Pergamon

0960-0760(95)00018-6

J. Steroid Biochem. Molec. Biol. Vol. 52, No. 6, pp. 505–515, 1995

0960-0760/95 $9.50 + 0.00

Tissue Distribution, Hormone Regulation and Evidence for a Human Homologue of the Estrogen-inducible *Xenopus laevis* Vitellogenin mRNA Binding Protein

Robin E. Dodson, Maria R. Acena and David J. Shapiro*

Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, U.S.A.

17β-estradiol induces the synthesis of massive amounts of the hepatic mRNA encoding the *Xenopus laevis* egg yolk precursor protein, vitellogenin. Vitellogenin mRNA exhibits a half life of approx. 500 h when 17β-estradiol is present, and 16 h after removal of 17β-estradiol from the culture medium. We recently reported that *Xenopus* liver contains a protein, which is induced by 17β-estradiol and binds with a high degree of specificity to a binding site in a segment of the 3′-untranslated region (3′-UTR) of vitellogenin mRNA implicated in 17β-estradiol stabilization of vitellogenin mRNA. To determine if this mRNA binding protein was specific to this system, or if it was present elsewhere, and regulated by other steroids, we examined the tissue distribution and androgen regulation of this protein. Substantial amounts of the vitellogenin 3′-UTR binding protein were found in several *Xenopus* tissues including testis, ovary and muscle. In the absence of hormone treatment, lung and intestine contained minimal levels of the mRNA binding protein. Testosterone administration induced the vitellogenin 3′-UTR RNA binding protein in several tissues. Additionally, we found a homologous mRNA binding protein in MCF-7, human breast cancer cells. Although the MCF-7 cell protein was not induced by 17β-estradiol, the MCF-7 cell mRNA binding protein appears to be closely related to the *Xenopus* protein since: (i) the human and *Xenopus* proteins elicit gel shifted bands with the same electrophoretic mobility using the vitellogenin mRNA 3′-UTR binding site; (ii) The human and *Xenopus* proteins exhibit similar binding specificity for the vitellogenin 3′-UTR RNA binding site; and (iii) RNA from MCF-7 cells is at least as effective as RNA from control male *Xenopus* liver in blocking the binding of the *Xenopus* and human proteins to the vitellogenin mRNA 3′-UTR binding site. Its broad tissue distribution and regulation by both 17β-estradiol and testosterone suggests that this mRNA binding protein may play a significant role in steroid hormone regulation of mRNA metabolism in many vertebrate cells.

J. Steroid Biochem. Molec. Biol., Vol. 52, No. 6, pp. 505–515, 1995

INTRODUCTION

The steps between gene transcription and protein translation involving RNA metabolism are increasingly seen as important regulatory points in many cellular processes [1, 2]. Regulation and control of RNA metabolism involves an emerging class of proteins, the RNA binding proteins [3–5], which are important in regulating virtually all aspects of RNA metabolism. RNA binding proteins appear to play critical roles in mRNA splicing [6, 7], nuclear export [6, 8], mRNA trafficking [9, 10], storage and translation [11, 12] stabilization and degradation [13–15]. RNA binding proteins may also play a role in cell division [16], differentiation [17] and the maintenance of the differentiated state [16, 18–21]. A number of diseases have been found to involve disruption of RNA binding proteins [22–25] and RNA binding proteins are important in viral etiology [26].

The control of mRNA stability and degradation represents a crucial step in the coupling (or uncoupling) of gene transcription and protein production [27]. The levels of a substantial number of RNAs are regulated at the level of RNA stability [28], and an increasing number of agents have been shown to regulate this process including steroid hormones [29–35],

*Correspondence to D. J. Shapiro.
Received 26 Sep. 1994; accepted 21 Dec. 1994.

cAMP [36–38], growth factors [39–41] and other cellular factors [42–53]. RNA binding proteins which are thought to control mRNA stability have been identified in only a few of these systems [39, 54–61]. Only in the case of iron regulation of transferrin-receptor mRNA degradation has the mRNA binding protein been cloned and characterized [reviewed in 14].

To study steroid hormone regulation of mRNA stability, our laboratory has developed a model system based on 17β-estradiol control of the stability of the hepatic mRNA encoding the *Xenopus laevis* egg yolk precursor protein vitellogenin [13]. Pharmacological levels of 17β-estradiol both induce an increase of at least several thousand-fold in the rate of vitellogenin gene transcription [62, 63], and stabilize vitellogenin mRNA against cytoplasmic degradation [30]. When 17β-estradiol is present in the culture medium, hepatic vitellogenin mRNA exhibits the unusually long half life of 500 h, or about 3 weeks. On removal of 17β-estradiol from the culture medium, vitellogenin mRNA is degraded with a half life of 16 h [30]. We recently identified an mRNA binding protein which binds to a segment of the vitellogenin mRNA 3′-UTR implicated in 17β-estradiol-mediated stabilization [54]. We showed that the vitellogenin 3′-UTR binding protein was induced by 17β-estradiol in *Xenopus* liver and bound with high specificity to a region centered around a 27 nucleotide segment of the 3′-UTR. This represented the first example of a steroid hormone-regulated protein binding to an mRNA 3′-UTR. Since vitellogenin mRNA is found only in liver, in the present study we determined whether the vitellogenin mRNA 3′-UTR binding protein was restricted to this tissue. Using RNA gel mobility shift assays to survey *Xenopus* tissues, we found that the mRNA binding protein was present in almost all of the tissues we examined. This suggested that the mRNA binding protein might have a broader role in mRNA metabolism than controlling the stability of vitellogenin mRNA, and led us to demonstrate its regulation by testosterone. It was also of interest to see if the mRNA binding protein was present in other species. We identified a homologous binding protein in the estrogen receptor-containing human breast cancer cell line, MCF-7 cells. The presence of the protein in these cells led us to ask if the human mRNA binding protein could be regulated by 17β-estradiol, and if endogenous MCF-7 RNA contained binding sites for the amphibian and human proteins.

MATERIALS AND METHODS

Animals and tissue collection

Adult male *Xenopus laevis* (Xenopus One, Ann Arbor, MI) were injected with either 17β-estradiol (1 mg), testosterone (1 mg) or vehicle (propylene glycol, 100 μl) bilaterally into the dorsal lymph sac. Estrogen was administered as previously described [54, 64]. Testosterone was administered 4 and 2 days before animals were killed. Tissues were collected and frozen directly on dry ice. For liver cube culture experiments, *Xenopus* were injected with 17β-estradiol (1 mg) 5 months and again 48 h prior to sacrifice.

Preparation of proteins

Salt extracts of crude polysomal preparations were prepared as recently described [54]. Whole tissue homogenates were prepared as follows. Tissues were cut into small pieces and placed in 1 ml of high salt buffer (10 mM Tris [pH 7.6], 2.5 mM MgOAc, 500 mM KCl, 1 mM DTT, 200 U/ml RNasin (Promega), 10% glycerol, 0.1 mM EGTA, 0.1 mM EDTA, 50 μg/ml leupeptin, 1 μg/ml pepstatin, 5 μg/ml aprotinin, 5 μg/ml phenylmethyl sulfonylfloride, 20 μg/ml benzamidine). Tissues were homogenized in a polytron for approx. 5–10 s at a setting of 7. The homogenate was kept on ice for about 5 min then diluted with an equal volume of dilution buffer (same as the high salt buffer, but without KCl). The homogenate was centrifuged at 300,000 ***g*** for 30 min. The supernatant was aliquoted and stored at -70°C for use in the RNA gel mobility shift assay. Protein determinations were made using a BioRad protein assay kit.

