Proteins Associated with the Messenger Ribonucleoprotein Particle for the Estrogen-Regulated Apolipoprotein II mRNA†

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ABSTRACT: The stability of the mRNA for apolipoprotein (apo) II is regulated by estrogen [Gordon et al. (1988) *J. Biol. Chem. 263*, 2625-2631]. On the hypothesis that estrogen regulation of apoII mRNA stability is mediated through mRNA-protein interaction, we have examined the messenger ribonucleoprotein particle (mRNP) for apoII mRNA following release from chicken liver polyribosomes. Polyribosomes containing undegraded apoII mRNA were obtained when tissue was homogenized without detergent, and polyribosomes were isolated following simultaneous addition of detergent and magnesium to a 20000*g* supernatant. ApoII mRNP released by EDTA sedimented at 12-18 S in sucrose gradients, and banded at ρ = 1.4 g/mL in CsCl isopycnic centrifugation, indicative of a 3:1 ratio of protein to mRNA. A fraction in which apoII mRNP was enriched to 40-50% of total mRNP was prepared by successive size fractionation steps on sucrose gradients. Proteins associated with sucrose gradient enriched apoII mRNP were examined by iodination of UV-cross-linked proteins followed by SDS-polyacrylamide gel electrophoresis. Comparisons of proteins in highly enriched apoII mRNP to proteins in mRNP from non-estrogen-treated rooster liver did not reveal any differences. This result suggests that the major proteins associated with apoII mRNA are mRNP proteins also associated with the bulk of liver mRNAs.

A number of recent studies have focused on gene regulation at the posttranscriptional level. Examples include autoregulation of splicing (Bell et al., 1988), developmental or cell type specific changes in splicing (Leff et al., 1987) or polyadenylation (Leff et al., 1986), and cytoplasmic iron-responsive factors which regulate translation or mRNA stability (Casey et al., 1988; Owen & Kühn, 1987; Brown et al., 1989). It is increasingly apparent that mRNA stability plays a crucial role in the regulation of a number of genes, including histones (Graves et al., 1987), tubulin (Yen et al., 1988), and genes involved in cell cycle regulation and proliferation, c-*myc* (Dean et al., 1986), c-*fos* (Shyu et al., 1989), and GM-CSF (Shaw & Kamen, 1986).

Apolipoprotein (apo)[1] II mRNA encodes an apolipoprotein component of very low density lipoprotein particles which transport lipid from the liver to the ovary for deposition in developing egg yolk. The hepatocyte apoII gene is transcriptionally activated by estrogen in the laying hen or in roosters given exogenous estrogen (Chan et al., 1976; Wiskocil et al., 1980). In addition to its effects on transcription, estrogen also selectively alters the stability of apoII mRNA. Depending on the period of prior estrogen treatment, apoII mRNA is subject to either slow ($t_{1/2}$ = 13 h) or rapid decay ($t_{1/2}$ = 1.5 h) upon hormone withdrawal (Gordon et al., 1988). The change in $t_{1/2}$ does not involve a shift in the polyribosomal apoII mRNA profile or an accumulation of mRNP in the postpolyribosomal fraction (ibid). Analysis of truncated apoII mRNA fragments suggests that mRNA degradation in vivo occurs via endonucleolytic cleavages at specific sites in the 3'-untranslated region (Binder et al., 1989, 1990).

In the present study, we describe a procedure for the isolation of apoII mRNP containing undegraded apoII mRNA. ApoII mRNP released from intact polyribosomes by EDTA has a buoyant density characteristic of a protein:RNA ratio of 3:1. Biochemical characterization of an mRNP fraction enriched for apoII mRNA by ultracentrifugation on successive sucrose gradients revealed that many of the proteins cross-linked to apoII mRNA by UV irradiation are the same as those associated with non-estrogen-regulated mRNPs.

Experimental Procedures

Animals and Hormone Treatment. Estrogen was administered to 2-3-week-old White Leghorn roosters (SPAFAS, Norwich, CT) by subcutaneous implantation of constant-release pellets containing 100 mg of 17-β-estradiol (Innovative Research of America). Animals were killed 72 h later, and livers were frozen in liquid nitrogen prior to storage at -70 °C.

Isolation of Polyribosomes. Solutions were prepared from autoclaved stocks and RNase-free components, and treated with diethyl pyrocarbonate before use (Palmiter, 1974). Frozen liver was coarsely ground under liquid nitrogen. In method 1, the magnesium precipitation method of Palmiter (1974), 1 g of ground liver was homogenized by hand in a Dounce homogenizer at 0 °C in 9 mL of buffer A (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 2.5 mM $MgCl_2$, 2% Triton X-100, 1 mg/mL heparin, 100 μg/mL PMSF, 5 μg/mL aprotinin, and 5 μg/mL leupeptin). After being cleared at 20000*g* for 5 min at 0 °C, the supernatant was diluted with an equal volume of buffer B (4 volumes of buffer A plus 1 volume of 1 M $MgCl_2$). Polyribosomes were allowed to aggregate for 1 h at 0 °C, were collected by centrifugation for 15 min at 20000*g* through a 0.5 M sucrose pad in buffer A

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[1] Abbreviations: apo, apolipoprotein; mRNP, messenger ribonucleoprotein particle; $t_{1/2}$, half-life; GTC, guanidinium isothiocyanate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; kDa, kilodalton(s).

