Estradiol Up-regulates AUF1p45 Binding to Stabilizing Regions within the 3′-Untranslated Region of Estrogen Receptor α mRNA*

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Estradiol up-regulates expression of the estrogen receptor α gene in the uterus by stabilizing estrogen receptor α mRNA. Previously, we defined two discrete minimal estradiol-modulated stability sequences (MEMSS) within the extensive 3′-untranslated region of estrogen receptor α mRNA with an in vitro stability assay using cytosolic extracts from sheep uterus. We report here that excess MEMSS RNA inhibited the enhanced stability of estrogen receptor α mRNA in extracts from estradiol-treated ewes compared with those from control ewes. Several estradiol-induced MEMSS-binding proteins were characterized by UV cross-linking in uterine extracts from ewes in a time course study (0, 8, 16, and 24 h after estradiol injection). The pattern of binding proteins changed at 16 h post-injection, concurrent with enhanced estrogen receptor α mRNA stability and the highest rate of accumulation of estrogen receptor α mRNA. The predominant MEMSS-binding protein induced by estradiol treatment was identified as AUF1 (A + U-rich RNA-binding factor 1) protein isoform p45 (a product of the heterogeneous nuclear ribonucleoprotein D gene). Immunoblot analysis indicated that only two of four AUF1 protein isoforms were present in the uterine cytosolic extracts and that estradiol treatment strongly increased the ratio of AUF1 isoforms p45 to p37. Non-phosphorylated recombinant AUF1p45 protected estrogen receptor α mRNA in vitro in a dose-dependent manner. These studies describe estrogenic induction of AUF1p45 binding to the estrogen receptor α mRNA as a molecular mechanism for post-transcriptional up-regulation of gene expression.

Estrogens are a family of hormones with important roles in reproduction as well as in cardiovascular, bone, and brain function. Estrogens potently regulate physiology by altering gene expression in sensitive tissues (1). The estrogen responsiveness of a tissue can be determined by its expression of the two genes encoding estrogen receptors, ERα and ERβ (2). Estrogens are best known for their ability to activate transcription of genes (1). However, nontranscriptional mechanisms of estrogen regulation of gene expression have been elucidated more recently, and many of them involve the actions of kinases and phosphatases (1, 3).

Expression of the ERα gene is tightly regulated by steroid hormones from the ovary in responsive tissues. In the uterus, expression of the ERα gene is 10-fold greater than that of ERβ (4). Studies with knock-out mice and with ERα- and ERβ-specific ligands indicate that the uterine effects of estradiol (E2) depend primarily upon expression of the ERα gene (5, 6). Therefore, the up-regulation of ERα gene expression in the uterus was the focus of our studies. Both mRNA and protein products of the ERα gene are short-lived, so that changes in their rates of synthesis and degradation rapidly alter tissue concentrations of ERα (7–9). The preovulatory surge of E2 up-regulates concentrations of ERα mRNA and protein in the uterus. This regulation occurs in several E2-sensitive tissues across diverse species ranging from fish to mammals (10–12). E2 is the only cellular signal known to up-regulate ERα gene expression.

Our laboratory discovered that E2 up-regulates expression of the ERα gene in sheep uterus by stabilizing ERα mRNA. In ovariectomized ewes injected with a physiological dose of E2, ERα mRNA concentrations increased 5-fold in 24 h (13). Although E2 treatment did not alter the rate of ERα gene transcription, it specifically increased the half-life of ERα mRNA 3.5-fold (14). E2 increased ERα mRNA concentrations throughout the endometrium and myometrium, with the most robust response in the deep endometrial glands and the adjacent myometrium (15), which we used exclusively in this report of studies of the molecular mechanism of E2 stabilization of ERα mRNA.

Hormones commonly autoregulate the expression of their receptor genes by regulating the stability of the mRNA encoding their receptor protein (16, 17). In general, hormone receptor mRNAs are inherently unstable because they have very long 3′-UTRs that contain destabilizing sequence elements such as the A + U-rich element (ARE) (8, 18, 19). Human and sheep ERα mRNAs carry 14 and 10 putative class I AREs, respectively (19), all of which are located within their extensive 3′-UTR sequences (18). Androgen receptor mRNA carries a class III ARE sequence that regulates its stability in response to andro-

untranslated region; hsp, heat shock protein; GST, glutathione S-transferase; NH, n hours previously treated with E2.

