Estrogen Induces the Assembly of a Multiprotein Messenger Ribonucleoprotein Complex on the 3′-Untranslated Region of Chicken Apolipoprotein II mRNA*

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UV cross-linking was used to identify estrogen-induced hepatocyte proteins that bind to apolI mRNA. Probes spanning the entire message revealed the presence of eight estrogen-induced proteins cross-linked to the 3′-untranslated region (UTR), but not to the coding region or the 5′-UTR. Two estrogen-induced proteins of 132 and 50 kDa were either absent or barely detectable in control animals, whereas six additional proteins of 93, 83, 74, 65, 58, and 45 kDa were clearly present in control animals and increased 2-5-fold by estrogen. A similar profile of estrogen-induced proteins was seen with the 3′-UTRs of the estrogen-regulated mRNAs for apoB and vitellogenin II, but not with the 3′-UTRs of the non-estrogen-regulated mRNAs for apoA-I and glycerolaldehyde-phosphate dehydrogenase. These findings indicate that the estrogen-induced proteins discriminate among mRNAs and suggest that they interact selectively with the family of estrogen-regulated mRNAs. The estrogen-induced proteins are found in the cytoplasmic fraction of liver extracts, and a subset of them are also found in adrenal glands, testes, heart, brain, and kidneys, but they are estrogen-induced only in the liver. Deletion analysis defined a 150-nucleotide region of the apoI 3′-UTR that is necessary for maximal binding of the estrogen-induced proteins. An internal deletion of endonucleolytic cleavage sites previously identified within the apolI 3′-UTR selectively reduced the binding of the 58-kDa protein. These findings reveal remarkable complexity in estrogen-stimulated protein-RNA interactions within the 3′-UTRs of estrogen-regulated mRNAs. These proteins may participate in the mRNA degradation process or in other aspects of cytoplasmic mRNA metabolism that accompany estrogen-stimulated vitellogenesis.

Regulation of cytoplasmic mRNA metabolism is known to play a major role in determining the expression levels of cellular proteins. The types of regulation are varied and include hormonally and developmentally induced changes in mRNA stability (1, 2), recruitment of translationally quiescent mRNAs via regulated cytoplasmic polyadenylation (3–6), and the locali-
these proteins are regulated by estrogen. The results from UV cross-linking analyses indicate that estrogen induces the assembly of an mRNP complex of eight proteins on the apoII 3'-UTR as well as on the 3'-UTRs of other estrogen-regulated mRNAs. Within the apoII 3'-UTR, deletion of one cluster of endonucleolytic cleavage sites selectively reduced the binding of a previously identified 58-kDa protein (35). These results reveal remarkable complexity in estrogen-stimulated protein-RNA interactions within the 3'-UTRs of estrogen-regulated mRNAs. These proteins may participate in the mRNA degradation process or in other aspects of cytoplasmic mRNA metabolism that accompany estrogen-stimulated vitellogenesis.

**EXPERIMENTAL PROCEDURES**

Preparation of Extracts—White Leghorn cockerels (2–3 week old; SPAFAS, Norwich, CT) were treated with estrogen for 2 days by subcutaneous implantation of a 100-μg 17β-estradiol constant-release pellet (5 mg/day; Innovative Research of America, Toledo, OH) (32). Control animals received placebo pellets. Cytosolic extracts from various tissues were prepared according to Ratnasabapathy et al. (35) using 1 g of tissue/7 ml of buffer. Extracts from adrenal glands and testes were made by homogenization of the entire organ in 1 ml of buffer. Nuclear extracts were prepared according to the procedure of Dingram et al. (36). All buffers contained 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml apropin. Protein concentrations were determined with the Bio-Rad microprotein assay using γ-globulin as standard.