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lacking Triton X-100, and were resuspended in 20 mM HEPES, pH 7.5, containing 0.2 mg/mL heparin, 100 μg/mL PMSF, 5 μg/mL aprotinin, and 5 μg/mL leupeptin.

In method 2, 1 g of ground liver was homogenized at −5 °C in 9 mL of buffer C [0.35 M sucrose, 200 mM Tris–acetate, pH 8.5, 50 mM KCl, 10 mM magnesium acetate, 6 mM 2-mercaptoethanol, 1 mg/mL heparin, 100 μg/mL PMSF, 5 μg/mL aprotinin, and 5 μg/mL leupeptin (Shore & Tata, 1977)] using five strokes of a motor-driven Teflon and glass homogenizer. The homogenate was centrifuged at 20000*g* for 8 min at −5 °C. The supernatant was collected and diluted with an equal volume of buffer D (buffer C lacking sucrose, but containing 0.2 M $MgCl_2$ and 4% Triton X-100). Polyribosomes were allowed to aggregate at 0 °C for 30 min and were collected by centrifugation at −5 °C for 90 min at 40000*g* through a 0.5 M sucrose pad in buffer C. The polyribosome pellet was resuspended in 20 mM HEPES, pH 7.5, containing inhibitors as for method 1. Polyribosome yield was determined by optical density at 260 nm in 0.5% SDS.

Sucrose Density Ultracentrifugation. Five or 10 A_{260} units of polyribosomes were layered on 0.5–1.5 M sucrose gradients in buffer E (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 2.5 mM $MgCl_2$, 0.2 mg/mL heparin, 100 μg/mL PMSF, 5 μg/mL aprotinin, and 5 μg/mL leupeptin) and centrifuged 2.5 h in an SW41 rotor at 39 000 rpm at 4 °C. Gradients were collected from the top, and A_{254} was recorded with an Isco UA-5 flow-through spectrophotometer. Fractions were collected on ice for nucleic acid analysis. For EDTA mRNP gradients, polyribosomes in 20 mM HEPES were adjusted to 10 mM EDTA and loaded onto 0.15–0.6 M sucrose gradients in buffer E containing 10 mM EDTA. Centrifugation was for 14 h at 39 000 rpm at 4 °C in an SW41 rotor. Sedimentation values were interpolated using ribosomal subunits and RNAs as markers.

Preparative-scale gradients fractionated 20–25 A_{260} units of polyribosomes on 0.5–1.5 M sucrose gradients in buffer E by centrifugation for 5 h in an SW27 rotor at 27 000 rpm at 4 °C. Typically, 12 SW27 gradients were loaded with polyribosomes from estrogen-treated rooster liver. Gradients were collected from the top, and A_{254} was recorded. Fractions corresponding to 4 to 8 ribosomes per mRNA were collected on ice (about 50–60-mL total volume), sodium chloride was added to 0.3 M, 2 volumes of ethanol was added, and polyribosomes were allowed to precipitate for 30 min at −20 °C. Polyribosomes were collected by centrifugation, washed with ethanol, and lyophilized. Subsequent resuspension was facilitated if the polyribosome pellet was not taken to complete dryness. Polyribosomes were resuspended in 1 mL of buffer E containing 10 mM EDTA. Half of the material was loaded onto each of two 0.15–0.6 M sucrose gradients in buffer E containing 10 mM EDTA. Centrifugation was for 14 h at 39 000 rpm in an SW41 rotor at 4 °C.

CsCl Gradient Analysis. Polyribosomes diluted to 1 A_{260} unit/mL and 10 mM EDTA were fixed with formaldehyde and subjected to CsCl gradient ultracentrifugation according to the procedure of Henshaw (1979). CsCl concentration was determined by the refractive index.

RNA Analysis. Dot blots of gradient fractions were prepared using the method of White and Bancroft (1982). Nitrocellulose filters were prepared, hybridized, and washed according to Thomas (1980). The uniformly labeled probe for apoII mRNA was a 240-nucleotide cDNA representing most of the coding region (Gordon et al., 1988). The control cDNA probe was oligo(dT)-primed ^{32}P-cDNA from a non-estrogen-treated rooster liver RNA (and thus did not contain apoII mRNA). RNA was prepared by the guanidinium isothiocyanate method as described (Gordon et al., 1988). RNA samples for Northern blot analysis were electrophoresed on 1% agarose gels containing 6% formaldehyde according to Maniatis et al. (1982). RNA was electrotransferred to a Nytran membrane (Schleicher & Schuell) in 20 mM sodium phosphate, pH 6.8, and cross-linked to the membrane using a Stratalinker (Stratagene) according to the manufacturer's instructions. Hybridizations and washes were identical to those for dot blots except for the inclusion of 5% SDS in the hybridization buffer. Relative enrichment of apoII mRNA sequences to other liver mRNAs was assessed by comparing the ratio of hybridizable apoII mRNA to control mRNA in unfractionated polyribosomal RNA and in enriched fractions. Primer extension was as described (Shelness & Williams, 1985) using an end-labeled oligonucleotide primer complementary to the 3′ end of the apoII mRNA (nucleotides 643–664, Hwang et al., 1989).