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‡ The abbreviations used are: ER, estrogen receptor; ARE, A + U-rich element; E2, estradiol-17β; MEMSS, minimal estrogen modulated stability sequences; NRS, nonimmune rabbit serum; PR, progesterone receptor; UTR,
Estradiol Induces AUF1p45 to Bind Estrogen Receptor α mRNA

gens (20). For ERα, androgen receptor, and many other hormone receptor genes, changes in mRNA stability are the predominant way their expression is regulated (16). However, the detailed molecular mechanism for this post-transcriptional regulation remains to be defined.

E2 is the hormone that is best known for effects on the stabilities of specific mRNAs (16, 21). In frog liver, E2 stabilizes vitellogenin mRNA by inducing vigilin protein (150 kDa) to bind specific sequences in the 3′-UTR of vitellogenin mRNA that are thereby protected from cleavage (22, 23). In rat uteri, E2 stabilizes IER2 (immediate early response gene) mRNA 4.5-fold by inducing binding of AUF1 (the A + U-rich binding factor 1, also known as heterogeneous nuclear ribonucleoprotein D) to its 3′-UTR (24, 25). Intriguingly, the primary transcript of the AUF1 gene is alternatively spliced to produce four mRNAs that encode 37-, 40-, 42-, and 45-kDa proteins (26). All bind AREs, but AUF1p37 and AUF1p42 associate with destabilized mRNAs, which they direct to exosomes for degradation (27–31). In contrast, AUF1p40 and AUF1p45 carry a unique mRNA stabilizing domain and are found associated with stabilized mRNAs (19, 32–36). AUF1 proteins dimerize and associate with other proteins to bind ARE sequences within mRNAs (37, 38). For AUF1p40 and AUF1p45, their partners on stabilized mRNAs may include HuR, heat shock protein (hsp) 27 and hsp 70 (38–43). These examples lead us to hypothesize that E2 treatment of ewes induces such uterine proteins to bind ARE sequences within mRNAs.

EXPERIMENTAL PROCEDURES

Materials—Common reagents were obtained from Sigma. Molecular biology enzymes were from Promega (Piscataway, NJ), except for RNase A (Roche Applied Science), RNase T1 (Ambion; Austin, TX), and ExTaq polymerase (Takara Mirus Bio, Madison, WI). Radiolabeled [35S]UTP (1412 Ci/mmol) and [32P]UTP (3000 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Antibodies P1b and P3a were developed against synthetic peptides of AUF1 carried by specific AUF1 isoforms (44). The P1b peptide was within exon 2 (which encodes the mRNA stabilization domain in AUF1p40 and AUF1p45), whereas the P3a peptide spanned the exon 6:8 junction (contained in AUF1p37 and AUF1p40). Pan-specific antisera that was raised to native AUF1 proteins containing all four isoforms (P1b, P2a, P3a, and P4b) were developed against synthetic peptides of AUF1 carried by specific AUF1 isoforms (44). The P1b peptide was within exon 2 (which encodes the mRNA stabilization domain in AUF1p40 and AUF1p45), whereas the P3a peptide spanned the exon 6:8 junction (contained in AUF1p37 and AUF1p40). Pan-specific antisera that was raised to native AUF1 proteins containing all four isoforms was purchased from Upstate (Lake Placid, NY), along with positive control HeLa cell nuclear extract. Antibodies for HuR (3A2) and hsp 70 (W27) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), whereas antiseros to hsp 27 was obtained from Stressgen (Victoria, Canada). Secondary antibodies conjugated to horseradish peroxidase were purchased from Invitrogen. Supersignal West Pico chemiluminescent substrate was purchased from Pierce. Bradford reagent and prestained SDS-PAGE standards (low range) were purchased from Bio-Rad.

