in cockerel liver may be connected to changes in the protein composition of mRNP.

In eukaryotic cells, mRNA associates with a variety of proteins and exists *in vivo* as two classes of messenger ribonucleoprotein (mRNP), termed free mRNP or polysomal mRNP, depending on whether or not it is bound to polysomes. Although it is clear that free mRNP can serve as a direct precursor to polysomal mRNP (1), evidence for additional functional roles for free mRNP has been presented. Such roles include sequestration of different frequency classes of mRNA (2), storage of particular mRNAs in an inactive form (3), and efficient packaging of accessory factors required for protein synthesis (4). Proteins associated with free and polysomal mRNP have been examined in a variety of systems. In many cell types, two major proteins of $M_r \approx 50,000$ and $\approx 78,000$ are found associated with polysomal mRNP (5). There is less agreement concerning the proteins that constitute the free mRNP. Most investigators have reported additional or different proteins, or both, present in free mRNP. However, a recent report (6) describing ultraviolet crosslinking of labeled protein to mRNA *in vitro* suggests that there may be considerable overlap between the proteins of polysomal and free mRNPs and more complexity in the protein of polysomal mRNP than previously thought.

We examined the proteins of free and polysomal poly(A)⁺-mRNP isolated from cockerel liver, using two-dimensional electrophoresis combined with silver staining. In addition, we isolated mRNP from cockerel liver after treatment *in vivo* with estradiol, which stimulates protein synthesis 3-fold and induces the synthesis of specific proteins such as vitellogenin (7). Our

results suggest that numerous proteins are shared by free and polysomal mRNPs and that there are sets of proteins unique to free mRNP and to polysomal mRNP. Moreover, we find that estradiol treatment markedly affects at least 11 proteins of mRNP.

MATERIALS AND METHODS

Procurement of cockerels and treatment with estradiol were as described (8). Cockerels were sacrificed 3–4 days after injection with estradiol, at the peak of vitellogenin induction. Total cytoplasmic ribonucleoprotein (RNP) was isolated by Mg²⁺ precipitation following the method of Bast *et al.* (8) with some modification. Briefly, this method consists of precipitating RNP by adjusting the pH and Mg²⁺ concentration of a postmitochondrial supernatant to 5.5 and 0.1 M, respectively, followed by pelleting the precipitate at low speed into a Mg²⁺-free pad containing 1 M sucrose. The following modifications were introduced: (i) 10 mM vanadyl ribonucleosides were included in the homogenization buffer to control ribonuclease activity (9); (ii) sodium heparin (3 mg/ml; Fisher) was included in the sucrose-containing pad buffer (100 mM 4-morpholineethanesulfonic acid (MES)/300 mM KCl/1 mM dithiothreitol/1 M sucrose, pH 6.0 at 0°C) and resuspension buffer (pad buffer without sucrose). Phenylmethylsulfonyl fluoride (0.5 mM) was included in all buffers to control protease activity. To isolate total (free and polysomal) mRNP, a modification of the thermal elution procedure of Jain and Sarkar (10) was used. Resuspended total cytoplasmic RNP was adjusted to 0.4 M KCl, and polysomes were dissociated with puromycin (50 µg/ml; Sigma) by the method of Blobel and Sabatini (11). The mRNPs were stirred for 1 hr at 0°C with oligo(dT)-cellulose (type T3; Collaborative Research) previously equilibrated with binding buffer containing 10 mM 2-[[tris(hydroxymethyl)methyl]amino]ethane sulfonic acid (TES), 0.4 M KCl, 1 mM dithiothreitol, and 100 µg of sodium heparin per ml (pH 7.5 at 0°C). The cellulose was pelleted at low speed, resuspended in binding buffer, poured into a minicolumn (Isolab), and washed with binding buffer until no absorbance was detectable at 260 nm. The column was then transferred to a 45°C water bath and eluted with elution buffer (binding buffer containing only 10 mM KCl). The mRNPs were concentrated by ethanol precipitation and resuspended in sterile distilled water before use. To obtain polysomal and free fractions, the resuspended total mRNP fraction containing polysomes and free mRNP was layered on 20–50% sucrose gradients (8) and centrifuged overnight in the SW 27 rotor. Gradients were monitored with a Gilford recording spectrophotometer and divided into ≤80S and >80S fractions. The 80S monomer peak was determined to be almost entirely dissociable by 0.5

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Abbreviations: RNP, ribonucleoprotein; mRNP, messenger ribonucleoprotein.