Oligo(dT) Selection of Cross-Linked mRNP. Gradient fractions containing mRNP were photo-cross-linked in an open petri dish on ice at 0.2 J/cm^2 using a Stratalinker (Stratagene). Polyribosomes were diluted to 1 A_{260}/mL in buffer E containing 10 mM EDTA and cross-linked in the same manner. Proteins cross-linked to polyadenylated mRNA were isolated by two cycles of chromatography on oligo(dT)–cellulose in the presence of 0.5% SDS and protease inhibitors (Greenberg, 1980; Dreyfuss et al., 1984).

Protein Analysis. Polyacrylamide gels (10%) containing SDS were run using the buffer system of Laemmli (1970). Protein samples were iodinated using the Iodogen method (Pierce) and $Na^{125}I$ in phosphate-buffered saline containing 1% SDS and 100 μg/mL PMSF. One microgram of RNase A was added to iodinated samples, which were then precipitated with 10% trichloroacetic acid containing 50 mM NaI, washed once with 10% TCA/NaI, twice with 80% ethanol/20% 1 M Tris-HCl, pH 8, and once with ethanol, and lyophilized. Photo-cross-linked mRNP samples were digested thoroughly with RNases A and T2 before electrophoresis (Swiderski & Richter, 1988).

Results

In order to prepare an mRNP fraction enriched in apoII mRNA, we initially used a procedure (method 1) for polyribosome isolation in which liver is homogenized in buffer containing Triton X-100 (Palmiter, 1974). Following a short centrifugation, polyribosomes and ribonucleoprotein particles are recovered from the supernatant by precipitation with magnesium. Northern blot analysis of polyribosomal RNA prepared in this manner (Figure 1A, lane b) showed that apoII mRNA migrated as two bands with the lower molecular weight species representing up to 40% of total apoII mRNA. In contrast, apoII mRNA migrated as a single band when RNA was isolated directly from liver by the guanidinium isothiocyanate (GTC) method (lane a, Gordon et al., 1988). Northern blot and primer extension analyses (data not shown) indicate that apoII mRNA isolated from liver by the GTC method is approximately 660 nucleotides in length (Shelness & Williams, 1985) plus a poly(A) tail of 40–180 residues (Binder et al., 1989). By probing Northern blots of polyribosomal RNA with probes from the coding and 3′-untranslated region, it was found that the faster migrating band (lane b) represented the 5′ fragment of apoII mRNA that was cleaved within the 3′-untranslated region (data not shown). These cleavages probably reflect nuclease activity that is unmasked or brought into contact with apoII mRNA during polyribosome isolation by this method. This degradation of apoII

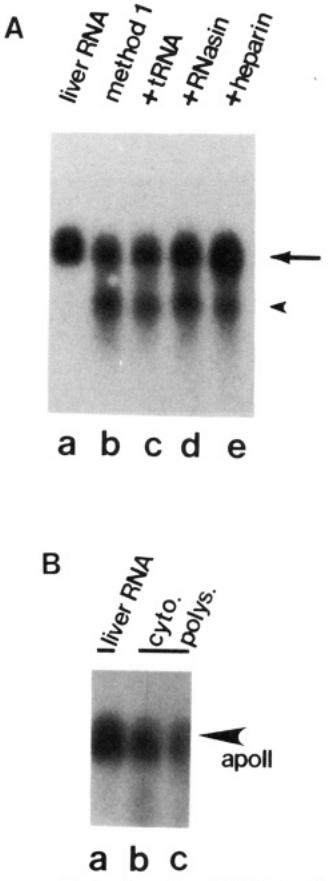

FIGURE 1: Northern analysis of apoII mRNA in polyribosomes. (A) The appearance of cleavages of apoII mRNA from polyribosomes made by method 1. Ten micrograms each of total RNA from estrogen-treated rooster liver (lane a) or RNA from polyribosomes made by method 1 (lanes b–e) was electrophoresed on an agarose–formaldehyde gel, transferred to Nytran, and probed for apoII mRNA as described under Experimental Procedures. The arrow indicates the mobility of intact apoII mRNA; the arrowhead indicates the mobility of specific degradation products of apoII mRNA. RNA from polyribosomes made by method 1 (lane b) or made by method 1 with the addition of 2 mg/mL yeast tRNA to buffers A and B (lane c), with the addition of 200 units/mL RNasin (Promega) in buffer A (lane d), or with the addition of 4 mg/mL heparin in buffer B (lane e) was loaded. (B) ApoII mRNA remains undegraded during preparation of polyribosomes by method 2. RNA was prepared directly from estrogen-treated rooster liver and polyribosomes prepared by method 2, and analyzed for apoII mRNA by Northern blot. RNA from rooster liver (lane a); RNA from 20000*g* supernatant from homogenate in buffer C (lane b); RNA from method 2 polyribosomes collected by centrifugation through a 0.5 M sucrose pad in buffer C (lane c). The mobility of intact apoII mRNA is indicated (arrow).

mRNA was not effectively eliminated by nonspecific inhibitors of ribonuclease (Figure 1A, lanes c–e). However, we noted when examining RNA at different steps in the procedure that degradation occurred up to, but not after, the addition of 0.1 M magnesium chloride (data not shown). Consequently, we employed a polyribosome isolation procedure (method 2) in which tissue is homogenized in buffer lacking detergent, followed by simultaneous addition of Triton X-100 and magnesium to a 20000*g* supernatant. As shown in Figure 1B, apoII mRNA isolated from the 20000*g* supernatant (lane b) or from polyribosomes after magnesium precipitation (lane c) exhibited the same mobility as apoII mRNA isolated directly from liver by the GTC method (lane a). This method typically yielded 70–75 A_{260} units of polyribosomes per gram of liver.