Animal Treatments and Sample Collection—Adult crossbred ewes (Ovis aries) with normal estrous cycles were ovarioectomized during the breeding season and rested 4 weeks. The ewes were split into four treatment groups (n = 5 ewes in each group) and received 0.5 ml of charcoal stripped corn oil (control ewes) or 0.5 ml of oil containing 50 µg E2. The treated ewes were hysterectomized at 8, 16, and 24 h after E2-injection, whereas controls were hysterectomized at 24 h post-injection. After removal of superficial endometrial tissue, the remaining deep endometrium and inner layer of myometrium (“uterine tissue”) was dissected from residual outer myometrium and perimetrium. This tissue was used to make Si100 uterine cytosolic extracts (18). All of the animal procedures were approved by the Texas A & M University Laboratory Animal Care and Use Committee.

RNAs and the in Vitro Stability Assay—The assay and radiolabeled MEMSS, 18 S rRNA, and non-E2-regulated RNAs were described previously (18). The MEMSS-I and -II used here were previously referred to as MEMSS-C and -D, respectively. The ERα mRNA distal 3′-UTR fragment (274 bases that contain MEMSS-II) was produced from a template generated by PCR with primers: 5′-TAATACGACTCACTATAGGGAGAGAATTAAATAGTG and 5′-AAGGTGTTGAGCTCCCTT7 promoter underlined. Sense strand RNAs were produced with in vitro transcription reactions containing [32P]UTP. As a negative control for E2-enhanced stability in vitro, we cloned the distal region of progesterone receptor (PR) mRNA (cognate of the ERα 3′-UTR RNA above). It was predicted from genomic sequences because it was missing from the human reference sequence (GenBank accession number NM_000926). That sequence was only 5 kb long with 750 bases of 3′-UTR, whereas the major endogenous PR mRNAs in mammalian uteri are 7.1–14 kb in length (45). Human and bovine genomic sequences containing the PR gene were scanned for polyadenylation sites with POLYADQ and ERPIN software programs (46, 47). Both predicted a strong polyadenylation site, conserved in human and bovine genomic sequences, that was consistent in location with the 3′ end of a 14-kb PR mRNA. BLAST results of the region identified numerous oligo(dT)-primed mammalian expressed sequence tags (e.g. DQY756947) that immediately extended 3′ to the predicted polyadenylation site, confirming its function. PCR primers were designed with bovine sequences: 5′-CTTGCTAAGGGTGAGCTC (sense) and 5′-CTCTGAAGTCTACCTAGT (antisense), to amplify a cDNA ending 182 bp upstream of the polyadenylation site. The 700-bp cDNA (GenBank accession number EU184862) was amplified from reverse transcribed mRNA from sheep uterus, cloned into pCR 2.1 (Invitrogen) and then subcloned with BamHI and XbaI into pBluescript II KS (Stratagene, La Jolla, CA). The pBS-P3′-UTR plasmid was linearized with XbaI prior to T3 RNA polymerase transcribing the sense cRNA 3′-UTR.

Gel-purified RNAs (10,000 cpm) were combined with 5 µg of cytosolic extract proteins in 10-µl reactions containing 10 mM Tris/HCl, pH 8, 0.4 mM MgAc, 60 mM KCl, 0.4 mM dithiothreitol, 80 mM spermine, 7% glycerol, and 5 µg of yeast RNA. After 15 min of incubation at 37 °C, RNAs were purified with proteinase K, phenol extraction, and ethanol precipitation prior to separation on denaturing 5% polyacrylamide/8 M urea gels alongside a sample of the input labeled RNA. Radioactive signals were recorded from the dried gels with a Typhoon 8600 variable mode imager (Molecular Dynamics, Sunnyvale, CA). The images within a figure were from the same gel/experiment and were adjusted for contrast identically. For competition assays,
UV Cross-linking—UV cross-linking was performed essentially as described by Thomson et al. (48). Extract proteins (18 μg) were bound to 32P-labeled MEMSS RNA (800,000 cpm) in 10-μl reactions containing 20 mM Tris/HCl, pH 7.5, 5 mM MgCl2, 100 mM KCl, 2 mM dithiothreitol, 8% glycerol, and 0.1 μg of yeast tRNA. After binding for 10 min at room temperature, the RNA was cross-linked to the bound proteins using UV irradiation (10 min at 2-cm distance) in a CL-1000 UV cross-linker (UVP Inc., Upland, CA) followed by digestion with 1 μg/μl RNase A and 0.3 unit/μl RNase T1. The proteins (labeled with the remaining 32P RNA tags) were then separated on 12.5% SDS-PAGE. The dried gels were imaged as described above. Competition reactions used less protein (10 μg) and labeled MEMSS RNA (100,000 cpm) and had a preincubation step with the protein and unlabeled competitor MEMSS RNA for 10 min at room temperature prior to the addition of 32P-labeled MEMSS RNA. Negative control competitor RNA (pGEM) was produced by in vitro transcription from pGEM11ZF+ linearized with EcoRI using T7 RNA polymerase.