RNA gel mobility shift assay

RNA probes were made by incorporating ^{32}P-UTP (ICN; 3000 Ci/mmole) into RNA transcribed from the SP6 promoter. pB1-15 UTR [54] was digested with Bam H1 and used as template. This construct contained 97 nucleotides of the vitellogenin B1 3′-UTR which encompasses the protein binding site and additional polylinker sequence. While the vitellogenin 3′-UTR binding protein exhibits specific binding to a 27 nucleotide region within this RNA fragment, binding is significantly reduced relative to the larger RNA [54]. Since additional sequences around the 27 nucleotide region appear to be necessary for optimal binding, the larger RNA probe was used. RNA gel mobility shift assays were carried out as previously described [54]. Briefly, ^{32}P-RNA probe (approx. 4 pmol ^{32}P-RNA) was incubated with protein extracts in 10 μl buffer (10 mM Tris [pH 7.6], 1 mM MgOAc, 1mM DTT, 10% glycerol, 1 mM EDTA, 0.04 mM EGTA, 70 mM KCl, 0.8 U/ul RNasin, 1 mg/ml yeast tRNA, 1 mg/ml heparin). The hybridization mixture was incubated for 2 h at 4°C and products were resolved on a 4% non-denaturing polyacrylamide gel. Band intensities were determined using a PhosphorImager (Molecular Dynamics).

Cell culture

XL110 cells [29] were maintained in 0.6 × Higuchi's Media with 10% fetal bovine serum, 2 mM $CaCl_2$, 25 U/ml penicillin and 25 U/ml streptomycin. MCF-7 cells were maintained in 1 × DMEM/F12, 6 ng/ml insulin, 5% calf serum, 25 U/ml penicillin, 25 U/ml streptomycin and 50 μg/ml gentomycin. MCF-7 cells

were treated with 17β-estradiol for 48 h, washed in phosphate buffered saline, trypsinized and washed with media containing serum. The cells were pelleted and stored at −70°C. The pellets were resuspended in high salt buffer and processed as described above.

RNA preparation

Total cellular RNA was prepared as previously described [65], by homogenizing tissue or cells and extracting with phenol–chloroform–isoamyl alcohol (24:24:1) five times followed by a chloroform extraction. RNA was precipitated with ethanol and resuspended in TE buffer (pH 7.6).

Xenopus liver cube cultures

Livers were removed aseptically and cut into pieces of approx. 1 mm^3. Cubes were cultured in 0.6 × DMEM/F12 with 25 U/ml penicillin, 25 U/ml streptomycin, 50 μg/ml gentamycin, 2000 U/ml nystatin and 5 μg/ml insulin for 48 h prior to treatment. 17β-estradiol, testosterone or dexamethasone (10^{-6} M) were added to cultures and vehicle (ethanol) was added to control plates. Liver cubes were cultured an additional 6 days and media was changed every 12 h. Half of the liver cubes were processed to obtain the RNA binding protein for use in the RNA gel mobility shift assay and the other half were processed for RNA as described above. Relative vitellogenin RNA levels were assayed by immobilization of 0.5 and 1 μg of total RNA on nitrocellulose filters and probed with vitellogenin DNA radiolabeled by random priming with ^{32}P-dCTP.

RESULTS

Levels of the vitellogenin 3′-UTR binding protein in Xenopus laevis tissues

To determine whether the vitellogenin mRNA binding protein was present in non-hepatic tissues, we prepared protein extracts from *Xenopus laevis* tissue homogenates. Equal amounts of protein from the several tissues tested were assayed for binding to the vitellogenin mRNA 3′-UTR binding site in RNA gel mobility shift assays. The intensity of the gel shifted band corresponding to the vitellogenin 3′-UTR binding protein bound to the RNA probe was quantified using a PhosphorImager. Relative binding for tissues in control *Xenopus laevis* are shown in Table 1 as are the number of animals from which separate samples were analyzed. The vitellogenin 3′-UTR binding protein is most abundant in liver, testis and muscle, and is present at moderate levels in kidney, spleen, and heart. The protein is present at very low levels in lung and is virtually absent in intestine. These data indicated that the vitellogenin 3′-UTR binding protein is not liver specific, and is widely distributed in *Xenopus*, and showed that its levels vary greatly in different tissues.

Table 1. Tissue distribution of the vitellogenin 3′-UTR RNA binding protein

Tissue*	Sample size†	Relative amount‡
Liver	4	7.8
Testis	4	12.0
Muscle	2	6.2
Kidney	3	4.4
Spleen	2	4.6
Heart	2	2.4
Lung	2	1.0
Intestine	3	0.1

*Protein extract was added at a concentration of 7.5 μg/assay.

†Sample size refers to the number of *Xenopus laevis* from which tissues were sampled.

‡Relative amounts refers to the number of PhosphorImager counts per unit area measured.

Effects of 17β-estradiol and testosterone on tissue levels of the vitellogenin 3′-UTR binding protein

Given the broad distribution of the protein, we postulated that the binding protein might play a wider role in mRNA metabolism and be subject to regulation by other steroid hormones. To test this hypothesis, male *Xenopus* were injected with vehicle, 17β-estradiol or testosterone. Tissues were also obtained from an untreated female. The tissues were collected, processed, and proteins were analyzed by RNA gel mobility shift assays (Fig. 1). The labeled RNA probe migrated as a single band near the bottom of the gel (Fig. 1, lane 1). When the probe was incubated with proteins made by salt extraction of liver polysomes, as expected, a single upshifted band resulting from binding of the vitellogenin 3′-UTR binding protein to the labeled probe was detected (Fig. 1, lane 2). When the probe was incubated with soluble protein from homogenates of whole liver in high salt buffer, the expected estrogen-inducible gel-shifted band containing the vitellogenin 3′-UTR binding protein and the labeled probe band was detected (Fig. 1, lanes 3 and 4). Testosterone administration moderately elevated levels of the vitellogenin mRNA 3′-UTR binding protein in liver to levels comparable to those in females (Fig. 1, lanes 5 and 6). The mRNA binding protein was present in high levels in the testis of the control male (Fig. 1, lane 7) and in the ovary of the female (Fig. 1, lane 10). Administration of 17β-estradiol or testosterone decreased levels of the mRNA protein in testis (Fig. 1, lanes 8 and 9).

When extracts prepared from whole tissues are compared to extracts from salt-extracted polysomes, additional upshifted bands are observed in the RNA gel mobility shift assays. For example, while the estrogen-treated *Xenopus* liver polysome extract results in the