Gradient Analysis of ApoII mRNP. We examined several features of polyribosomes isolated by method 2. Figure 2A shows the sucrose gradient sedimentation profile of liver polyribosomes (solid line) and of apoII mRNA (open circles) as determined by dot blot hybridization. ApoII mRNA was found associated with higher order polyribosomes corresponding to a ribosome density of 4–8 ribosomes/mRNA. Upon release by EDTA, apoII mRNA sedimented as a broad peak at 12–18 S (Figure 2B), suggesting the association of protein with apoII mRNA.

To examine the association of apoII mRNA with protein in polyribosomes, EDTA-treated apoII mRNP was cross-linked with formaldehyde and fractionated by buoyant density centrifugation in CsCl (Figure 3A). After centrifugation, the A_{254} profile was recorded, and apoII mRNA was detected by dot blot. Control gradients, run without formaldehyde, indicated that unfixed apoII mRNA pelleted to the bottom of the centrifuge tube (Figure 3B). Formaldehyde-fixed apoII mRNP had an average cross-linked density of about 1.4 g/mL in CsCl, corresponding to a particle composition of 75% protein/25% nucleic acid by weight (Preobrazhensky & Spirin, 1978). For comparison, ribosomal subunits band at 1.55 g/mL characteristic of a 1:1 protein/RNA composition (Perry & Kelly, 1966). Heterogeneity in the profiles may indicate variation in the association with protein or in the efficiency of cross-linking.

Sucrose Gradient Enrichment for ApoII mRNP. To biochemically characterize the apoII mRNA proteins, we used an enrichment scheme based on size fractionation of the mRNP on two successive sucrose gradients. In this scheme, 20–25 A_{260} units of estrogen-treated rooster liver polyribosomes were layered on each of 12 sucrose gradients for a total of 240–300 A_{260}. After centrifugation in an SW27 rotor for 5 h at 27 000 rpm and 4 °C, the gradients were fractionated, and the apoII-containing polyribosomes were pooled. Following concentration of this material by ethanol precipitation, apoII polyribosomes were dissociated with 10 mM EDTA, and 8–10 A_{260} were layered onto a second sucrose gradient containing EDTA. Centrifugation was for 14 h at 39 000 rpm in an SW41 rotor. The peak of enriched apoII mRNP was identified by RNA dot blot analysis and pooled for further analysis.

Figure 4A shows the A_{254} profile and the dot blot profile of apoII mRNA of the EDTA mRNP gradient of an enrichment experiment performed as outlined. Sedimentation of the EDTA-derived 40S ribosomal subunit on this second gradient was identical to that derived directly from rooster liver polyribosomes (data not shown). Similarly, Northern and dot blot analyses showed that sedimentation of the EDTA-released apoII mRNP was indistinguishable from that released directly from total polyribosomes, suggesting that recovery of apoII polyribosomes by ethanol precipitation had not dramatically altered the association of apoII mRNA with protein (data not shown). Electrophoretic analysis (Figure 4B) shows that the 40S peak contained 18S ribosomal RNA as expected, while fractions 4–6, enriched in apoII mRNA, were not appreciably contaminated with ribosomal subunits.

Fractions from the EDTA mRNP Gradient Are Enriched for ApoII mRNP. Dot blot hybridization analysis was performed to assess the enrichment of apoII mRNP relative to that of other non-estrogen-regulated mRNAs for the experiment described in Figure 4. As seen in Figure 5 (top), the EDTA-released apoII mRNP sedimented as a broad peak, such that fractions 4 and 8 contained 70% of the apoII mRNA. ^{32}P-Labeled total cDNA from poly(A)$^+$ RNA made from the liver of a non-estrogen-treated rooster was used as a control probe (Figure 5, middle). This probe does not contain apoII