M KCl and, thus, assigned to the free fraction. mRNP was obtained from the two fractions by thermal elution from oligo(dT)-cellulose as described.

Yields of mRNP were generally 0.3–0.4% of the input of A_{260} material applied to the oligo(dT)-cellulose. Control experiments (data not shown) established that poly(A)⁺RNA obtained from the total polysomal and free mRNP fractions could stimulate a nuclease-treated reticulocyte lysate (New England Nuclear) 5-fold more than endogenous incorporation of labeled amino acid into protein and that elution of the mRNP from oligo(dT)-cellulose with formamide did not alter the protein composition as judged by one-dimensional gel electrophoresis.

Two-dimensional nonequilibrium pH gradient electrophoresis was as described by O'Farrell *et al.* (12) with 10-cm first-dimension gels containing Ampholines pH 3.5–10; LKB. mRNP (12–17 μ g of protein) was treated with boiled RNase A (100 μ g/mL; 15 min at 37°C) in 50 μ L of distilled water. To prepare samples for gels, we added 50 mg of urea, 2 μ L of Ampholines, 2 μ L of Nonidet P-40, 5 μ L of 2-mercaptoethanol, and 0.5 μ L of 10% NaDodSO₄ and heated the mixture at 37°C until the urea dissolved. The gels were run at 600 V for 2 hr, equilibrated for 15 min in the stacking-gel buffer for the second dimension (5% 2-mercaptoethanol/2% NaDodSO₄/10% glycerol), and sealed with agarose onto 10% acrylamide gels (13) for the second-dimension electrophoresis. Silver staining was by the method of Oakley *et al.*, (14) with the addition of a brief treatment with 0.02 M Tris (pH 9) to wash out residual Ampholines, which stain heavily with silver.

Proteins were iodinated with Na¹²⁵I (New England Nuclear) by using the chloramine-T procedure as modified by Leister and Dawid (15). Autoradiography was performed with preflashed Kodak X-Omat AR film at –70°C and intensifying screens.

RNA was estimated by absorbance at 260 nm. Protein was estimated by the Bradford procedure (16) with bovine serum albumin as standard.

RESULTS

Several criteria indicate that the proteins we examined are specifically associated with poly(A)⁺mRNA. First, after elution of mRNP from oligo(dT)-cellulose, there were few if any proteins that appeared to be ribosomal proteins based on one-dimensional gels (Fig. 1). No ribosomal RNA was detectable in the mRNP RNA, whereas it appeared mostly intact in the unbound fraction when analyzed on denaturing gels (17). Second, when RNA was labeled *in vivo* with [³H]guanosine and the isolated mRNPs were hybridized to poly(U), >90% of the specific binding to oligo(dT)-cellulose was prevented. When isolated mRNPs were bound to oligo(dT)-cellulose a second time, the spectrum

FIG. 1. Comparison of proteins from RNP that specifically bound to oligo(dT)-cellulose and those that did not bind. The total polysomal fraction was dissociated with puromycin and high salt and bound to oligo(dT)-cellulose; 25 μ g of protein from RNP specifically bound to the column (lane 1) and 80 μ g of protein from unbound RNP (lane 2), mainly ribosomes, were electrophoresed on a 5–17.5% acrylamide gradient gel and stained with Coomassie blue. The proteins below the M_r 43,000 marker in lane 2 are mainly ribosomal proteins. M_r markers are shown $\times 10^{-3}$.

of proteins obtained was the same as before the second binding (data not shown).