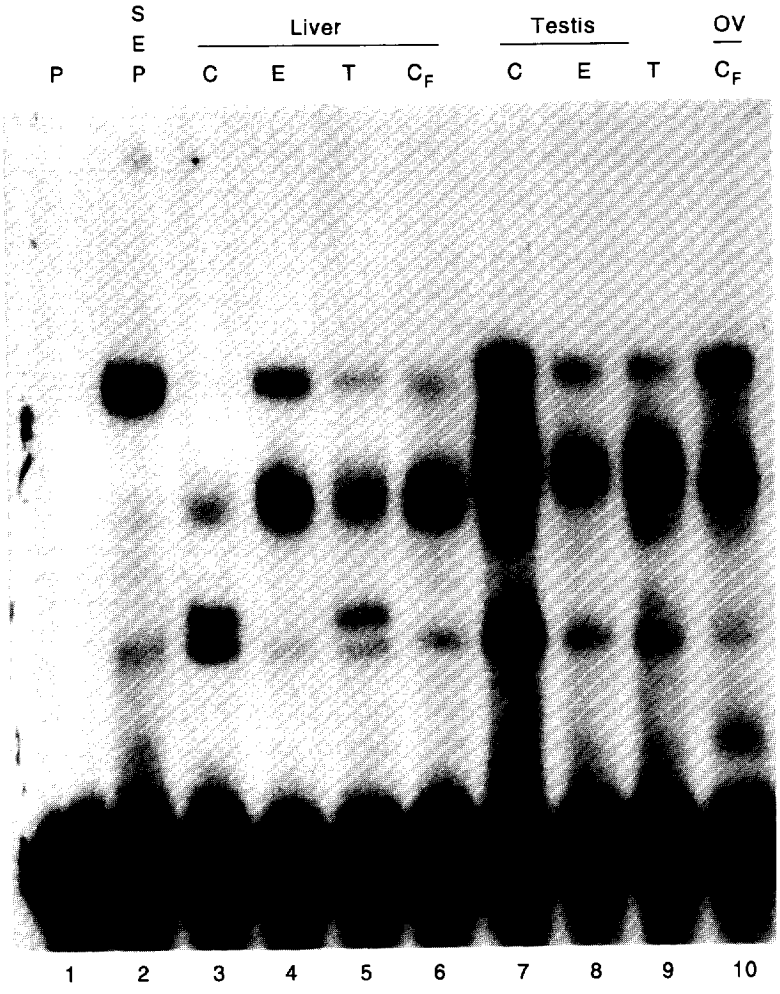

Fig. 1. Gonadal steroid hormone regulation of the vitellogenin 3′-UTR RNA binding protein in liver and testis. Autoradiogram of an RNA gel mobility shift of ^{32}P-vitellogenin 3′-UTR RNA and protein extracts from liver, testis and ovary (OV). The migration of the free probe (P) and vitellogenin 3′-UTR binding protein bands (SEP) are shown in the lanes labeled 1 and 2 respectively. Male *Xenopus laevis* were injected with vehicle (C), 17β-estradiol (E) or testosterone (T). An untreated female (C_F) was also included. Whole tissue extracts were made from liver, testis and ovary and used in the RNA gel shift.

appearance of a single major upshifted complex (Fig. 1, lane 2), an additional more rapidly migrating RNA–protein complex is observed using the whole liver cell extract (Fig. 1, lane 4). To determine if these bands represented sequence-specific binding to the vitellogenin 3′-UTR, we carried out a competition gel mobility shift assay (Fig. 2). The incubations contained either a specific competitor from the 3′-UTR of the homologous vitellogenin B2 mRNA, or a non-specific competitor of similar length, containing about 65 nucleotides at the 3′ end of the vitellogenin B2 mRNA coding region and 37 nucleotides of the 3′-UTR, which does not bind the protein [54]. These RNAs are approximately the same size as the B1 probe, and have been used previously to demonstrate specific binding of the protein to the vitellogenin 3′-UTR [54]. The competitors were incubated with protein extracts from liver of estradiol-treated *Xenopus* (Fig. 2, lanes 1–5) or testis from control animals (Fig. 2, lanes 6–10). The vitellogenin 3′-UTR binding protein from both liver and testis (Fig. 2, upper arrow) is competed by the specific competitor, but not by equivalent concentrations of the non-specific competitor. An additional upshifted band is present in extracts of liver from estrogen-treated male *Xenopus* (Fig. 2, lanes 1–5, lower arrow). This band is also competed by addition of the specific competitor but not by the non-specific competitor. In extracts from testis, two additional bands are present (Fig. 2, lanes 6–10, middle and lower arrows). The slower migrating band is competed specifically while the faster migrating band is competed by both specific and non-specific competitors and may represent a non-specific, low affinity binding protein present in testicular extracts. The complexes which are specifically competed may represent a cleavage product derived from the slower migrating vitellogenin mRNA 3′-UTR binding proteins, or may be due to binding by unrelated proteins.

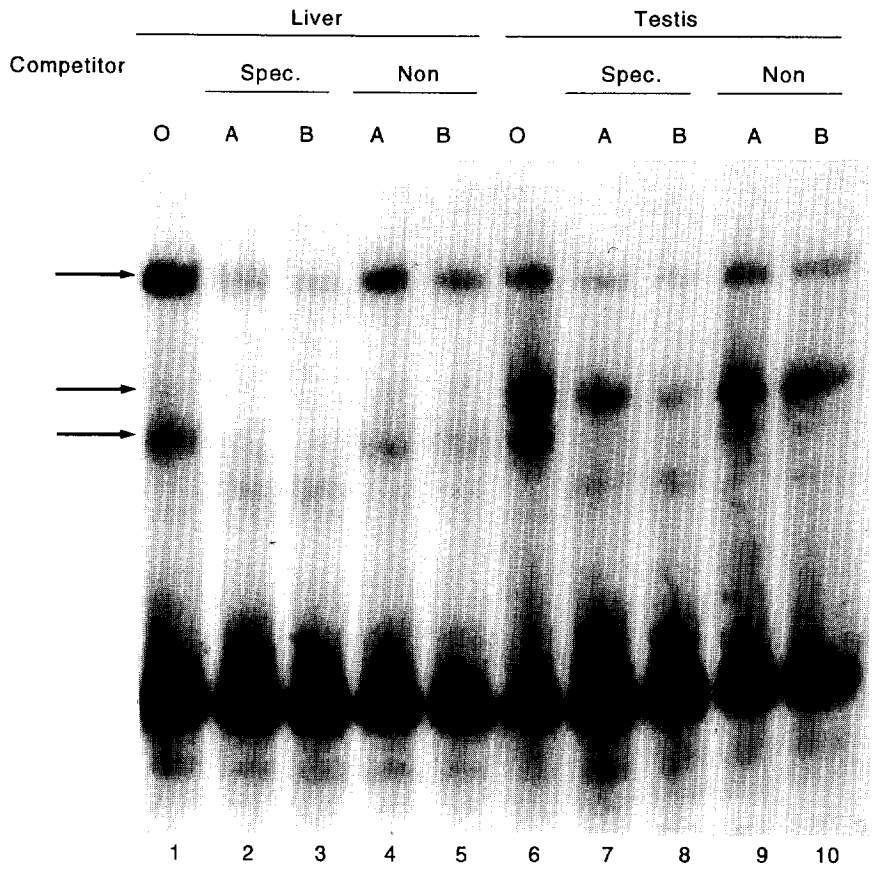

Fig. 2. Binding specificity of complexes between proteins present in whole tissue extracts and the vitellogenin 3′-UTR RNA. An autoradiogram of an RNA gel mobility shift assay showing competition for binding to proteins from whole tissue preparations of liver and testis by a specific (spec.) and a non-specific (non.) competitor. The specific competitor from the homologous vitellogenin B2 mRNA 3′-UTR [54] contained the same region used in our the binding assay, plus approx. 12 nucleotides 5′ to this sequence. Protein (10 μg) from liver of a *Xenopus* treated with 17β-estradiol or testis from a control animal were added to mixtures containing probe and either no competitor (0) or a 500-fold (A) or 1000-fold (B) excess of the competitor. The bands discussed in the text are indicated by arrows.

The effects of administration of 17β-estradiol or testosterone on the level of binding to the RNA probe were tissue specific. The complex of vitellogenin 3′-UTR binding protein and the labeled RNA is shown as the dark upshifted band (Fig. 3, lane 2). While administration of 17β-estradiol to males had little effect on levels of the vitellogenin 3′-UTR binding protein in intestine (Fig. 3, lanes 6 and 7) and muscle (Fig. 3, lanes 9 and 10), testosterone increased levels of the vitellogenin 3′-UTR binding protein in both of these tissues (Fig. 3, lanes 8 and 11). Exposure to 17β-estradiol or testosterone had little or no effect on levels of the mRNA binding protein in lung or spleen (Fig. 3). In different tissues, 17β-estradiol and testosterone had similar effects, divergent effects or no effect on levels of the mRNA binding protein. This suggested that the vitellogenin mRNA 3′-UTR binding protein may be part of a larger system modulating mRNA stability in response to steroid hormones, and perhaps other factors, in a variety of tissues.