Immunoblotting—Protein samples (20 μg) from three control ewes and three ewes treated with E2 24 h previously were separated on 7.5% SDS-PAGE. Electrophoresis was continued until the 34-kDa marker was at the gel bottom to separate the individual AUF1 isoforms for analyses. Replicate samples for HuR, hsp 27, and hsp 70 analyses were electrophoresed on 10% SDS-PAGE until the dye front reached the gel bottom. All of the gels were electroblotted to Hybond C membrane (Amersham Biosciences). After blocking, the blots were incubated with primary antibodies raised to human proteins. Antisera P1b and P3a, GST-HuR, hsp 27, and hsp 70 were subcloned from pcDNA3-AUF1p45 and pcDNA-AUF1p37 using PCR primers 5’-GGCCGATCCCACTATGGTCGGAGGAGCAGTTC (sense) and 5’-GGCGAATTCCGCTTAGATGTTGTGACTATTTTGATG (antisense) according to Accuprime polymerase (Invitrogen) instructions. The 1050- and 846-bp cDNAs were restricted with BamHI and EcoRI enzymes (sites underlined in primers) and subcloned into those sites in the pGEX2T vector (Amersham Biosciences). After clone validation in FB5α Escherichia coli cells, pGEX2T, pGEX2T-AUF1p45, pGEX2T-AUF1p37, and pGEX2T-HuR (49) were transformed into BL21 E. coli cells for expression of glutathione S-transferase (GST), GST-AUF1p45, GST-AUF1p37, and GST-HuR proteins, respectively. Purification of the recombinant proteins from 500-ml cultures grown at 30°C was performed on glutathione-agarose (50). Protein concentration was measured in a Bradford assay (Bio-Rad), and purity was assessed on 10% SDS-PAGE (see Fig. 6A). The recombinant proteins or their buffer (20 mM reduced glutathione, 100 mM Tris, pH 8, 10% glycerol) composed 20% of the in vitro stability assay reactions. The recombinant proteins were preincubated with uterine extract proteins from control ewes (1.8 μg) in the stability assay buffer for 10 min at 37°C prior to the addition of radiolabeled ERα RNA (10,000 cpm) and a 5-min incubation at room temperature.

Data Analyses—Quantitated data were analyzed by least squares analysis of variance using General Linear Models procedures of the Statistical Analysis System (51). Significance was taken as p < 0.05.