The proteins we analyzed probably represent those most tightly complexed within the mRNPs because of the presence of vanadyl ribonucleosides (10 mM) and heparin (3 mg/ml) during the preparation. These compounds are known to be competitors for RNA-binding proteins. Moreover, mRNPs were bound to oligo(dT)-cellulose in the presence of 0.4 M KCl, which is sufficient to dissociate nonspecific RNA–protein complexes (18). The protein/RNA ratio of the mRNP was 0.27 ± 0.06 ($n = 6$) μ g of protein per μ g of RNA. This is lower than that usually reported (19, 20). Ribosomes exposed to the same conditions of vanadyl ribonucleosides and heparin also showed a substantial reduction in protein/RNA ratio (0.5 μ g of protein per μ g of RNA).

FIG. 2. Effect of iodination on the migration of proteins in nonequilibrium pH gradient electrophoresis. (A) Silver-stained gel of protein from total (free and polysomal) mRNP from induced cockerel. A portion of this material was iodinated and included with the remainder of the sample. Arrowhead, albumin that was introduced as a contaminant during iodination. (B) Autoradiogram of the gel shown in A. M_r markers are shown $\times 10^{-3}$ and are β -galactosidase, phosphorylase A, bovine serum albumin, and ovalbumin (each at 100 ng).

FIG. 3. Representative autoradiograms of iodination experiments used to identify proteins shared between different mRNA fractions. In both A and B, the sample contained iodinated polysomal protein from mRNA of induced cockerel liver. (A) The iodinated polysomal protein was coelectrophoresed with stainable amounts of polysomal protein from mRNA of uninduced liver (see Fig. 4B). (B) The iodinated polysomal protein was coelectrophoresed with protein from free mRNA of induced liver (see Fig. 4C).

We considered four mRNA fractions for analysis of protein content: (i) free mRNA from estradiol-treated (induced) and untreated (uninduced) cockerels and (ii) polysomal mRNA from induced and uninduced birds. To identify spots common to gels of different mRNA fractions, we performed several experiments in which radioiodinated proteins from mRNA were included in the gel samples. We first ascertained that radioiodination did not affect the migration of protein in either dimension. The iodinated protein from mRNA was coelectrophoresed with a large excess of unlabeled protein from the same preparation. As shown in Fig. 2, those proteins visible on the autoradiogram could be superimposed on the stained gel. Therefore, it was possible to determine similarities and differences between samples by the inclusion of trace amounts of labeled protein from

one fraction with stainable amounts of protein from another fraction in the same gel. We next radioiodinated free or polysomal protein from mRNA of an induced bird. The labeled free mRNA protein was included in gels of unlabeled polysomal mRNA protein from induced birds and in gels of unlabeled free mRNA protein from uninduced birds. Similarly, the labeled polysomal mRNA protein was included in gels of unlabeled free mRNA protein from induced birds and in gels of unlabeled polysomal mRNA protein from uninduced birds.

Representative autoradiograms of iodinated polysomal protein from mRNA of induced birds are shown in Fig. 3. The iodinated sample was coelectrophoresed with stainable amounts of protein from polysomal mRNA of uninduced liver (Fig. 3A; stain shown in Fig. 4B) and was coelectrophoresed with stain-

FIG. 4. Silver-stained gels of free (A and C) and polysomal (B and D) protein from mRNA. (A and B) Free and polysomal fractions, respectively, from livers of uninduced cockerels. (C and D) Free and polysomal fractions, respectively, from livers of cockerels injected 4 days previously with estradiol. *M_r* markers were as in Fig. 2. Spot nomenclature is described in the text.