Synthesis of RNA Probes—Templates for the synthesis of labeled RNA probes were prepared by polymerase chain reaction amplification of various plasmid DNAs using 18–23-mer primer pairs. Each upstream primer also included the T7 RNA polymerase promoter sequence GGCTCGAAATAATTACGACTCACTATAGGAGGA at its 5'-end. The reaction mixture (100 μl) contained 1–10 ng of template, 0.1 mM dNTPs, 1 × buffer (polymerase chain reaction kit, Perkin-Elmer), 2.5 units of AmpliTaq DNA polymerase, and 30 pmol of each primer. After 25–30 cycles, the DNA product was purified through a 2% agarose gel (NuSieve GTG, FMC Bioproducts). High specific activity RNA probes were synthesized by incubation for 15 min at 37°C of a 10-μl reaction mixture containing 40 μl Tris-HCl (pH 8.0), 25 mM NaCl, 8 mM MgCl2, 2 mM spermidine (HCl), 0.4 mM GTP, 0.4 mM ATP, 0.4 mM CTP, 10 mM diethiothreitol, 1 unit/μl RNA guard (Pharmacia Biotech Inc.), 10–20 ng of DNA template, 200 μCi of [3H]UTP (800 Ci/mmol; Amersham Corp.), and 50 units of T7 RNA polymerase (Life Technologies, Inc.). The RNA was purified through a Quick Spin column (Boehringer Mannheim). This method generates an RNA having 6 extra nucleotides (GGGAGA) at its 5'-end. The integrity of the labeled probe was checked on a 5% sequeling gel. The probes were stable for 1 week. The conditions for synthesis of large amounts of unlabeled RNA (for use in competition experiments) were identical, except that the reaction mixture (50 μl) contained all four unlabeled nucleotides at 0.5 mM, 0.2 μg of DNA template, 100 units of T7 RNA polymerase, and a trace of [3H]UTP (10 μCi, 36.2 Ci/mmol) to allow for quantitation of the synthesized RNA.

ApoII mRNA probes are labeled A–F with numbers (e.g. D1, etc.) using (−) 32P-deoxythymidine triphosphate (−dATP). The 3'-end of cDNA was labeled by the method of Feinberg and Vogelstein (37) with 100Ci of [α-32P]dCTP (6000 Ci/mmol; Amersham Corp.). The labeled cDNA was purified through a Quick Spin column (Boehringer Mannheim). The labeled cDNA was digested to completion with restriction enzymes that recognize sequences within the apoII 3'-UTR (38). The DNA fragments were separated on a 2% agarose gel, and the fragment containing exon 5 (representing the apoII 3'-UTR) was purified with a spinning column (Boehringer Mannheim) and purified further through a 5% sequencing gel (NuSieve GTG, FMC Bioproducts). The RNA was labeled at its 5'-end using T4 RNA polymerase (Promega) with [α-32P]ATP (1000 Ci/mmol; Amerham Corp.) and 50 units of T4 RNA polymerase (Life Technologies, Inc.). This labeled RNA was purified through a Quick Spin column (Boehringer Mannheim) and resolved on a 2% agarose gel to ensure the removal of any digestion fragments.

UV Cross-linking of Proteins—Protein cross-linking was carried out in a 10-μl reaction containing 10 μl Hepes (pH 7.9), 40 mM KCl, 3 mM MgCl2, 1 mM diethiothreitol, 15 μg of yeast RNA, 1.5 ng of labeled probe, and 20–60 μg of protein extract. The mixture was incubated for 10 min at 26°C and cross-linked at full power for 30 min in a UV Stratalinker 1800 (Stratagene). After addition of 0.2 μl of RNaseVI (0.7 unit/μl; Pharmacia Biotech Inc.) and 0.8 μl of RNase A (10 μg/ml), the mixture was incubated for 30 min at 37°C followed by 30 min at 55°C.

Proteins were resolved by electrophoresis through a 9% SDS-polyacrylamide gel using the buffer system of Laemmli (44). Fixation was in 7% methanol and 7% acetic acid for 1 h before drying and autoradiography. Protein bands were quantified with a GS-670 imaging densitometer (Molecular Analyst/PC, Bio-Rad). We have referred to the bands detected by autoradiography as protein bands even though they represent proteins cross-linked to RNA fragments. Protein molecular mass represents the average from five independent experiments using prestained protein standards (Life Technologies, Inc.) whose molecular masses have been corrected against [3H]-labeled protein standards (Life Technologies, Inc.).