RESULTS

In Vivo E2 Treatment Stabilizes ERα mRNA in Uterine Extracts in Vitro—In the mRNA stability assay we developed (18), radiolabeled ERα mRNA was much more stable during incubations with uterine extracts from E2-treated ovarioctomized ewes compared with extracts from control ewes. Fig. 1 demonstrates that a significantly larger percentage of the 3′-UTR fragment of ERα mRNA (pictured in the [en] extract lane at left) remained intact during duplicate incubations with extracts from two ewes treated with E2 24 h previously (E2-1 and E2-2 in the top panel) than during incubations with extracts from control ewes (Con-1 and Con-2). In the latter two, the ERα mRNA fragment was degraded to smaller RNA products (data not shown and Ref. 18). In contrast, the majority of the cognate 3′-UTR fragment of PR mRNA was unstable in extracts from both E2-treated and control ewes (Fig. 1, middle panel). This was expected because the instability of endogenous PR mRNA in sheep uteri was not affected by E2 treatment (14). 18 S rRNA was relatively stable in all uterine extracts, consistent with previous reports of 18 S rRNA and β-globin mRNA (18). A small degree of E2-enhanced stability for PR mRNA and 18 S rRNA appeared here and in previous data (18). However, when data
from four control and four E2-treated ewes were compared, the difference in percentages of intact RNA remaining in extracts from E2-treated ewes compared with controls was by far the greatest (80-fold compared with control) for the ERα 3′-UTR and much less for PR 3′-UTR (4-fold) and 18 S rRNA (2-fold). The E2-enhanced stability of ERα mRNA in Fig. 1 was similar to that in extracts from superficial endometrium, whereas there was no E2 effect detected in liver extracts from the same ewes (18). All together, we concluded that the uterine extracts in the in vitro assay reflected the E2-enhanced stability of ERα mRNA in vivo (13, 14), and the assay was a physiologically relevant model for investigation of the underlying molecular mechanism.

Inhibition of E2-enhanced ERα mRNA Stability by Excess MEMSS RNAs Implicates One or More E2-induced Binding/Stabilizing Protein(s)—Previously, we identified two 82-base-long sequences named MEMSS within the 4354-base-long ERα mRNA 3′-UTR that 1) retained enhanced stability in extracts from E2-treated ewes and 2) conferred E2-enhanced stability when transferred to heterologous RNAs (18). Uterine proteins from E2-treated ewes were implicated in the enhanced stability of ERα mRNA in vitro, because this function was ablated by pretreatment with proteinase K or heat (70 °C for 15 min). These data suggested two possible mechanisms for E2 stabilization of ERα mRNA: 1) E2 induces a sequence-specific mRNA stabilizing factor or 2) E2 inhibits an endoribonuclease that cleaves ERα mRNA specifically. To distinguish between these, excess amounts of unlabeled MEMSS RNA were added to the in vitro degradation assay. If the first proposed mechanism was correct, excess MEMSS RNA was predicted to sequester the E2-induced ERα mRNA-binding protein and destabilize ERα mRNA in extracts from E2-treated ewes. However, if the second was correct, excess MEMSS RNA competitor was predicted to sequester the endonuclease and stabilize ERα mRNA in extracts from control ewes. The data in Fig. 2 support the first mechanism. The addition of excess unlabeled MEMSS-II RNA destabilized radiolabeled ERα 3′-UTR in the extract from a ewe treated with E2 24 h previously. The effect was dose-dependent, so that the high dose of MEMSS RNA reduced the stability of ERα mRNA in the extract from the E2-treated ewe to the level of instability demonstrated in the extract from the control ewe. Similar results were obtained with extracts from other control and E2-treated ewes, as well as in assays using MEMSS-I RNA as the unlabeled competitor (not shown). The effect of the MEMSS competitor was specific, because the addition of an ERα mRNA fragment with stability not regulated by E2 treatment had no effect. The molar excess concentrations of unlabeled MEMSS added to the stability assays were large, similar to those employed by other laboratories to sequester mRNA-binding proteins (52). The high concentrations were required because of the dynamic nature of the stability assay, with degradation of both radiolabeled and unlabeled ERα mRNA increasing with sequestration of mRNA stabilizing proteins. The data lead us to postulate that E2 treatment induces one or more titratable proteins to bind MEMSS and stabilize ERα mRNA.