Estrogen directly effects levels of the vitellogenin 3′-UTR binding protein in liver

In vivo studies do not directly access whether the hormone acts directly on the tissue in question or acts indirectly via another mechanism. To determine whether 17β-estradiol could act directly on liver tissue to induce or maintain the vitellogenin 3′-UTR binding protein, liver cubes were prepared from male *Xenopus* which had been previously injected with 17β-estradiol. Cultures were exposed to 17β-estradiol and levels of the binding protein were assayed after 6 days. Since estradiol directly induces vitellogenin mRNA in liver cubes [30], vitellogenin mRNA levels were measured as a control. Although the animals were pre-treated with 17β-estradiol, it none the less elicited a further 7-fold elevation in vitellogenin mRNA in the liver cube cultures (Table 2), while testosterone and dexamethasone did not induce the mRNA. 17β-estradiol exposure also led to a 2-fold increase in the level of the

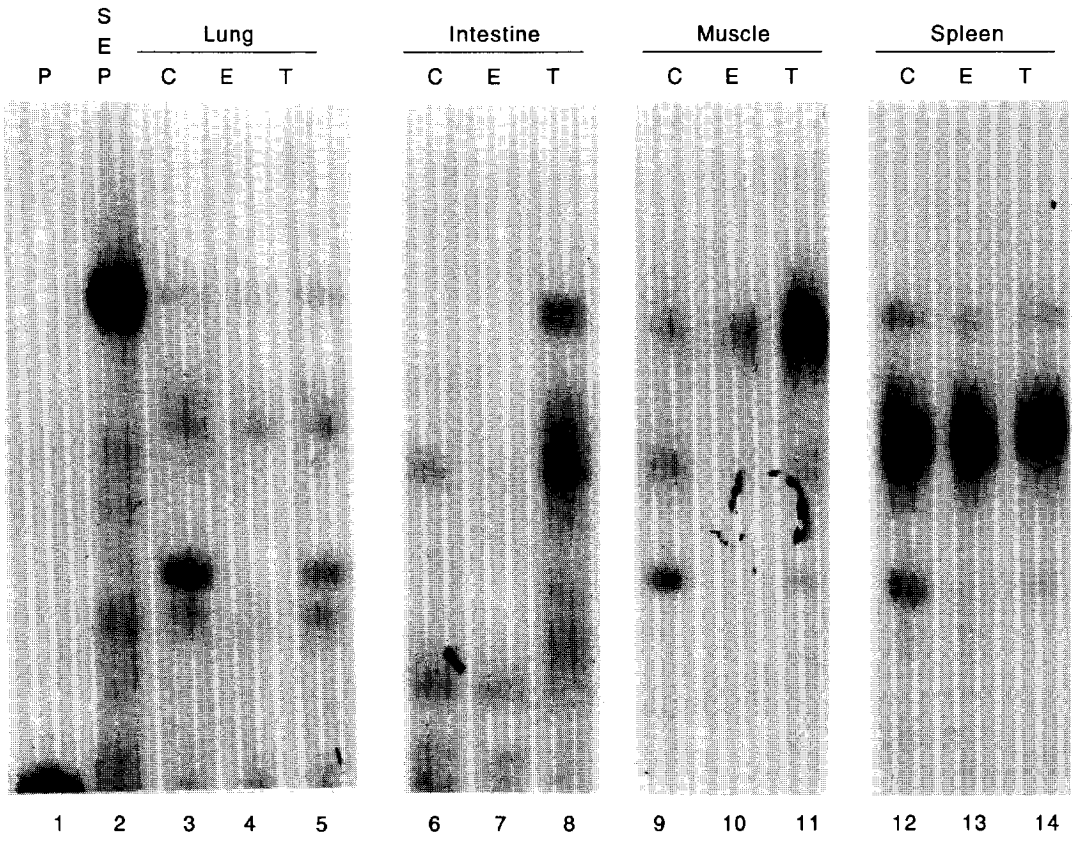

Fig. 3. Gonadal steroid effects on levels of the vitellogenin 3′-UTR RNA binding protein in tissues from *Xenopus laevis*. Autoradiogram of an RNA gel mobility shift assay. Bands corresponding to free probe and the vitellogenin 3′-UTR RNA binding protein are shown in lanes of probe incubated in the absence of protein (P), or in the presence of proteins from salt-extracted polysomes (SEP) from *Xenopus* liver. Tissues (lung, intestine, muscle and spleen) from control animals treated with vehicle (C) or from animals treated with estrogen (E) or testosterone (T) were collected, and whole tissue homogenates were used in the RNA gel shift assay.

vitellogenin 3′-UTR binding protein indicating that 17β-estradiol can act directly on liver to either induce or maintain levels of the binding protein.

The vitellogenin 3′-UTR binding protein is present in Xenopus and human cell lines

To further explore the distribution of the vitellogenin 3′-UTR binding protein, it was of interest to determine if the protein was present in tissue culture cells and if a mammalian homologue existed. Two cell lines were selected. First, the XL110 cell line from *Xenopus* liver was chosen because this cell line stabilizes vitellogenin mRNA in the presence of 17β-estradiol and estrogen receptor [29]. Secondly, MCF-7 human breast cancer cells [66] were used because these cells contain endogenous estrogen receptor [67]. Extracts were prepared from both cell lines and used in an RNA gel mobility shift assay (Fig. 4). The arrow indicates the vitellogenin 3′-UTR binding protein band (Fig. 4, lane 1). An upshifted band with the electrophoretic mobility characteristic of the vitellogenin mRNA 3′-UTR binding protein is present in extracts from XL110 cells (Fig. 4, lane 2). The MCF-7 cell extract (Fig. 4, lane 3) also shows a band which upshifts the labeled vitellogenin mRNA 3′-UTR probe to the same position as protein from the liver extract, indicating this may be a mammalian homologue of the vitellogenin mRNA 3′-UTR RNA binding protein.

Table 2. Regulation of vitellogenin mRNA and the vitellogenin mRNA 3′-UTR binding protein by steroids in Xenopus laevis liver cube cultures

	Ratio of steroid value/control value*	
	Vitellogenin mRNA	Vitellogenin 3′-UTR binding protein
Control	1	1
17β-Estradiol	7.1	1.9
Testosterone	0.5	0.6
Dexamethasone	0.3	0.8

*Ratio of the steroid-induced and control levels of the binding protein as determined from Phosphorlmager counts of RNA dots, or from the intensity of gel shifted bands.

Binding specificity of the MCF-7 RNA binding protein

To test the binding specificity to the 3′-UTR probe of the MCF-7 cell RNA binding protein, competition RNA gel mobility shift assays were performed. The labeled probe and increasing concentrations of the

Fig. 4. The vitellogenin 3′-UTR RNA binding protein is present in a *Xenopus laevis* liver cell line and in MCF-7 cells. RNA gel mobility shift using whole cell extracts (15 μg) from *Xenopus laevis* liver (liver), XL110 cells (XL110) or MCF-7 cells (MCF7). The band on the autoradiogram corresponding to the vitellogenin 3′-UTR RNA binding protein–RNA complex is indicated by the arrow.

competitors described above were added to extracts from MCF-7 cells or *Xenopus* liver. As shown in Fig. 5, the specific competitor competed for binding with the labeled RNA probe in both the MCF-7 and *Xenopus* liver extracts, while non-specific competitor did not compete over the same range of concentrations. The competition curves for both the MCF-7 and liver proteins are quite similar, indicating both proteins have a similar specificity for the protein binding site in the vitellogenin mRNA 3′-UTR.