**RESULTS**

Estrogen-induced Proteins Bind to the 3'-UTR of ApoII mRNA—Cytosolic proteins that bind to apoII mRNA were detected via UV cross-linking using four uniformly labeled RNA probes that span the entire mRNA (Fig. 1, lower panel). The influence of hormone treatment was determined by comparing cytosolic extracts from control birds with those receiving 17β-estradiol for 2 days via a constant-release implant. Note that...
radiolabeled bands detected by this cross-linking assay were abolished by protease treatment and were dependent on UV irradiation (data not shown). The results indicate that probes A, B, and C, which cover nucleotides 1–510, cross-link with several proteins (Fig. 1, lanes 1–6). Although there is variation in band intensity with these probes, the cross-linking pattern is qualitatively similar, and no difference was observed between extracts from estrogen-treated or control animals. In contrast, probe D, which covers most of the 3'-UTR, shows several significant differences. First, in extracts from control birds (lanes 1, 3, 5, and 7), a number of bands that cross-link weakly to probes A–C were prominently detected with probe D, suggesting that these proteins selectively recognize the 3'-UTR. Second, estrogen treatment enhanced the cross-linking of several proteins to probe D, including bands of 93, 83, 74, 65, 58, and 45 kDa (compare lanes 7 and 8). Densitometric scanning of autoradiograms from seven independent experiments indicated that the mean ± S.E. increase in band intensity was 3.9 ± 0.6-, 5.7 ± 1.1-, 4.6 ± 0.8-, 4 ± 0.7-, 1.8 ± 0.2-, and 2.1 ± 0.2-fold for the 93-, 83-, 74-, 65-, 58-, and 45-kDa bands, respectively. Third, a very intense band of 132 kDa and a band of 50 kDa were often seen only in extracts of estrogen-treated birds. In these instances in which faint 132- and 50-kDa bands were seen in control liver extracts, the -fold induction in estrogen-treated liver extracts ranged from 14- to 25-fold for the 132-kDa band and from 3- to 29-fold for the 50-kDa band. Thus, the 132- and 50-kDa proteins may be induced de novo by estrogen or may simply be increased by estrogen to a much greater degree than the other proteins noted above. These data define a set of proteins that bind selectively to the 3'-UTR of apoII mRNA and that are markedly increased by estrogen treatment. We will refer to these as estrogen-induced proteins although this is not meant to imply anything about the mechanism of the estrogen-stimulated increase in cross-linking activity.

Two additional points should be noted. First, the 116-kDa protein that cross-links to probe D (Fig. 1, lanes 7 and 8) was not included among the estrogen-induced proteins because it does not reproducibly show estrogen induction. This band is only occasionally resolved from a minor estrogen-induced band of 107 kDa (for example, see Figs. 2, 4, 6, and 7), which may account for the irreproducibility of estrogen induction of the 116-kDa band. Because the 107-kDa band was not reproducibly resolved, we have not considered it further. Second, several cross-linked bands in the 30-42-kDa range (Fig. 1, lanes 7 and 8) appear to show differences with estrogen. However, since these differences were not reproducible, these bands were not considered further.

Fig. 2. Estrogen-induced proteins bind the 3'-UTRs of estrogen-regulated mRNAs. The 3'-UTRs from the indicated mRNAs were cross-linked to cytosolic liver extracts (40 μg of protein) from a control (-) or an estrogen-treated (+) animal. An autoradiogram of the RNA-protein complexes separated by SDS-polyacrylamide gel electrophoresis is shown. Odd-numbered lanes contain extracts from control animals, and even-numbered lanes contain extracts from estrogen-treated animals. Identical results were obtained with three different control and three different estrogen-treated animals. The RNA sequence used forapolI was probe D (RNA-(481–659)), while the entire 3'-UTR was used for the other mRNAs (see “Experimental Procedures”). GAPDH, glyceraldehyde-phosphate dehydrogenase.

Estrogen-induced Proteins Recognize the 3'-UTR of Estrogen-regulated mRNAs—To test the ability of the estrogen-induced proteins to discriminate among mRNAs, we compared the cross-linking pattern of probe D with eight probes generated from the 3'-UTRs of five different mRNAs as well as with a RNA probe generated from the pGEM vector. Among the mRNAs tested are several known to be estrogen-regulated (apolI, apoB, and VTGII) and several known not to be estrogen-regulated (apoA-I, glyceraldehyde-phosphate dehydrogenase, and pGEM). Fig. 2 shows that probes from the 3'-UTRs of apolI, apoB, and VTGII mRNAs generate similar patterns, each showing prominent cross-linking of estrogen-induced proteins of 132, 83, 74, 65, 58, and 45 kDa (lanes 2, 6, and 10). Several differences are also apparent. For example, the 93- and 83-kDa proteins cross-link less well to VTGII mRNA (lane 10), and the 50-kDa protein is not easily seen with apoB mRNA (lane 6). Nevertheless, the cross-linking patterns are remarkably similar for the three estrogen-regulated mRNAs. In contrast, the cross-linking patterns for the three non-estrogen-regulated mRNAs are markedly different (lanes 4, 8, and 18) and show no evidence of estrogen-induced proteins, with the possible exception of the 45-kDa band (lane 3 versus 4, lane 7 versus 8, and lane 17 versus 18). The estrogen-induced proteins of 132, 93, 83, 74, 65, 58, and 50 kDa are clearly absent with probes from apoI, glyceraldehyde-phosphate dehydrogenase, and pGEM RNAs.