E2 Treatment Induces Several Proteins to Bind MEMSS RNA Specifically—The initial characterization of E2-induced proteins that bind to MEMSS was performed by UV cross-linking radiolabeled MEMSS to uterine extract proteins from ovariec-tomized ewes treated with E2 in a time course experiment. Fig. 3A shows data from two ewes from each of the four time point groups. The pattern of MEMSS-binding proteins was similar in extracts from three of the four Con and 8hE ewes and was distinct from the pattern generated by extracts from all four 16hE and 24hE ewes. Protein bands from Con and 8hE ewes included one major MEMSS-binding protein of 29 kDa and two fainter bands at 39 and 56 kDa that were more apparent than in the 16hE and 24hE ewe extracts. In contrast, 16hE and 24hE ewe extracts had one predominant protein band of 48 kDa and weaker bands of 34 and 27 kDa. In addition, a 70-kDa protein was common to all extracts but was more strongly labeled in the 16hE and 24hE extracts. When bovine serum albumin was used in the assay instead of uterine extract proteins, it was not labeled by UV cross-linking to MEMSS-II RNA. UV cross-linking with MEMSS-I RNA generated similar patterns of binding proteins with the uterine extracts from the time course ewes (not shown). These data demonstrate that E2 treatment of ewes altered the pattern of proteins in uterine extracts that bind MEMSS RNAs, including the induction of several binding proteins by 16 h post-injection. This time correlated with the in vitro stability of ERα mRNA in extracts from all ewes in the time course study; Con and 8hE extracts were not different and had
Estradiol Induces AUF1p45 to Bind Estrogen Receptor α mRNA

E2 Treatment Increases Binding of Specific Proteins to MEMSS RNA

7.0 ± 2.7% intact ERα RNA remaining, whereas 16hE extracts had 15.8 ± 1.7% remaining, and 24hE extracts had 20.2 ± 1.1% remaining. The 16-h time after E2 injection also correlates with the time of greatest accumulation of ERα mRNA in vivo (13).

The specificity of UV cross-linking of extract proteins to MEMSS RNA was demonstrated by adding excess unlabeled MEMSS RNA as a competitor. Increasing concentrations of unlabeled MEMSS-II RNA inhibited the UV cross-linking of extract proteins from a 24hE ewe to radiolabeled MEMSS-II RNA in a dose-dependent manner (Fig. 3B). All of the bands appeared equally affected, probably because the radiolabeled RNA and unlabeled competitor were the same sequence. The negative control pGEM RNA was added at the same concentrations but did not reduce the labeling of proteins by UV cross-linking to MEMSS RNA. The inhibitory concentrations were similar to those used by others to interfere with AUF1 binding to MYC mRNA in these static assays of RNA-protein interactions (53). These results indicate that the binding of proteins to MEMSS RNA is specific and saturable. Excess unlabeled MEMSS-I RNA was also an effective competitor of protein labeling by UV cross-linking to either radiolabeled MEMSS-I or -II (not shown), further suggesting that MEMSS-I and -II share common E2-induced binding proteins.

AUF1p45 and AUF1p37 in Uterine Extracts from E2-treated Ewes Bind MEMSS RNA—Because of one report of AUF1 proteins being involved in E2-stabilized mRNAs in the rat uterus (25), we investigated whether AUF1 proteins were among the MEMSS-binding proteins in uterine extracts from ewes treated with E2 16 h previously. Extract proteins were UV cross-linked to radiolabeled MEMSS-II RNA in replicate large scale reactions. Then the labeled proteins were subjected to immunoprecipitation with AUF1 isoform-specific antisera P1b and P3a and pan-specific antisera. In the leftmost lane of Fig. 4, a sample of UV cross-linked proteins (10% of that used in immunoprecipitations) demonstrates the large number of MEMSS-binding proteins in the extract. The P1b and P3a antisera immunoprecipitated protein bands that migrated at 48 and 40 kDa, respectively. The pan-specific αAUF antiserum bound two proteins with those sizes. The 48- and 40-kDa sizes are consistent with reported sizes of the AUF1p45 and AUF1p37 proteins, respectively (54–56). In addition, the 48-kDa size is consistent with that of the predominant E2-induced protein band in Fig. 3A. The 40-kDa protein band relating to AUF1p37 comigrated with a faint band in the sample lacking immunoprecipitation that was not E2-induced in Fig. 3A. In the negative control, in which immunoprecipitation was performed with NRS, no labeled proteins were apparent, even on very long exposures of the dried gel (not shown). Immunoprecipitation with antisera to HuR detected no cross-linked proteins (not shown). These data provide direct proof that AUF1p45 is among the uterine proteins induced by E2 treatment to bind MEMSS RNA.