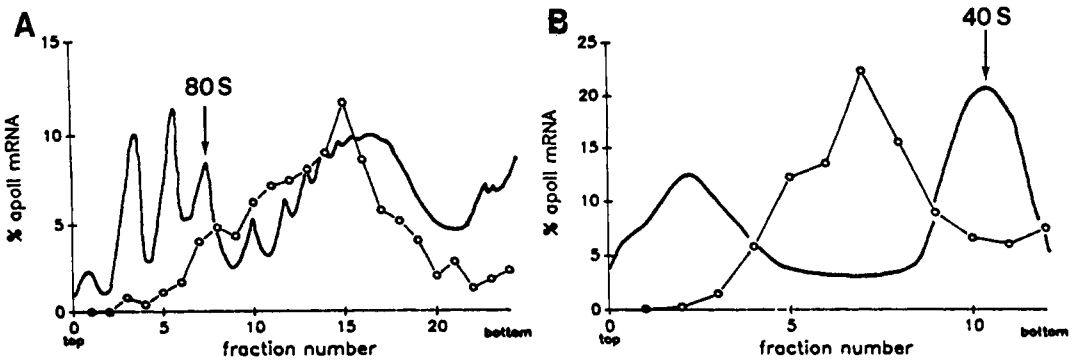

FIGURE 2: (A) Sucrose gradient profiles of polyribosomes prepared from estrogen-treated rooster liver. Five A_{260} units of polyribosomes were loaded onto a 0.5–1.5 M sucrose gradient and centrifuged for 2.5 h as described under Experimental Procedures. The gradient was collected from the top, and the A_{254} profile was recorded (heavy line). Fractions were collected and assayed for apoII mRNA by dot blot (open circles). The direction of centrifugation was from left to right, and the position of the 80S ribosome monomer is indicated with an arrow. ApoII mRNA in each fraction is expressed as the percent of total apoII mRNA recovered in the gradient. (B) Sucrose gradient of EDTA-released polyribosomes prepared from estrogen-treated rooster liver. Five A_{260} units of polyribosomes were made 10 mM in EDTA and loaded onto a 0.15–0.6 M sucrose gradient containing EDTA and centrifuged for 14 h as described under Experimental Procedures. The gradient was collected and assayed for apoII mRNA as described for panel A. The position of the 40S ribosomal subunit is indicated by the arrow.

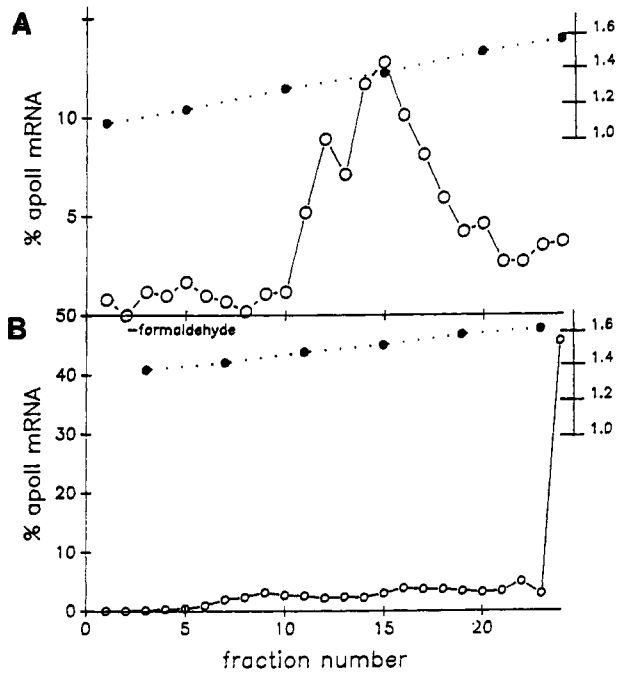

FIGURE 3: CsCl gradient analysis of apoII mRNP in polyribosomes. (A) Formaldehyde-fixed apoII mRNP. Rooster liver polyribosomes were diluted to 1 A_{260}/mL in buffer containing EDTA and formaldehyde, and loaded onto a CsCl pad containing formaldehyde. After centrifugation overnight, fractions were collected, and the A_{254} profile was recorded as for Figure 1. CsCl concentration in grams per milliliter is shown at the right (dotted line). A dot blot profile for the apoII mRNA is shown (solid line). (B) Unfixed mRNP. Rooster liver polyribosomes were prepared as for panel A except that formaldehyde was omitted. Fractions were collected and apoII mRNA detected as above.

sequences, and thus reveals a representative range of non-apoII cellular mRNAs. In contrast to the apoII probe, the cDNA probe revealed that cellular mRNPs were less abundant at the top of the gradient, gradually reached a peak at fraction 6, and remained at a high level throughout the rest of the gradient. Relative enrichment of apoII mRNP was estimated by comparing the ratio of hybridized radioactivity using the apoII cDNA probe and the control cDNA probe for each fraction (Figure 5, bottom). Fractions 4 and 5 were greatly enriched for apoII mRNP relative to other regions of the gradient. Fractions 6 through 8, while abundant for the apoII mRNP, also have a significant content of other cellular mRNPs.

Table I: Typical ApoII mRNP Enrichment[a]

sample	apoII/cDNA	enrichment
polyribosomes	4.37	1.0×
apoII polyribosomes	17.20	3.9×
apoII mRNP	32.05	7.3×

[a] ApoII mRNP was enriched by successive sucrose gradient centrifugations as in Figures 4 and 5. RNA was prepared from polyribosomes, apoII polyribosomes, and enriched apoII mRNP, and analyzed by dot blot for apoII mRNA and control cDNA (see Experimental Procedures). Columns are the ratio of cpm hybridized with apoII mRNA to cpm hybridized to control cDNA (apoII/cDNA) and the enrichment of apoII mRNA relative to that in polyribosomes (enrichment).

Fractions 4 and 5 were pooled as apoII mRNP, and enrichment was assessed relative to total estrogen-treated rooster liver polyribosomal RNA (Table I). As indicated, mRNPs in fractions 4 and 5 are enriched about 7-fold for apoII mRNA over bulk polyribosomal mRNA and about 2-fold over the size-fractionated apoII polyribosomes.