Upon first inspection, the results with the PEPCK probes are puzzling. PEPCK-2 shows little cross-linking and no indication of estrogen-induced proteins (Fig. 2, lanes 13 and 14). With control liver extract, PEPCK-1 (lane 11) shows some bands in common with the estrogen-regulated mRNAs (lanes 1, 5, and 9) and slightly increased cross-linking of some bands upon estrogen treatment (lane 12). In contrast, in control liver extracts, PEPCK-3 (lane 15) shows most bands in common with the estrogen-regulated mRNAs and clear evidence of estrogen induction with extracts from hormone-treated birds (lane 16). Since estrogen regulation has not been reported for the cytosolic form of PEPCK, we carried out Northern blot analysis to examine this possibility. Analysis of the blot with a PhosphorImager indicated that PEPCK mRNA was increased 3.1-fold (arbitrary units: control, 1500 ± 333 (S.E., n = 5); estrogen-treated, 4640 ± 1070 (n = 5); p = 0.02) following 2 days of estrogen treatment. Thus, although not dramatically induced, PEPCK mRNA abundance appears to be under estrogenic control. Taken together, these data indicate that the estrogen-induced proteins show specificity in their binding as they appear to distinguish among mRNAs that are estrogen-regulated (apolI, apoB, VTGII, and PEPCK) and mRNAs that are not estrogen-regulated (apoA-I, glyceraldehyde-phosphate dehydrogenase, and pGEM).

Binding Specificity of the Estrogen-induced Proteins—The specificity of the estrogen-induced proteins was further tested by competition of probe D1 (RNA-(510–659)) with increasing amounts of unlabeled probes derived from the 3'-UTRs of five mRNAs. The results (Fig. 3) indicate that apolI (lane 4), apoB (lane 10), and VTGII (lane 13) 3'-UTRs compete effectively for
the apoI probe, whereas the 3'-UTRs of apoAI (lane 7) and glyceraldehyde-phosphate dehydrogenase (lane 15) mRNAs are ineffective at a 250-fold excess. These competition data confirm the specificity of the estrogen-induced proteins for the estrogen-induced mRNAs as seen with the direct cross-linking assay (Fig. 2).

Cellular and Tissue Distribution of the Estrogen-induced Proteins—To determine the cellular location of the estrogen-induced proteins, nuclear and cytosolic liver extracts from chickens treated for 2 days with estrogen were cross-linked with probe D1 (RNA-(510–659)). Fig. 4 shows that the cross-linking pattern is distinct for nuclear (lanes 1 and 3) and cytosolic (lanes 2 and 4) extracts. Estrogen-induced proteins were detected exclusively in cytosolic extracts (lanes 1 and 3 versus 2 and 4). The estrogen-induced proteins of 132, 93, 83, and 50 kDa appear to have no nuclear counterparts of similar mobility, whereas proteins of 74, 65, 58, and 45, although not estrogen-induced, were found at low levels in nuclear extracts (lanes 1 and 3). Whether these nuclear proteins are the same as the cytosolic proteins of equivalent mobility is unknown.

The tissue distribution and estrogen inducibility of the apoI 3'-UTR-binding proteins was determined by cross-linking probe D1 (RNA-(510–659)) to cytosolic extracts prepared from tissues of three control and three estrogen-treated animals. The results for one representative tissue from each group are shown in Fig. 5. Several points are of interest. First, of the 10 tissues tested, only the liver shows estrogen-induced proteins that cross-link to the apoI 3'-UTR probe. Second, each tissue exhibits a unique pattern of cross-linked proteins. Third, some of the estrogen-induced liver proteins may be present in other tissues as judged by similar mobilities, whereas some are absent in other tissues. For example, the 58-kDa protein is detected only in the liver and possibly the kidney (lanes 11 and 12), but appears to be absent in all other tissues tested. Similarly, the 132-kDa protein is not seen in the intestine (lanes 9 and 10), kidney (lanes 11 and 12), lungs (lanes 13 and 14), muscle (lanes 15 and 16), and spleen (lanes 17 and 18), but may be present in the adrenal gland (lanes 3 and 4), brain (lanes 5 and 6), heart (lanes 7 and 8), and testes (lanes 19 and 20). In addition, some proteins such as the 74- and 65-kDa proteins appear to be present in all tissues, albeit at quantitatively different levels. Whether proteins in different tissues that cross-link to the apoI 3'-UTR and that have the same electrophoretic mobility are identical will require further study.