Table 1. Fraction assignments and response to estradiol of proteins from mRNP

Spot	Uninduced		Induced	
	Free	Polysomal	Free	Polysomal
4-21*	+	+	+	+
3-23	+	m	+	+
5-27*	-	+	-	+
6-32*	m	-	+	-
2-34*	m	m	E	+
6-37*	m	-	+	-
2-39*	m	-	+	-
2-41*	-	m	+	+
2-42*	+	+	+	+
6-43*	+	+	+	+
6-45*	+	+	+	+
7-48*	+	m	+	+
2-49*	+	+	+	+
7-50*	m	m	E	E
2-51*	m	m	+	+
7-53*	m	m	E	E
7-56*	-	-	+	+
2-61	-	+	-	+
7-64	-	-	+	+
3-68	-	+	-	+
2-72	-	+	-	+
6-77*	-	-	+	+
2-78	-	+	-	+
3-86	+	-	-	-
4-93	+	-	-	-
5-104*	m	m	E	E

Analysis of the gels is described in the text. m, Marginal; +, present; -, absent; E, increased relative intensity with respect to the gel of the corresponding fraction from uninduced birds.

* Identified by iodination.

able amounts of protein from free mRNP of induced liver (Fig. 3B; stain shown in Fig. 4C). In this way, 19 proteins were positively identified as shared by two or more mRNP fractions. An additional eight spots had sufficiently characteristic staining patterns to allow identification in different gels (see below). Thus, we were able to analyze 27 proteins from mRNP with respect to their location in the free or polysomal fraction and their response to estradiol stimulation.

Fig. 4 shows silver-stained gels of free and polysomal mRNP proteins isolated from induced or uninduced birds. The spot coordinates were derived from analysis of two independent sets of gels of this type. For example, spot 2-49 was most commonly found in the second centimeter of the first-dimension gel (numbered from the acidic end) and had a mean M_r of about 49,000 based on eight gels.

Analysis of the stained gels was done in the following manner. Gels from a number of preparations were crudely scored on the basis of relative spot intensity within a gel and compared. To minimize staining variability, assignments to polysomal and free fractions were made from gels that were stained with the same ammoniacal silver solution. Differences in relative intensity between gels of protein from mRNP of induced and uninduced birds were confirmed similarly. Differences in relative intensity were considered significant only if obvious and consistent in all preparations examined. Spots were classified as "marginal" for a given fraction if they did not appear in every preparation, and, when present, were near the threshold of detection. All 10 marginal spots were confined to fractions from uninduced cockerels, and, with 2 exceptions (spots 7-50 and 7-53), were present in at least 50% of the gels of that particular fraction. All of the marginal proteins (Table 1) are shown in Fig. 4 A and B.

The distribution of proteins among the four mRNP fractions is summarized in Table 1. In all four fractions examined, 12 spots were present of which 6 were marginal as defined above. Seven spots were specific to polysomal mRNP, of which one was marginal. Five spots were specific to free mRNP, of which three were marginal. Eleven spots showed changes in relative intensity or distribution correlated with estradiol induction. Three spots were not detected in uninduced cockerels (spots 7-56, 7-64, and 6-77). Spot 6-77 may well be the poly(A)⁺-binding protein found in many cell types. Other spots were occasionally detectable in uninduced cockerels but had much greater relative intensity after estradiol induction (spots 7-50, 7-53, and 5-104). Two spots specific to free mRNP were not detected in preparations from induced cockerels (spots 3-86 and 4-93). Three additional spots showed less pronounced changes. Spots 2-41 and 3-68, both of which were confined to polysomal mRNP in uninduced birds, also appeared in the free fraction after estradiol induction. Spot 2-34, an inconspicuous marginal spot common to free and polysomal fractions in uninduced cockerels, became quite prominent in the free fraction in estradiol-stimulated birds. In all, six spots from uninduced birds showed large increases in relative intensity after estradiol induction, three spots were undetectable before induction, and two were undetectable after induction. Of the spots considered, 15 were common to free and polysomal fractions, of which 3 were present only after estradiol treatment (Table 1).