To estimate the abundance of apoII mRNA in the enriched fraction, we first needed to know the actual abundance of apoII mRNA in total liver mRNA. ApoII mRNA abundance was measured in total liver mRNA by solution hybridization of [^{32}P]-labeled apoII cDNAs from total liver cDNA (C. C. MacDonald and D. L. Williams, unpublished data). [^{32}P]-Labeled cDNA was prepared from total liver poly(A)$^+$ mRNA. This cDNA was hybridized to completion with a single-stranded M13 clone containing apoII sequences in the sense orientation. The percent of radiolabeled cDNA protected from digestion by S1 nuclease by the M13–apoII clone was taken to represent the abundance of apoII mRNA in total poly(A)$^+$ mRNA, and averaged 6–7% in several experiments. This value is in agreement with estimates of approximately 10% based on R_0t analysis (Wiskocil et al., 1980), and similar estimates based on the frequency of apoII cDNA clones in a liver cDNA library (Protter et al., 1982). The base value for apoII mRNA abundance in total liver mRNA of 6–7% was used in our assessments of apoII mRNA enrichment in the present study and indicates that the enriched apoII mRNP fraction contains 43–51% apoII mRNA by weight.

Proteins Cross-Linked to Enriched ApoII mRNP. To confirm the identity of polypeptides cosedimenting with the enriched apoII mRNP as authentic mRNP proteins, we used UV cross-linking to isolate mRNP proteins associated with poly(A)$^+$ mRNA. The enriched mRNP fraction was pooled,

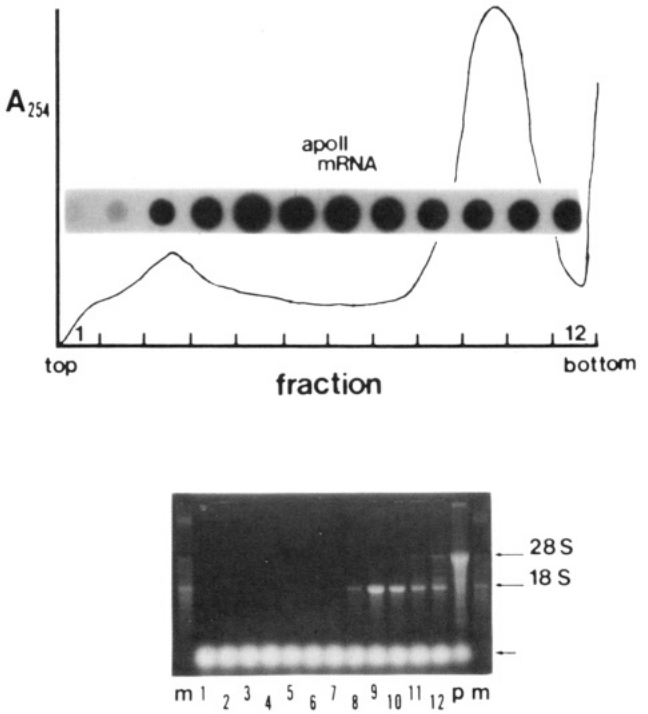

FIGURE 4: ApoII mRNA in EDTA mRNP gradient fractions. 300 A_{260} units of polyribosomes were enriched for apoII mRNP as described under Experimental Procedures. Fractions were collected from the second EDTA mRNP gradient for analysis. Sedimentation is from left to right. (A) A_{254} profile of the EDTA mRNP gradient. Nine A_{260} units of EDTA-treated apoII polyribosomes were loaded onto each of two EDTA mRNP gradients, and centrifuged for 14 h in an SW41 rotor at 39 000 rpm at 3 °C. Gradients were collected from the top while a continuous A_{254} recording was made. Twelve fractions of approximately 1 mL each were collected and pooled with the corresponding fraction from the second gradient for further analysis. Superimposed on the A_{254} profile is an autoradiogram of dot blot analysis of apoII mRNA in the mRNP gradient. RNA was prepared from aliquots of each fraction of the EDTA mRNP gradient by phenol/chloroform extraction, and analyzed by dot blot probed for apoII mRNA. Lanes 1–12 represent fractions, from top to bottom, of the gradient. (B) Formaldehyde gel analysis of RNA in EDTA mRNP gradient fractions. RNA was prepared from aliquots of each fraction of the mRNP gradient in panel A, displayed by formaldehyde–agarose gel electrophoresis, and stained with ethidium bromide. Lanes m represent rooster liver RNA as size markers; RNA from gradient fractions in panel A, lanes 1–12; RNA from the resuspended pellet from panel A, lane p. Sizes of 28S and 18S ribosomal RNAs are indicated.