RNA-binding Site(s)—To determine which region of the apoI mRNA 3'-UTR is recognized by the estrogen-induced proteins, various deletions of probe D (RNA-(481–659)) were generated by progressive 5'-end deletions and tested with liver cytosolic extracts from control and estrogen-treated chickens (Fig. 6). Deletion of the first 29 nucleotides (probe D1) modestly but reproducibly increased the cross-linking of the estrogen-induced proteins. Further deletions revealed that most of the binding of the 132-, 93-, 83-, and 50-kDa estrogen-induced proteins was lost with deletion to nucleotide 550 (probe D3). Densitometric scanning of the autoradiogram indicated that...
were cross-linked with 40 μg of protein from liver extracts from estrogen-treated (+) or control (−) animals. The RNA-protein complexes were separated by SDS-polyacrylamide gel electrophoresis. An autoradiogram of the gel is shown. Upper panel: lanes 1 and 2, probe D (RNA-(481–659)); lanes 3 and 4, probe D1 (RNA-(510–659)); lanes 5 and 6, probe D2 (RNA-(530–659)); lanes 7 and 8, probe D3 (RNA-(550–659)); lanes 9 and 10, probe D4 (RNA-(593–659)). Odd-numbered lanes are with extracts from control animals, and even-numbered lanes are with extracts from estrogen-treated animals. Lower panel: diagram of the deletions aligned with the apoII 3′-UTR.

The 58-kDa Protein—The 150-nucleotide domain necessary for maximal binding of the estrogen-induced proteins includes the domain previously reported to bind a protein of ~60 kDa (35). To test whether the 58-kDa protein noted in the present study corresponds to the previously reported 60-kDa protein, we examined proteins cross-linked to probe E (nucleotides 550–642), which is a very similar RNA domain as previously used (nucleotides 568–643) to identify the 60-kDa protein (35). The results show that probe E cross-links to one predominant protein (Fig. 8A, lane 2) that comigrates with the estrogen-induced 58-kDa protein (lane 3) detected with probe D. The cross-linking pattern seen with probe E is nearly identical to that seen previously (35), suggesting that the 58-kDa protein is the same protein as the previously reported 60-kDa protein. Note also that with probe E little estrogen inducibility is seen for the 58-kDa protein or the other proteins (lane 2 versus 4) in comparison with the strong estrogen inducibility seen with probe D (lane 1 versus 3). This is consistent with the deletion analysis (Fig. 6) showing that the 5′-sequence between nucleotides 510 and 550 is required to detect the full extent of estrogen induction.

The domain necessary for maximal binding of the estrogen-induced proteins also contains a cluster of endonucleolytic cleavage sites (nucleotides 597–602) that were detected in vivo and that represent putative target sites for apoII mRNA degradation (33). It was therefore of interest to determine whether this region played a role in binding the estrogen-induced proteins. Probe F, which was obtained by deletion of nucleotides 550–607 from probe D1, was cross-linked with cytosolic extracts from estrogen-induced or control chickens. In control

FIG. 6. Binding sites of the estrogen-induced proteins: 5′-end deletions. A series of 5′-end deletions of probe D (RNA-(481–659)) were cross-linked with 40 μg of protein from liver extracts from estrogen-treated (+) or control (−) animals. The RNA-protein complexes were separated by SDS-polyacrylamide gel electrophoresis. An autoradiogram of the gel is shown. Upper panel: lanes 1 and 2, probe D (RNA-(481–659)); lanes 3 and 4, probe D1 (RNA-(510–659)); lanes 5 and 6, probe D2 (RNA-(530–659)); lanes 7 and 8, probe D3 (RNA-(550–659)); lanes 9 and 10, probe D4 (RNA-(593–659)). Odd-numbered lanes are with extracts from control animals, and even-numbered lanes are with extracts from estrogen-treated animals. Lower panel: diagram of the various deletions aligned with the apoII 3′-UTR.