E2 Treatment Up-regulates AUF1p45 Protein Concentrations in Uterine Extracts—To determine which AUF1 protein isoforms were present in the uterine cytosolic extracts and whether E2 treatment altered their concentrations, replicate immunoblot analyses were performed on samples from three control and three E2-treated ewes (24 h post-injection). HeLa cell nuclear extract was used as a positive control to confirm the migration positions of the AUF1 protein isoforms. The P1b antiserum detected one band in all uterine samples, migrating at a size consistent with that of AUF1p45 (Fig. 5A). The HeLa nuclear extract contained that protein and a higher molecular weight protein that generated a nonspecific band seen by others using the P1b antiserum (44, 57). The P3a antiserum identified a single protein band in uterine extracts that was consistent with the size of AUF1p37, whereas HeLa nuclear extract demonstrated both AUF1p37 and AUF1p40 bands. For comparison with the works of others, the most commonly used AUF1 antisera (raised to native AUF1 proteins) was also used. This pan-specific AUF1 antiserum confirmed that uterine extracts contained only AUF1p45 and AUF1p37 isoforms, whereas the HeLa nuclear extract contained all but the AUF1p42 isoform.
Semiquantitative analyses of the blots developed with P1b and P3a antisera indicated that E2 treatment increased concentrations of AUF1p45 6-fold, whereas those of AUF1p37 decreased 33% (Fig. 5B). An explanation for why E2-induced differences were not reflected in the blot developed with the pan-specific AUF1 antiserum is that it probably binds both phosphorylated and nonphosphorylated forms of AUF1, perhaps responsible for the doublet bands of AUF1p37. The total concentrations of AUF1 proteins did not appear to change with E2 treatment. In contrast, P1b and P3a antisera were generated with nonphosphorylated, synthetic peptides (44), and therefore, they may bind only the nonphosphorylated AUF1 protein isoforms (see “Discussion”). The P1b and P3a antisera allowed detection of a strong increase in the ratio of AUF1p45 to AUF1p37 in the uterine cytosolic extracts that was induced by E2 treatment.

Concentrations of other putative, E2-induced MEMSS-binding proteins were assessed on three more replicate immunoblots of the uterine extract samples. Proteins HuR (36 and 34 kDa), hsp 27 (27 kDa), and hsp 70 (70 kDa) were suspected to be E2-induced MEMSS-binding proteins because of their sizes (consistent with E2-induced bands in Fig. 3A) and because others have identified them within ribonucleoprotein complexes on stabilized mRNAs (38–43, 58, 59). The immunoblot results indicated that all three proteins were present in the uterine cytosolic extracts (Fig. 5A). However, their concentrations were unaffected by E2 treatment. Thus, E2 treatment specifically upregulated AUF1p45 in uterine extracts.

Recombinant GST-AUF1p45 Stabilizes ERα mRNA in Vitro—To determine whether increasing amounts of nonphosphorylated AUF1p45 could stabilize ERα mRNA in the presence of nucleases from control ewe extracts, the recombinant fusion protein GST-AUF1p45 was produced in E. coli alongside GST (a negative control), GST-AUF1p37, and GST-HuR (60, 61). The recombinant protein preparations appeared pure, with predominant protein bands for GST, GST-AUF1p45, GST-AUF1p37, and GST-HuR of expected sizes (27.5, 75.5, 67.5, and 61.5 kDa, respectively; Fig. 6A). In mRNA stability assays, increasing amounts of GST-AUF1p45 stabilized the radiolabeled ERα mRNA 3′-UTR in a dose-dependent manner (Fig. 6B, upper panel). Quantitation of the bands indicated that addition of the highest amount of GST-AUF1p45 stabilized 47% of the ERα mRNA put into the reaction compared with 0.7% in the reactions containing no recombinant protein or the highest amount of GST (Fig. 6B, upper panel). The addition of increasing amounts of GST-AUF1p37 and GST-HuR demonstrated much lower stabilizing effects on ERα mRNA (Fig. 6B, lower panel). Similar results were obtained when the experiment was repeated with extracts from two other control ewes (not

![Figure 5](image1.png)