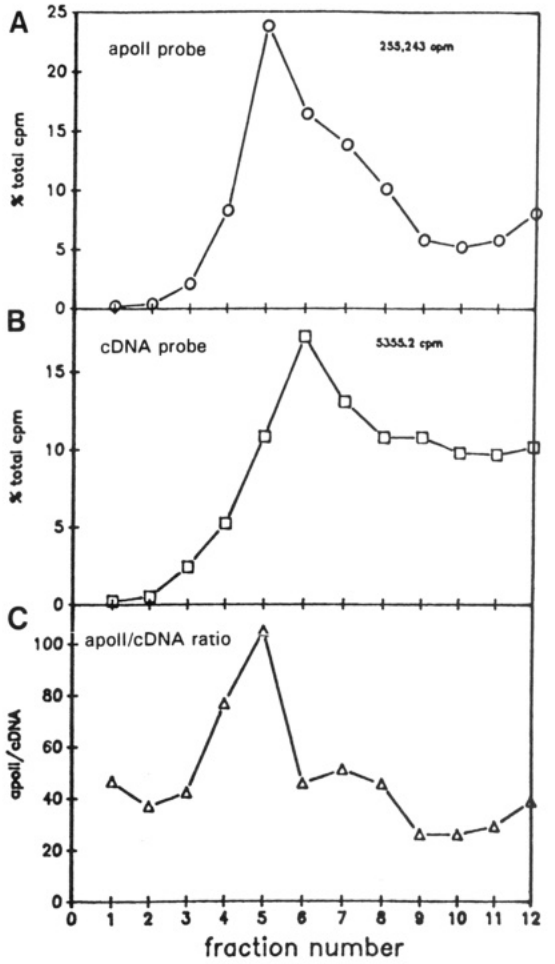

FIGURE 5: Enrichment for the apoII mRNP. ApoII mRNA was enriched from polyribosomes as described in the text. Gradient fractions were prepared for dot blot analysis as described under Experimental Procedures. (A) Dot blot profile of apoII mRNA from the enriched EDTA mRNP gradient. The dot blot was probed for apoII mRNA. Dots were excised, radioactivity was determined by liquid scintillation spectrometry, and apoII mRNA was displayed as a percentage of the total counts per minute over the entire gradient. (B) Dot blot profile of non-apoII mRNA from the enriched EDTA mRNP gradient. The dot blot was probed with ^{32}P-labeled cDNA from non-estrogen-treated rooster liver (see Experimental Procedures). Radioactivity was determined as for panel A, and non-apoII mRNA was displayed as a percentage of the total counts per minute over the entire gradient. (C) ApoII:cDNA ratio. As an arbitrary indication of the enrichment of apoII mRNA relative to non-apoII mRNA, the ratio of apoII cpm (panel A) to non-apoII cpm (panel B) is shown.

photo-cross-linked with UV light, selected on oligo(dT)–cellulose in the presence of SDS, and labeled with ^{125}I (Figure 6B). Total polyribosomal proteins were also labeled and examined for comparison (Figure 6A). After radioiodination, mRNP was treated with ribonuclease and subjected to electrophoresis on SDS–10% polyacrylamide gels. In comparing iodinated proteins of total polyribosomes (panel A) to those of UV-cross-linked mRNP (panel B), it is clear that few, if any, of the mRNP proteins correspond to proteins abundant in total polyribosomes, further confirming the absence of ribosomal contamination in the enriched mRNP fractions. Control experiments (not shown) confirmed that in the absence of UV cross-linking, no labeled protein was detected after chromatography on oligo(dT)–cellulose. Therefore, we have independent assurance that (a) the fraction examined in uncontaminated by ribosomal protein and (b) the proteins examined are covalently linked to polyadenylated mRNAs from that fraction.

For comparison to enriched apoII mRNP, identical EDTA mRNP gradients were loaded with EDTA-treated polyribosomes either from estrogen-treated rooster liver (lane b) or from non-estrogen-treated rooster liver (lane c). Gradient fractions from these polyribosomes were treated as for enriched apoII mRNP. Radioiodinated mRNP proteins cross-linked to mRNA in fractions enriched for apoII mRNA appear identical to mRNP proteins in total polyribosomal mRNP (lanes a and b). An identical protein profile was seen with polyribosomal mRNP isolated from a rooster not treated with estrogen (lane c). In all samples, prominent cross-linked proteins of 64, 53, 32, and 29 kDa can be identified, as well as a 22-kDa band near the bottom of the gel. The most prominent polypeptides detected by UV cross-linking appear to be those of 64 and 32 kDa.

Discussion

When released from polyribosomes by EDTA treatment, apoII mRNA was associated with protein as judged by its sedimentation at 12–18 S in sucrose gradients and its buoyant

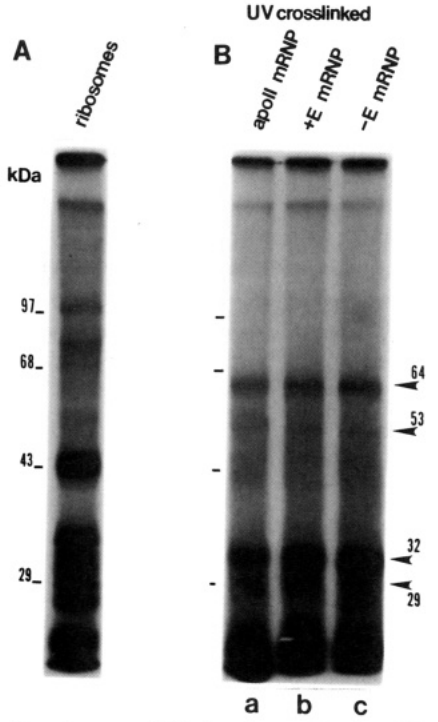

FIGURE 6: Proteins cross-linked to the enriched apoII mRNP. (A) Ribosomal proteins. Polyribosomes from estrogen-treated rooster liver were labeled with ^{125}I and displayed by SDS–10% polyacrylamide gel electrophoresis and autoradiography. (B) Proteins cross-linked to mRNP. ApoII mRNP was enriched from polyribosomes by the scheme described in the text. Fractions from the enriched peak of apoII mRNP were pooled, cross-linked with UV light, selected by oligo(dT)–cellulose chromatography, radioiodinated, and subjected to SDS–10% polyacrylamide gel electrophoresis and autoradiography (lane a). For comparison, identical fractions from EDTA mRNP gradients which had been loaded with unenriched estrogen-treated polyribosomes (lane b) or with non-estrogen-treated polyribosomes were treated in parallel. Molecular size markers (in kilodaltons) are indicated on the left of the panel; sizes of prominent polypeptides are indicated to the right.