FIG. 7. Binding sites of the estrogen-induced proteins: 3′-end deletions. A series of 3′-end deletions of probe D (RNA-(481–659)) were cross-linked with 40 μg of protein from liver extracts from estrogen-treated (+) or control (−) animals. The RNA-protein complexes were separated by SDS-polyacrylamide gel electrophoresis. An autoradiogram of the gel is shown. Upper panel: lanes 1 and 2, probe D (RNA-(481–659)); lanes 3 and 4, probe D5 (RNA-(481–642)); lanes 5 and 6, probe D6 (RNA-(481–613)); lanes 7 and 8, probe D7 (RNA-(481–575)); lanes 9 and 10, probe D8 (RNA-(481–531)). Odd-numbered lanes are with extracts from control animals, and even-numbered lanes are with extracts from estrogen-treated animals. Lower panel: diagram of the various deletions aligned with the apoII 3′-UTR.
liver extracts, deletion of nucleotides 596–607 strongly diminished the cross-linking of the 58-kDa protein, whereas the other proteins were unaffected (Fig. 8B, compare lanes 1 and 2). A similar and selective reduction in the cross-linking of the 58-kDa protein was also seen in extracts from estrogen-treated birds (compare lanes 3 and 4). These results indicate that full binding of the 58-kDa protein requires the local domain that includes endonucleolytic cleavage sites. This result is consistent with the 3'-deletion series noted above in which deletion from nucleotides 613 to 575 eliminated the estrogen induction of the 58-kDa protein (Fig. 7, lane 6 versus 8).

RNA Secondary Structure Is Important for Binding of the Estrogen-induced Proteins—To test whether RNA secondary structure is important for binding of the estrogen-induced proteins, probe D1 was denatured for 5 min at 90°C and rapidly cooled on ice. Fig. 9 shows that this denaturation protocol had no effect on the cross-linking of proteins in control liver extracts (lane 1 versus 2). In contrast, probe denaturation eliminated cross-linking of all of the estrogen-induced proteins with the exception of the 74- and 45-kDa proteins (lane 3 versus 4). The 45-kDa protein appeared to be unaffected by probe denaturation, whereas the 74-kDa protein, although reduced, was still clearly increased in estrogen-treated as compared with control liver extracts (lane 2 versus 4). These data indicate that RNA secondary structure has a major influence on the binding of the estrogen-induced proteins.

**DISCUSSION**

UV cross-linking analysis has identified a set of estrogen-regulated liver cytosolic proteins that selectively recognize the 3'-UTR of apolI mRNA as compared with the coding or 5'-noncoding regions of the mRNA. Upon estrogen treatment, two proteins of 132 and 50 kDa were strongly induced over barely detectable levels in control extracts, while an additional six proteins of 45–93 kDa were increased 2–5-fold over levels measured in control liver extracts. Specificity in these interactions was demonstrated by competition studies and by localization of binding sites to a subdomain of the apolI mRNA 3'-UTR. This set of estrogen-regulated proteins also recognized the 3'-UTRs of other estrogen-regulated mRNAs including those encoding apoB, VTGII, and PEPCK. In contrast, with the exception of the 45-kDa protein, the estrogen-induced proteins did not show significant cross-linking to the 3'-UTRs of apoA-I and glyceraldehyde-phosphate dehydrogenase mRNAs or an RNA probe derived from the pGEM vector. These findings demonstrate that the estrogen-induced proteins discriminate among different mRNAs and suggest that they selectively recognize the 3'-UTRs of estrogen-regulated mRNAs. We refer to these proteins as induced by estrogen, but at present, it is not known whether this induction reflects an action of estrogen to increase the abundance of these proteins or to activate the binding activities of pre-existing proteins, or a combination of these effects. Nevertheless, it is clear that estrogen treatment enhances the formation of an mRNP complex in which these proteins assemble onto a defined domain of the 3'-UTR of apolI mRNA.