17β-Estradiol does not alter the level of the MCF-7 cell mRNA binding protein

The vitellogenin mRNA 3′-UTR binding protein is induced by 17β-estradiol in *Xenopus* liver [54], and it was of interest to determine if the MCF-7 cell binding protein could be induced in MCF-7 cells. MCF-7 cells contain high levels of endogenous estrogen receptor [67]. A range of concentrations of 17β-estradiol was added to the cultures, and the cells were harvested 48 h later. Protein extracts were prepared as described above, and used in RNA gel mobility shift assays. The band corresponding to the RNA binding protein was quantified using a PhosphorImager and the results are presented in Table 3 as percent of the control (no added 17β-estradiol) value. Increasing amounts of 17β-estradiol had no effect on the intensity of the upshifted band, indicating that under these conditions, 17β-estradiol does not induce the MCF-7 cell mRNA binding protein.

Endogenous RNA from MCF-7 cells and from Xenopus liver and the vitellogenin 3′-UTR mRNA compete for binding to the Xenopus and MCF-7 cell mRNA binding proteins

The vitellogenin mRNA 3′-UTR binding site used to identify the human mRNA binding protein was derived from an amphibian mRNA. If the MCF-7 cell proteins play a role in mRNA metabolism, we would expect MCF-7 cell RNA to contain comparable protein binding sites. To test for the presence of these binding sites in MCF-7 cell RNA, we examined the ability of RNA extracted from MCF-7 cells to compete with the labeled vitellogenin 3′-UTR mRNA for binding of the *Xenopus* and human mRNA binding proteins. Binding of the *Xenopus* liver vitellogenin mRNA 3′-UTR RNA binding protein to the probe in the absence of competitor was defined as 100% binding. The histogram shown in Fig. 6 demonstrates that 0.4 μg of total MCF-7 cell RNA, from 17β-estradiol treated cells, was at least as effective as 0.4 μg of total RNA from 17β-estradiol treated male *Xenopus* liver (Fig. 6) or control male *Xenopus* liver, which does not contain vitellogenin mRNA (data not shown) in competing for binding by both the MCF-7 cell and *Xenopus* liver vitellogenin mRNA 3′-UTR binding proteins. In contrast, addition of 0.4 μg of tRNA, a non-specific competitor, had no effect on binding. These data indicate that RNA from MCF-7 cells and *Xenopus* liver can effectively compete for binding by the binding protein from both the original cells and from the other species. This provides additional data supporting the view that MCF-7 cells contain a functional mRNA binding protein system related to the one seen in *Xenopus* liver.

DISCUSSION

We previously demonstrated that control male *Xenopus* liver contained low levels of the vitellogenin mRNA 3′-UTR binding protein and that binding was induced 4–5-fold after administration of 17β-estradiol. Since vitellogenin mRNA is undetectable in control male *Xenopus* liver [30, 65, 68], it seemed likely that the binding protein had additional functions beyond participation in the 17β-estradiol-mediated stabilization of vitellogenin mRNA. The observation that RNA from control *Xenopus* liver competes for binding by the vitellogenin mRNA 3′-UTR binding protein provides additional support for the view that this protein binds to a diverse set of liver mRNAs. Our observation that low levels of the vitellogenin 3′-UTR binding protein were present in control male *Xenopus* liver [54] led us to investigate whether other tissues and organisms contained proteins which bind to the vitellogenin

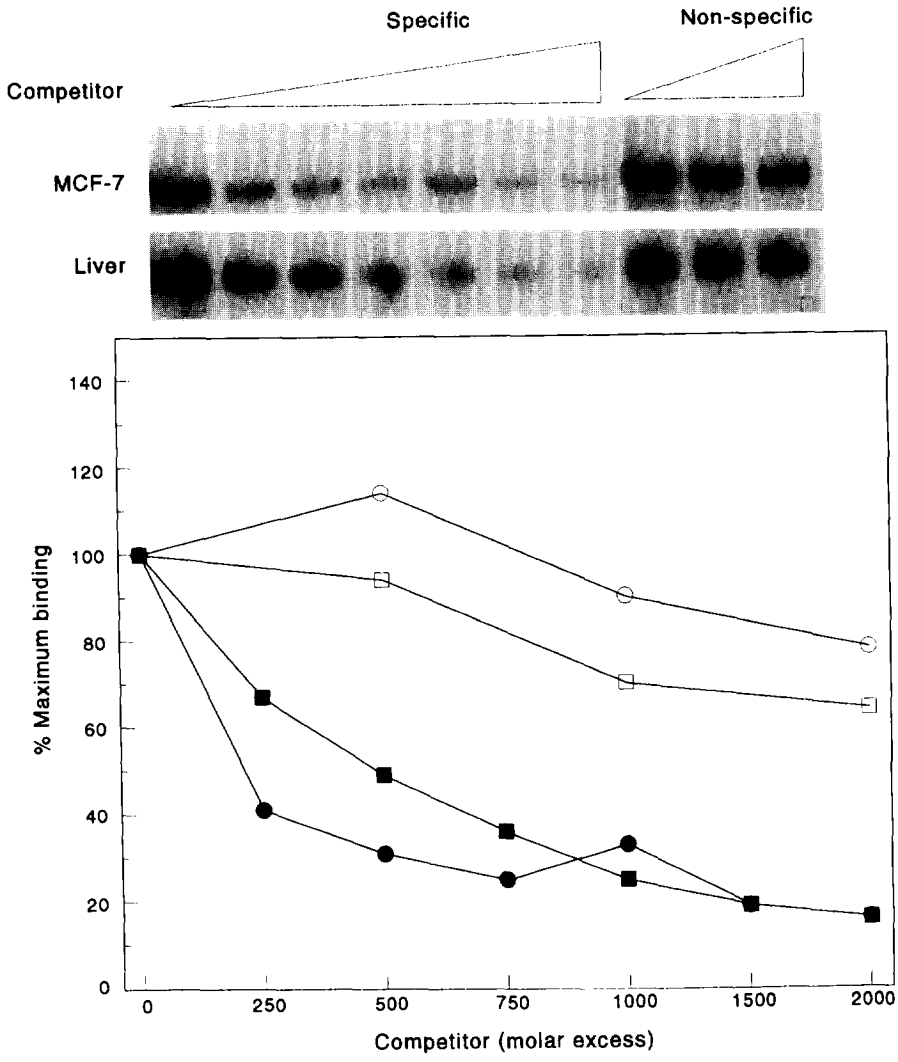

Fig. 5. RNA binding specificity to the MCF-7 RNA binding protein. An autoradiogram of an RNA gel mobility shift assay is shown at the top of the figure using protein from either MCF-7 cells (MCF-7; 4.4 μg protein) or *Xenopus laevis* liver (liver; 15 μg protein). Bands corresponding to the vitellogenin 3′-UTR RNA binding protein are shown. Increasing amounts of a specific or non-specific competitor RNA were added as indicated by the triangles at the top. Band intensities were quantified using a PhosphorImager and values are plotted in the graph at the bottom of the figure as percent of maximum binding versus molar excess of competitor added. Circles indicate the competition curve using MCF-7 protein and squares indicate competition using the liver protein. Filled symbols indicate values when specific competitor was added and open symbols indicate addition of non-specific competitor.

mRNA 3′-UTR binding site. Here we report that the vitellogenin mRNA 3′-UTR binding protein is present in a variety of *Xenopus* tissues, and an established *Xenopus* cell line, and that a homologous protein is present in a human breast cancer cell line. This suggests a broad role in mRNA metabolism for this protein. Our observation that the protein is differentially regulated by 17β-estradiol and by testosterone lends additional credence to the view that this protein is important in mRNA metabolism in diverse tissues. Both 17β-estradiol and testosterone slightly suppressed levels of the mRNA binding protein in testis. Since exogenous gonadal steroids act to decrease testicular function by suppressing gonadotropin levels [69], diminished testicular levels of the protein may result. A direct comparison of relative effects of 17β-estradiol and testosterone is not possible since we have not investigated the doses of the two hormones required to elicit maximum responses in the different tissues.