density in CsCl gradients. Formaldehyde-fixed apoII mRNP had an average density of 1.4 g/mL, corresponding to a particle composition of 75% protein/25% RNA by weight. This composition is typical of bulk mRNP as well as the few specific mRNPs that have been examined (Dreyfuss, 1986; Ruzdijic et al., 1985; Preobrazhensky & Spirin, 1978; Greenberg, 1977). Fractionation of liver polyribosomes and mRNP on successive sucrose gradients yielded a 7-fold enrichment of apoII mRNP relative to other mRNPs. The enriched mRNP fraction appeared to be free of contaminating ribosomal subunits as judged as the absence of detectable 18S ribosomal RNA. On the basis of the 7-fold enrichment for apoII mRNA and the determination that apoII mRNA represented 6–7% of total liver poly(A)$^+$ mRNA, apoII mRNP accounts for 40–50% by mass of the total mRNP in the enriched fraction. In order to examine the proteins associated with mRNA in the enriched fraction, proteins were photocross-linked to RNA by UV irradiation and isolated as poly(A)$^+$ mRNP by selection on oligo(dT)–cellulose. Following radioiodination, proteins were examined by SDS–polyacrylamide gel electrophoresis and autoradiography. This approach provided a stringent criterion for the identification of mRNP proteins.

Analysis of proteins cross-linked to poly(A)$^+$ mRNA in the apoII-enriched fraction identified prominent protein bands of 64, 53, 32, 29, and 22 kDa. Proteins of the same molecular weights and of similar abundance were seen with size-fractionated poly(A)$^+$ mRNP released from total polyribosomes isolated from estrogen-treated or non-estrogen-treated rooster liver. This result suggests that the major proteins cross-linked to apoII mRNA are mRNP proteins that are associated with the bulk of liver mRNAs.

It is of interest that previous studies found no striking differences between the mRNP proteins cross-linked to bulk cellular mRNA in different cell types grown in culture (Greenberg, 1980; Dreyfuss et al., 1984; Dreyfuss, 1986) or in the highly specialized reticulocyte (Greenberg & Carroll, 1985). Similarly, in vesicular stomatitis virus infected cells, where viral message becomes the predominant mRNA on treatment of cells with actinomycin D (Dreyfuss et al., 1984), in polyadenylated histone H4 mRNAs (Ruzdijic et al., 1985), or in proteins cross-linked to mRNAs microinjected into *Xenopus* oocytes (Swiderski & Richter, 1988), an identical pattern of mRNP proteins was detected by cross-linking. These findings, as well as the results of the present study, suggest that the major mRNP proteins associated with EDTA-released mRNP and detected by UV cross-linking are common to many cellular mRNAs, and likely participate in aspects of mRNA metabolism common to most mRNAs.

The similarity in cross-linked mRNP proteins between the enriched apoII mRNP and other cellular mRNPs may indicate that aspects of mRNA regulation specific to apoII mRNA are not mediated by specific mRNP proteins. However, the present results do not eliminate this potential mechanism. It is possible, for example, that an mRNP protein specific to apoII mRNA was not detected here because (1) the protein does not efficiently cross-link to the mRNA, or is not efficiently iodinated, (2) the protein is present on only a small fraction of apoII mRNA molecules at any one time, or (3) the protein has an electrophoretic mobility identical to one of the major mRNP proteins detected. A previous study suggested many differences in mRNP proteins in liver polyribosomes from estrogen-treated as compared to control roosters (Johnson & Ilan, 1982). However, because of the relaxed chromatographic conditions employed in mRNP isolation and the absence of cross-linking as a stringent criterion for identification of mRNP proteins, it is not possible to assess which of the numerous proteins detected in that study were authentic mRNP proteins.

The functions of the major mRNP proteins detected in the enriched apoII mRNP fraction or in total mRNP (Figure 6) are not known. Similarly, the regions of apoII mRNA and other mRNAs with which these proteins associate are not known. In a recent study, label transfer reactions via UV cross-linking were used to identify liver cytosolic proteins which interact with synthetic [^{32}P]apoII mRNA fragments (Ratnasabapathy et al., 1990). Among the proteins detected were two of approximately 60 and 34 kDa which were shown to bind to independent domains in the 3′-untranslated region of apoII mRNA. Two prominent proteins of similar molecular masses (64 and 32 kDa, Figure 6) were seen in EDTA-released mRNP and in apoII mRNP-enriched fractions prepared from rooster liver polyribosomes. While the present results do not determine the relationship between the proteins detected in these studies or the regions of apoII mRNA associated with specific proteins, it should be possible to do so in future studies using monoclonal antibodies raised against mRNP proteins. The preparation of mRNP fractions enriched to 40–50% purity for apoII mRNP and containing intact apoII mRNA should yield suitable antigen for the generation of monoclonal antibodies.

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