Our results reveal remarkable complexity in the process by which estrogen treatment increases the interaction of eight cytosolic proteins with the 3'-UTR of apolI mRNA. The minimal RNA domain necessary for maximal cross-linking of all proteins is a 150-nucleotide region extending from nucleotide 510 to the site of poly(A) addition. The binding of these proteins within this domain could be regulated by estrogen in two ways. In the first, each protein is regulated independently by estrogen. In the second, one or a few proteins are estrogen-regulated, and these serve to recruit other proteins or to stabilize the interaction of other proteins with the mRNA. The data suggest that both of these processes may occur. For example, the 58-kDa protein was selectively diminished upon deletion of nucleotides 596–607 (Fig. 8B), but was not lost with 5'- and 3'-deletions that greatly reduced or eliminated many of the other proteins (Figs. 6 and 7). Similarly, binding of the 74- and 45-kDa proteins was relatively unaffected by denaturation of the RNA probe, whereas the binding of the other estrogen-induced proteins was reduced to levels seen in control liver extracts (Fig. 9). We have also been able to resolve the 58-kDa
protein from the others by ammonium sulfate fractionation and to resolve the 74-, 58-, and 45-kDa proteins from the others by polyethyleneimine fractionation. Thus, the 58-kDa protein binds independently of the estrogen-induced proteins, and the 74- and 45-kDa proteins appear to bind independently of the 132-, 83-, and 50-kDa proteins. In contrast, the 132-, 83-, and 50-kDa proteins appear to behave in concert in 5'- and 3'-deletion analyses and upon probe denaturation, raising the possibility that these proteins bind to apol mRNA as a multiprotein complex. Thus, within a 150-nucleotide domain of the apoII 3'-UTR, estrogen induces the assembly of a ribonucleoprotein complex consisting of eight proteins, some of which bind independently and some of which may bind as a multiprotein complex.

A multiprotein messenger ribonucleoprotein complex, the α-complex, consisting of three proteins has recently been shown to assemble on the 3'-UTR of human α-globin mRNA and to be associated with mRNA stabilization (45). Interestingly, the α-complex also was found in non-erythroid cells despite the selective expression of α-globin mRNA in erythroid cells, suggesting that these 3'-UTR-binding proteins interact with other mRNAs and may have functions common to non-globin mRNAs or additional functions unrelated to mRNA metabolism. This is similar to the present situation in which the estrogen-induced 3'-UTR-binding proteins, although clearly not ubiquitous and not estrogen-regulated in nonhepatic tissues, appear to be present in some tissues other than the liver. Since apol mRNA as well as the other estrogen-regulated liver mRNAs are not expressed in many of these tissues, it follows that these proteins participate in some aspects of mRNA metabolism that are not unique to estrogen-regulated mRNAs. Whether this is the case will require confirmation of the presence of the hepatic 3'-UTR-binding proteins in other tissues by additional means.

The cytosolic localization of these proteins, their induction by estrogen, and their interaction with the 3'-UTR of apol mRNA as well as the 3'-UTRs of other estrogen-regulated mRNAs are consistent with the idea that the estrogen-induced proteins participate in aspects of mRNA metabolism that are important for vitellogenesis. In addition to the selective and dramatic induction of egg yolk protein mRNAs, estrogen has many effects on cytoplasmic mRNA metabolism, including increases in ribosome number (23, 28), a shift of ribosomes into higher order polyribosomes (28), increases in the hepatocyte content of rough endoplasmic reticulum membranes (23, 26), post-translational modifications of ribosomal proteins (46), and alterations in the translational elongation rates of the average hepatocyte protein as well as of vitellogenin (24, 25). In addition to these changes in the translational apparatus, vitellogenesis involves increases in lipid synthesis (30, 31) and in the enzyme activities (29, 47) necessary for estrogen-stimulated production of triglyceride-rich very low density lipoprotein particles that transport lipids to the developing egg yolk. Estrogen also has complex effects on the turnover of apol and VTGII mRNAs. Whereas estrogen has no effect on the decay of these mRNAs after short-term (1-3 days) hormone treatment, long-term (5-14 days) hormone treatment leads to the rapid and selective destabilization of apol and VTGII mRNAs when hormone is withdrawn (32). Thus, there are a variety of potential roles that the estrogen-induced mRNA-binding proteins could play in the regulation of translation or mRNA turnover or in processes such as the transport of mRNAs from the nucleus to the cytoplasm.