Table 3. Regulation of the mRNA binding protein by 17β-estradiol in MCF-7 cells

17β-Estradiol (M)	% of control*
0	100
10^{-9}	106
10^{-8}	100
10^{-7}	98
10^{-6}	104

*Control value is the level with no added hormone (0).

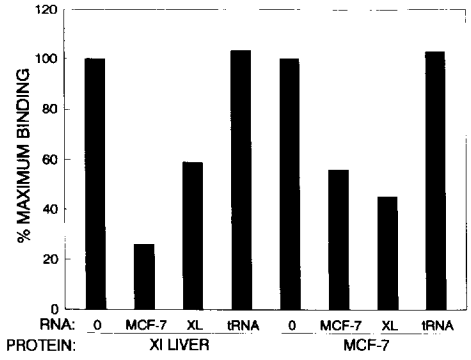

Fig. 6. Displacement of the MCF-7 and *Xenopus laevis* liver 3′-UTR RNA binding protein by endogenous MCF-7 and *Xenopus* liver RNA. Yeast tRNA (tRNA) or total RNA extracted from MCF-7 cells (MCF-7) or *Xenopus laevis* liver (XL) was used to compete for binding to ^{32}P-vitellogenin 3′-UTR RNA probe in an RNA gel mobility shift assay. Protein in the assay was from MCF-7 cells (4.4 μg), or extracts from salt extracted polysomes from *Xenopus* liver (2.6 μg) as indicated. The band corresponding to the vitellogenin 3′-UTR RNA binding protein was quantified using a PhosphorImager and values were graphed as percent of maximum binding. The value in the absence of added competitor RNA was used as maximum binding. Competitor RNA (0.4 μg) was added to each reaction.

Nevertheless, the observation that the two hormones elicited divergent effects in some tissues suggests a different action on the mRNA binding protein. In muscle and intestine, 17β-estradiol had no effect while testosterone elevated levels of the mRNA binding protein. Since androgen receptors are present in muscle, testosterone may have had a direct effect to elevate levels of the RNA binding protein. There is insufficient information on the androgen responsiveness of amphibian intestine to enable us to account for the appearance of this protein in intestine in response to testosterone.

The differences in levels of the vitellogenin 3′-UTR binding protein in different tissues and among various treatment groups within a given tissue appear to be due to real differences in levels of the protein, and not due to differences in nuclease activity which would result in differences in the level of the RNA probe, rather than of the binding protein. While the crude extracts most likely contain nucleases, we find little evidence of nuclease activity. This is probably because the binding reaction mixtures contain substantial amounts of tRNA, heparin and RNasin, which act to inhibit nucleases. The full-length probe band is seen as the major band on the gel with relatively little or no radioactivity migrating faster than it. Additionally, the vitellogenin 3′-UTR binding protein–RNA complex migrates as a single, compact band with little smearing or tailing. When substantial amounts of RNase T1 are added to the incubations, the probe band is completely degraded and the vitellogenin 3′-UTR binding protein–RNA complex exhibits a higher electrophoretic mobility due to digestion of portions of the probe not protected by the protein (data not shown).

The vitellogenin mRNA 3′-UTR binding site we define provides a convenient high affinity binding site with which to search for the mRNA binding protein in other tissues. The absence of the liver-specific vitellogenin mRNA in control male *Xenopus* liver, and in all of the other tissues in which the protein has been detected, indicates that these tissues contain RNAs with binding sites for the protein. To directly test this idea, we investigated the ability of RNAs extracted from control male *Xenopus* liver and from MCF-7 cells to compete for binding with the vitellogenin mRNA 3′-UTR binding site. RNA from both sources competed effectively indicating that both of these types of cells contain RNAs with binding sites for the protein. Scanning the GenBank database for mRNA sequences homologous to the 27 nucleotide sequence [54] which contains the vitellogenin 3′-UTR binding protein recognition sequence did not reveal a significant number of RNAs with a high homology to the primary sequence. This was not surprising since RNAs can assume a variety of 3-dimensional structures and sequence homology need only be a small part of the protein recognition site. This is exemplified by the multiple iron response elements important in iron control of ferritin and transferrin receptor mRNA which exhibit only a modest level of sequence homology. The protein appears to recognize a 3-dimensional RNA structure in which only about 6 non-contiguous nucleotides are highly conserved [14, 70]. A structural analysis of the vitellogenin mRNA 3′-UTR is likely to be necessary to identify the exact requirements for binding of the protein.

We have also identified a human homologue of the vitellogenin 3′-UTR RNA binding protein. A protein is present in MCF-7 cell extracts which binds to the vitellogenin mRNA 3′-UTR and an upshifted band is seen in the RNA gel mobility shift assay at the same position as the protein from the *Xenopus* liver extract. The MCF-7 cell RNA binding protein also has the same specificity as the *Xenopus* protein, in that it binds to a specific segment of the vitellogenin mRNA 3′-UTR and not to the directly adjacent region of the vitellogenin mRNA 3′-UTR. The view that the MCF-7 protein represents the human homologue of the *Xenopus* protein is supported by our observation that the competition curves for the two proteins are similar. Since vitellogenin mRNA is not present in mammals and RNA from MCF-7 cells can compete for vitellogenin binding to the MCF-7 protein, there are RNAs present in MCF-7 cells which are recognized by both the *Xenopus* and human mRNA binding proteins. There have been a few reports of the regulation of the stability of specific mRNAs in MCF-7 cells [40, 71, 72]. Although our data must be qualified because we have not exhaustively examined the regulation of the protein at a variety of times after addition of

17β-estradiol to the culture medium, or the level of estrogen receptor remaining in the cells, the mRNA binding protein does not appear to be regulated by 17β-estradiol in MCF-7 cells. Additional studies will be required to identify both the specific MCF-7 cell mRNAs containing binding sites for the protein and agents which regulate its level in MCF-7 cells.

We have shown that this protein which was previously only shown to be implicated in the control of *Xenopus* vitellogenin mRNA stability is broadly distributed and that a homologous protein is found in human cells. The differential regulation of the mRNA binding protein in different tissues by testosterone and by 17β-estradiol, coupled with the observation that RNAs other than vitellogenin containing binding sites for the protein are found in amphibian and human cells, suggests that this protein may have a broad role in mRNA metabolism. The identification of this steroid hormone regulated mRNA binding protein system extends the actions of steroid hormones to a new area of regulation, the control of the activity of mRNA binding proteins.

Acknowledgements—We are grateful to Professor B. S. Katzenellenbogen and members of her laboratory for useful advice on working with MCF-7 human breast cancer cells. This research was supported through grants from the National Institute of Child Health and Development (HD16720) and the National Science Foundation (DCB9008504).