DISCUSSION

The most striking features of our results are the large number of proteins reproducibly associated with mRNP, particularly the polysomal fraction, and the substantial qualitative and probably quantitative effects associated with estradiol stimulation. The large number of proteins observed is attributable to the high sensitivity of the electrophoretic system used and is comparable to that seen for (nonpolysomal) globin mRNP of duck erythroblasts (21) and rabbit reticulocytes (22) when examined by two-dimensional gel electrophoresis. Recent evidence suggests that numerous proteins can be crosslinked *in vitro* to the polysomal poly(A)⁺mRNA of mouse L cells (6). In addition, the proteins of polysomal mRNP seem to exchange with a cytoplasmic pool of proteins (23). This may suggest a role for these proteins in the translation process because it is known that cytoplasmic RNA-binding proteins, which are likely candidates for those proteins with which the proteins from mRNP exchange, can substitute for initiation and elongation factors in translation (4, 24, 25). In turn, this could influence the loading of ribosomes on mRNA and the rate of polypeptide elongation, both of which are affected by estradiol induction in cockerel liver (8, 26, 27). It is conceivable that mRNA could transiently associate with a considerable variety of proteins during translation.

It could be argued that the protein population from mRNP is the same before and after estradiol treatment but that the hormone changes the spectrum of mRNP bound to oligo(dT)-cellulose by affecting the poly(A) tails—e.g., by altering their size or the sequence representation in poly(A)⁺mRNP. This explanation seems unlikely to us because, if this were the case, proteins such as those in spots 6-77 and 5-104, which show dramatic increases after estradiol treatment, should appear in the oligo(dT)-cellulose runthrough from samples of uninduced liver if they were present in RNP form. They were never observed even on silver-stained gels.

Although our isolation procedure and experimental system differ in many respects from those of others, several features are consistent with other reports. Vincent *et al.* (21), using a nonequilibrium pH gradient electrophoresis technique, found

in duck erythroblasts a prominent mRNP protein with very similar electrophoretic characteristics in both dimensions to the protein in spot 6-77. Using nucleases, they identified this protein as the poly(A)-binding protein common to many cell types, and comparable preliminary experiments in our laboratory indicate that protein 6-77 binds to the poly(A)-containing segment of mRNA. Because the presence of protein 6-77 depends upon prior estradiol treatment, our results suggest that its association with mRNA can be regulated. In duck erythroblasts (21) and rabbit reticulocytes (28), the poly(A)-binding protein apparently associates only with polysomal mRNP, whereas in embryonic and cell culture systems, it is found in the free mRNP as well (5). Several other laboratories have found a prominent single protein (19) or doublet (21, 29) in the M_r range of 48,000 to 52,000 when analyzing protein from mRNP of avian material. Spots 6-43 and 6-45 may be the equivalent(s) in our system because their behavior in both dimensions is similar (21). When analyzed on 5-17.5% gradient gels, they possess M_r of 46,500 and 48,700, respectively ($n = 6$).

Although no function for proteins from mRNP is yet known with certainty, it seems likely that the observed changes in the mRNP protein population are related to the known effects of estradiol on the liver's transcriptional and translational apparatus. Control over the synthesis of liver proteins is known to be exerted at several points. For example, the accumulation of vitellogenin mRNA after estradiol induction is due both to induction of mRNA synthesis and to an estrogen-dependent increase in mRNA stability (30). The amount of vitellogenin protein synthesized is a function both of the amount of mRNA present and of the specific rate of vitellogenin polypeptide chain elongation, which changes after estradiol induction (27). Estradiol induction also results in a large average increase in the number of ribosomes per polysome (8). The absolute amount of chicken serum albumin mRNA does not seem to change after estradiol treatment (31), although the rate of albumin polypeptide chain elongation does change in a manner different from that of vitellogenin (27). All of these effects may be related to the protein population of specific mRNP after induction.

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