With regard to apol mRNA turnover, the 150-nucleotide domain necessary for maximal binding of the estrogen-induced proteins includes two regions previously shown to contain prominent sites of endonucleolytic cleavage in vivo (33). One region contains a cluster of cleavage sites at nucleotides 597–602 within a single-stranded domain and cleavage sites at nucleotides 636 and 637 within the polyadenylation signal, also in a single-stranded domain (33, 34). Interestingly, deletion of the region containing the cleavage sites at nucleotides 597–602 in probe F led to the selective loss of the 58-kDa protein in the profile of cross-linked proteins (Fig. 8B). In addition, in 3'-deletions, the estrogen-induced 58-kDa protein was partially lost upon deletion to nucleotide 613 and completely lost upon deletion to nucleotide 575 (Fig. 7). These results suggest that the 58-kDa protein may be associated with two regions within the nucleotide 575–659 domain, one of which corresponds closely to the cluster of endonucleolytic cleavage sites at nucleotides 597–602 and the other of which includes the cleavage sites at nucleotides 636–637. The precise correspondence between the endonucleolytic cleavage sites and the binding sites of the 58-kDa protein remains to be tested with more detailed analyses. Nevertheless, the suggestion from the current results is that the 58-kDa protein is associated with these sites and may function in the process of mRNA turnover, possibly as a nuclease or a factor that protects against nuclease cleavage.

A novel feature of our results is the coordinate increase in the binding of a large group of proteins to the 3'-UTR of several mRNAs upon estrogen treatment. This appears to differ from previous reports of mRNA-binding proteins interacting with other estrogen-regulated mRNAs. A single estrogen-induced chick liver protein of 66 kDa has been reported to cross-link to the 12-nucleotide 5'-UTR of chicken vitellogenin mRNA and to protect this RNA fragment from degradation in cell-free extracts (48). This protein shows specificity for the 12-nucleotide vitellogenin 5'-UTR when compared with ribosomal RNA, tRNA, poly(A), or tobacco mosaic virus RNA, but binding to other chick liver mRNAs was not tested. In the present study, we did not observe an estrogen-induced protein that cross-linked to the 5'-UTR of apol mRNA. In a recent report, gel shift assays were used to show that estrogen treatment increases by 4-5-fold Xenopus liver proteins that recognize homologous regions of either the vitellogenin B2 or B1 mRNA 3'-UTR (49). UV cross-linking identified two proteins of 141 and 71 kDa that bound to this RNA fragment, although it was not established that the proteins identified with the cross-linking assay are estrogen-induced and represent the binding activity observed with the gel shift assay. The specificity of the Xenopus liver binding activity toward other liver mRNAs or estrogen-induced mRNAs was not tested, although it was shown to discriminate among various domains of the vitellogenin 3'-UTR and to show a reduced affinity for tRNA (49).

In another recent study, an estrogen-regulated 64–66-kDa endoribonuclease with specificity for cleavage of serum albumin mRNA was purified from Xenopus liver (50). This nuclease has been proposed to be responsible for the estrogen-mediated destabilization of albumin mRNA that occurs during vitellogenesis in frogs. The relationship of these Xenopus proteins to the apol mRNA-binding proteins described in the present study is unknown, although it is possible that their homologues are among the larger set of estrogen-induced proteins we have described.

Several lines of evidence suggest that the binding of the estrogen-induced proteins to the 3'-UTR is sensitive to RNA secondary structure. First, sequence comparisons of the 3'-UTRs of apol, apoB, and VTGII mRNAs show no major sequence elements (>10 nucleotides) in common. Second, heat denaturation of the RNA completely eliminates binding of the

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the single-stranded loop in the vicinity of nucleotide 600 that is distal to the domains required for binding of the 132-, 83-, and 50-kDa proteins. The partial loss of the 58-kDa protein with 3'-deletions to nucleotide 613 may also indicate one or more binding sites for the 58-kDa protein in the domain more 3' to nucleotide 613. The binding of these proteins within the domain of the apoll 3'-UTR that is subject to endonucleolytic cleavage in vivo (33) may reflect a functional association with the mRNA degradation process. Although the details of these interactions remain to be tested, it is interesting to note that structure mapping of apoll mRNA has shown that the 3'-UTR is very similar in naked RNA (34) or in polyribosomal mRNPs, with the exception of a cobra venom nuclease cleavage site at nucleotide 550 in the mRNP (33). The presence of this site in the mRNP as compared with the mRNA could be due to a difference in base pairing or to a difference in accessibility, either of which could reflect a structural change in the mRNA due to binding of the estrogen-induced proteins in the nucleotide 510–550 region. Additional studies will be required to establish the structural features of the mRNA required for protein binding and to test the functional activity of the mRNP complex on the 3'-UTR.

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