REFERENCES

1. Jackson R. J.: Cytoplasmic regulation of mRNA function: the importance of the 3′ untranslated region. *Cell* 74 (1993) 9–14.
2. Manley J. L. and Proudfoot N. J.: RNA 3′ ends: formation and function-meeting review. *Genes Dev.* 8 (1994) 259–264.
3. Burd C. G. and Dreyfuss G.: Conserved structures and diversity of functions of RNA-binding proteins. *Science* 265 (1994) 615–621.
4. Mattaj I. W.: RNA recognition: a family matter? *Cell* 73 (1993) 837–840.
5. Birney E., Kumar S. and Krainer A. R.: Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors. *Nucl. Acids Res.* 21 (1993) 5803–5816.
6. Dreyfuss G., Matunis M. J., Pinol-Roma S. and Burd C. G.: hnRNP proteins and the biogenesis of mRNA. *A. Rev. Biochem.* 62 (1993) 289–321.
7. Hodgkin J.: Drosophila sex determination: a cascade of regulated splicing. *Cell* 56 (1989) 905–906.
8. Maquat L. E.: Nuclear mRNA export. *Curr. Op. Cell Biol.* 3 (1991) 1004–1012.
9. Ding D. and Lipshitz H. D.: Localized RNAs and their functions. *BioEssays* 15 (1993) 651–658.
10. Steward O. and Banker G. A.: Getting the message from the gene to the synapse: sorting and intracellular transport of RNA in neurons. *Trends Neurosci.* 15 (1992) 180–186.
11. Kozak M.: Regulation of translation in eukaryotic systems. *Rev. Cell Biol.* 8 (1992) 197–225.
12. Melefors O. and Hentze M. W.: Translational regulation by mRNA/protein interactions in eukaryotic cells: ferritin and beyond. *BioEssays* 15 (1993) 85–90.
13. Neilsen D. A. and Shapiro D. J.: Insights into hormonal control of messenger RNA stability. *Molec. Endocr.* 4 (1990) 953–957.
14. Klausner R. D., Rouault T. A. and Harford J. B.: Regulating the fate of mRNA: the control of cellular iron metabolism. *Cell* 72 (1993) 19–28.
15. Sachs A. B.: Messenger RNA. degradation in eukaryotes. *Cell* 74 (1993) 413–421.
16. Taylor S. J. and Shalloway D.: An RNA-binding protein associated with Src through its SH2 and SH3 domains in mitosis. *Nature* 368 (1994) 867–871.
17. Bandziulis R. J., Swanson M. S. and Dreyfuss G.: RNA-binding proteins as developmental regulators. *Genes Dev.* 3 (1989) 431–437.
18. Robinow S., Campos A. R., Yao K-M. and White K.: The elav gene product of drosophila, required in neurons, has three RNP consensus motifs. *Science* 242 (1988) 1570–1572.
19. King P. H., Levine T. D., Fremeau R. T. Jr and Keene J. D.: Mammalian homologs of drosophila elav localized to a neuronal subset can bind *in vitro* to the 3′-UTR of mRNA encoding the id transcriptional repressor. *J. Neurosci.* 14 (1994) 1943–1952.
20. Buckanovich R. J., Posner J. B. and Darnell R. B.: Nova, the Paraneoplastic R. antigen, is homologous to an RNA-binding protein and is specifically expressed in the developing motor nervous system. *Neuron* 11 (1993) 657–672.
21. Kelley R. L.: Initial organization of the drosophila dorsoventral axis depends on an RNA-binding protein encoded by the squid gene. *Genes Dev.* 7 (1993) 948–960.
22. Ashley C. T. Jr, Wilkinson K. D., Reines D. and Waren S. T.: RNR1 protein: Conserved RNP family domains and selective RNA binding. *Science* 262 (1993) 563–566.
23. Ma K., Inglis J. D., Sharkey A., Bickman, W. A., Hill R. E., Prosser E. J., Speed R. M., Thomson E. J., Jobling M., Taylor K., Wolfe J., Cooke H. J., Hargreave T. B. and Chandley A. C.: AY chromosome gene family with RNA-binding protein homology: Candidates for the azospermia factor AZF controlling human spermatogenesis. *Cell* 75 (1993) 1287–1295.
24. Szabo A., Daimau J., Manley G., Rosenfeld M., Wong E., Henson J., Posner J. B. and Furneaux H. M.: HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal. *Cell* 67 (1991) 325–333.
25. Siomi H., Siomi M. C., Nussbaum R. L. and Dreyfuss G.: The protein product of the fragile X gene, FMR-1, has characteristics of an RNA-binding protein. *Cell* 74 (1993) 291–298.
26. Gait M. J. and Karn J.: RNA recognition by the human immuno-deficiency virus Tat and Rev proteins. *Trends Biochem.* 18 (1993) 255–259.
27. Shapiro D. J., Blume J. E. and Nielsen D. A.: Regulation of messenger RNA stability in eukaryotic cells. *BioEssays* 5 (1987) 221–226.
28. Belasco J. and Brawerman G.: *Control of Messenger RNA Stability*. Academic Press Inc., San Diego (1993).
29. Nielsen D. A. and Shapiro D. J.: Estradiol and estrogen receptor-dependent stabilization of a mini-vitellogenin mRNA lacking 5,100 nucleotides of coding sequence. *Molec. Cell. Biol.* 10 (1990) 371–376.
30. Brock M. L. and Shapiro D. J.: Estrogen stabilizes vitellogenin mRNA against cytoplasmic degradation. *Cell* 34 (1983) 207–214.
31. Noteborn M. H., Bakker M. O., Dejonge M. A. W., Gruber M. and Ab G.: Differential estrogen responsiveness of the vitellogenin and apo very low density lipoprotein II genes in the rooster liver. *J. Steroid Biochem.* 24 (1986) 281–285.
32. Paek I. and Axel R.: Glucocorticoids enhance stability of human growth hormone mRNA. *Molec. Cell. Biol.* 7 (1987) 1496–1507.
33. Palmiter R. D. and Carey N. H.: Rapid inactivation of ovalbumin messenger ribonucleic acid after acute withdrawal of estrogen. *Proc. Natn. Acad. Sci. U.S.A.* 71 (1974) 2357–2361.
34. Schoenberg D. R., Moskaitis J. E., Smith L. H. and Pastori R. L.: Extranuclear estrogen-regulated destabilization of *Xenopus laevis* serum albumin mRNA. *Molec. Endocr.* 3 (1989) 805–814.
35. Petersen P. D., Koch S. R. and Granner D. K.: 3′ non-coding region of phosphoenolpyruvate carboxykinase mRNA contain a glucocorticoid-responsive mRNA stabilizing element. *Proc. Natn. Acad. Sci.* 86 (1989) 7800–7804.
36. Heaton J. H., Rathju S. and Gelehrter T. D.: Transcriptional and post-transcriptional regulation of type 1 plasminogen activator inhibitor and tissue-type plasminogen activator gene expression in HTC rat hepatoma cells by glucocorticoids and cyclic nucleotides. *Molec. Endocr.* 6 (1992) 53–60.
37. Bretano S. T., Block S. M., Lin D. and Miller W. L.: cAMP post-transcriptionally diminishes the abundance of adrenodoxin reductase mRNA. *Proc. Natn. Acad. Sci. U.S.A.* 89 (1992) 4099–4103.

38. Hod Y. and Hanson R. W.: Cyclic AMP stabilizes the mRNA for phosphoenolpyruvate carboxykinase (GTP) against degradation. *J. Biol. Chem.* **263** (1988) 7747–7752.
39. Amara F. M., Chen F. Y. and Wright J. A.: A novel transforming growth factor-β1 responsive cytoplasmic trans-acting factor binds selectively to the 3′-untranslated region of mammalian ribonucleotide reductase R2 mRNA: role in message stability. *Nucl. Acids Res.* **21** (1993) 4803–4809.
40. Stephens J. M., Carter B. Z., Pekala P. H. and Malter J. S.: Tumor necrosis factor alpha-induced glucose transporter (Glut-1) mRNA stabilization in 3T3-L1 adipocytes. *J. Biol. Chem.* **267** (1992) 8336–8341.
41. Nishizawa K.: NGF-induced stabilization of GAP-43 mRNA is mediated by both 3′-untranslated region and a segment encoding the carboxy-terminus peptide. *BBRC* **200** (1994) 789–796.
42. Herrick D. J. and Ross J.: The half-life of cmyc mRNA in growing and serum stimulated cells: influence of the coding and 3′ untranslated regions and role of ribosome translocation. *Molec. Cell Biol.* **14** (1994) 2119–2128.
43. Casey J. L., Koeller P. M., Ramin V. C., Klausner R. D. and Harford J. B.: Iron regulation of transferrin receptor mRNA levels requires iron-responsive elements and a rapid turnover determinant in the 3′-untranslated region of the mRNA. *EMBO J.* 8 (1989) 3693–3699.
44. Krane I. M., Spindel E. R. and Chin W. W.: Thyroid hormone decreases the stability and the poly(A) tract length of rat thyrotropin β-subunit messenger RNA. *Molec. Endocr.* 5 (1991) 469–475.
45. Murphy D., Pardy K., Seah V. and Carter D.: Posttranscriptional regulation of rat growth hormone gene expression: increased message stability and nuclear polyadenylation accompany thyroid hormone depletion. *Molec. Cell. Biol.* 12 (1992) 2624–2632.
46. Saceda M., Knabbe C., Dickson R. B., Lippman M. E., Bronzert D., Lindsey R. K., Gottardis M. M. and Martin M. B.: Post-transcriptional destabilization of estrogen receptor mRNA in MCF-7 cells by 12-O-tetradecanoylphorbol-13-acetate. *J. Biol. Chem.* **266** (1991) 17,809–17,814.
47. Zhu Y-Y., Schwartz R. J. and Crow M. T.: Phorbol esters selectively down regulate contractile protein gene expression in terminally differentiated myotubes through transcriptional repression and message destabilization. *J. Cell. Biol.* **115** (1991) 745–754.
48. Klett C., Rainer N., Gierschik P. and Hackenthal E.: Angiotensin II stimulates the synthesis of angiotensinogen in hepatocytes by inhibiting adenylcyclase activity and stabilizing angiotensinogen mRNA. *J. Biol. Chem.* **268** (1993) 25,095–25,107.
49. Seiser C., Teixeira S. and Kuhn L. C.: Interleukin-2-dependent transcriptional and post-transcriptional regulation of transferrin receptor mRNA. *J. Biol. Chem.* **268** (1993) 13,074–13,080.
50. Lopez-Barahona M., Minano M., Mira E., Iglesias T., Stunnenberg H. G., Rodriguez-Pena A., Benal J. and Nunoz A.: Retinoic acid posttranscriptionally up-regulates proteolipid protein gene expression in C6 glioma cells. *J. Biol. Chem.* **268** (1993) 25,617–25,623.
51. Busam K. J., Geiser A. G., Roberts A. B. and Sporn M. B.: Synergistic increase of phorbol ester-induced c-fos mRNA expression by retinoic acid through stabilization of the *c-fos* message. *Oncogene* **8** (1993) 2267–2273.
52. Nair A. P. K., Hahn S., Banholzer R., Hirsch H. H. and Moroni C.: Cyclosporin A inhibits growth of autocrine tumor cell lines by destabilizing interleukin-3 mRNA. *Nature* **369** (1994) 239–242.
53. Vacher J., Camper S. A., Krumlauf R., Compton R. S. and Tilgman S. M.: raf regulates the postnatal repression of the mouse alpha-fetoprotein gene at the posttranscriptional level. *Molec. Cell Biol.* **12** (1992) 856–864.
54. Dodson R. E. and Shapiro D. J.: An estrogen-inducible protein binds specifically to a sequence in the 3′-untranslated region of estrogen-stabilized vitellogenin mRNA. *Molec. Cell Biol.* **14** (1994) 3130–3138.
55. Kanamori H., Suzuki H., Siomi H., Nosaka T., Sato A., Sabe H., Hatanaka M. and Honjo T.: HTLV-1 p27rex stabilizes human interleukin-2 receptor alpha chain mRNA. *EMBO J.* **9** (1990) 4161–4166.
56. Port J. D., Huang L-Y. and Malbon C. C.: β-adrenergic agonists that down regulate receptor mRNA up-regulate a Mr 35,000 protein(s) that selectively binds to β-adrenergic receptor mRNAs. *J. Biol. Chem.* **267** (1992) 24,103–24,108.
57. Lee F-J. S. and Moss J.: An RNA-binding protein gene (RBP1) of *Sacchoromyces cerevisiae* encodes a putative glucose-repressible protein containing two RNA recognition motifs. *J. Biol. Chem.* **268** (1993) 15,080–15,087.
58. Nachaliel N., Jain D. and Hod Y.: A cAMP-regulated RNA-binding protein that interacts with phosphoenol pyruvate carboxy kinase (GTP) mRNA. *J. Biol. Chem.* **268** (1993) 24,203–24,209.
59. Chen F. Y., Amara F. M. and Wright J. A.: Mammalian ribonucleotide reductase R1 mRNA stability under normal and phorbol ester stimulating conditions: involvement of a *cis–trans* interaction at the 3′ untranslated region. *EMBO J.* **12** (1993) 3977–3986.
60. Liang H. and Jost J-P.: An estrogen-dependent polysomal protein binds to the 5′-untranslated region of the chicken vitellogenin mRNA. *Nucl. Acids Res.* **19** (1991) 2289–2294.
61. Maata A. and Penttinen R. P. K.: A fibroblast protein binds the 3′-untranslated region of pro-alpha 1 collagen mRNA. *Biochem. J.* **295** (1993) 691–698.
62. Brock M. L. and Shapiro D. J.: Estrogen regulates the absolute rate of transcription of the *Xenopus laevis* vitellogenin genes. *J. Biol. Chem.* **258** (1982) 5449–5455.
63. Wolffe A. P., Glover J. F., Martin S. C., Teniswood M. P. R., Williams J. L. and Tata J. R.: Deinduction of transcription of *Xenopus* 74-kD albumin genes and destabilization of mRNA *in vivo* and in hepatocyte cultures. *Eur. J. Biochem.* **146** (1985) 489–496.
64. Blume J. E. and Shapiro D. J.: Ribosome loading, but not protein synthesis, is required for estrogen stabilization of *Xenopus laevis* vitellogenin mRNA. *Nucl. Acids Res.* 17 (1989) 9003–9014.
65. Baker H. J. and Shapiro D. J.: Kinetics of estrogen induction of *Xenopus laevis* vitellogenin messenger RNA as measured by hybridization to complementary DNA. *J. Biol. Chem.* **252** (1977) 8434–8438.
66. Soule J. D., Vazquez J., Long A., Albert S. and Breanan M. A.: A human cell line from a pleural effusion derived breast carcinoma. *J. Natn. Cancer Inst.* 51 (1973) 1409–1416.
67. Lippman M. E., Bolan G. and Huff K.: The effects of estrogen and antiestrogen on hormone responsive human breast cancer in long term tissue culture. *Cancer Res.* **36** (1976) 4595–4601.
68. Baker H. J. and Shapiro D. J.: Accumulation of vitellogenin messenger RNA during secondary estrogen stimulation of *Xenopus laevis*. *J. Biol. Chem.* **253** (1978) 4521–4524.
69. Knobil E. and Neill J. D. (Eds): *The Physiology of Reproduction.* Raven Press, NY (1988).
70. Henderson B. R., Menoti E., Aonnard C. and Kuhn L. C.: Optimal sequence and structure of iron-responsive elements. *J. Biol. Chem.* **269** (1994) 17,481–17,489.
71. Levy J. R. and Hug V.: Regulation of insulin receptor gene expression: cell cycle-mediated effects on insulin receptor mRNA stability. *J. Biol. Chem.* **267** (1992) 25,289–25,295.
72. Sheikh M. S., Shao Z-M., Chen J-C., Clemmons D. T., Roberts Jr C. T., LeRoith D. and Fontana J. A.: Insulin-like growth factor binding protein-5 gene expression is differentially regulated at a post-transcriptional level in retinoic acid-sensitive and resistant MCF-7 human breast carcinoma cells. *Biochem. Biophys. Res. Commun.* **188** (1992) 1122–1130.