**FIGURE 5.** E2 treatment increases the ratio of AUF1p45 to AUF1p37 in uterine cytosolic extracts. Replicate immunoblots containing samples of uterine extract proteins (20 μg) from three control ewes and three ewes treated with E2 24 h previously were bound by antisera to AUF1 proteins described in Fig. 4 as well as antisera to HuR (αHuR), hsp 27 (αhsp27), and hsp 70 (αhsp70). The HeLa cell nuclear extract was included for identification of AUF1 isoforms AUF1p45, AUF1p40, and AUF1p37. Images of the chemiluminescent signals are shown with antisera indicated at left and protein identifications at the right of each panel. A, the signals for AUF1p45 and AUF1p37 are quantitated from the blots developed with P1b and P3a antisera, respectively. They are presented graphically as the means and standard errors for the control (Con) and E2-treated (E2) ewes.

![Figure 6](image2.png)

**FIGURE 6.** Nonphosphorylated recombinant GST-AUF1p45 increases ERα mRNA stability in vitro. A, samples of recombinant GST, GST-AUF1p45, GST-AUF1p37, and GST-HuR were analyzed on 10% SDS-PAGE followed by Coomassie staining. Protein bands of expected sizes are apparent for each when compared with the migration distances of standards (indicated at left). B, increasing amounts (7.5, 15, and 30 ng/μl) of the indicated recombinant protein (rProtein) were added to duplicate in vitro stability reactions containing radiolabeled ERα mRNA 3′-UTR and uterine extract proteins from a control ewe. The input radiolabeled RNA is shown in the leftmost lane of each panel (arrow).
Estradiol Induces AUF1p45 to Bind Estrogen Receptor α mRNA

FIGURE 7. A model for estrogen induction of stabilizing proteins that bind ERα mRNA. A cartoon of ERα mRNA is shown with the coding sequence (cds) 5′ to the extensive (4354 base long) 3′-UTR. Within the latter, there are two 62-base MEMSS: I and II, shown as simple stem loop structures. Estrogen increases levels of AUF1p45 protein (p45), which binds MEMSS along with other proteins (ovals). The estrogen-induced ribonucleoprotein complex stabilizes ERα mRNA against degradation by ribonuclease (RNase), which results in increased ERα gene expression.

shown). These data indicate that nonphosphorylated AUF1p45 is a limiting factor that stabilizes ERα mRNA in uterine extracts and is likely to stabilize endogenous ERα mRNA within sheep uteri in response to E2 in vivo.

DISCUSSION

The data presented in this report provide the first characterization of the proteins that are induced by E2 treatment to bind stabilizing sequences of ERα mRNA. The predominant one was identified as AUF1p45, which associates with several distinct proteins in ribonucleoprotein complexes on mRNAs that are stabilized in response to cellular signals (30, 32, 35). The simplest mechanistic model for E2 stabilization of ERα mRNA based on our data is presented in Fig. 7. E2 treatment increases the concentration of AUF1p45, which binds to MEMSS along with other proteins to form stabilizing ribonucleoprotein complexes on the 3′-UTR of ERα mRNA, thereby reducing its degradation by RNase. This indirect mechanism of E2 action is consistent with the two other well characterized examples of E2 stabilization of specific mRNAs: vitellogenin mRNA in frog liver and IER2 mRNA in rat uterus (22, 25). Our discovery that E2 induces AUF1p45 binding to ERα mRNA indicates that the ERα gene shares post-transcriptional regulation with early response genes that bear AREs, including IER2 and c-Fos mRNAs (8, 62). The specific binding of AUF1p45 to both MEMSS-I and -II implies that they contain functional AREs that are most similar to class III AREs because they are U-rich (18, 19). Characterization of the E2 stabilization of ERα mRNA in the sheep uterus will help further define the functions of these ARE cis-elements and the AUF1-binding proteins.

Regulation of the expression of the AUF1 gene is important to normal physiology and disease (38, 63–69). In the sheep uterus (this report) and rat uterus (25), E2 treatment increased concentrations of stabilizing AUF1 protein isoforms AUF1p45 and AUF1p40, respectively. In rat uteri, the pan-specific AUF1 antisera detected E2 induction of a rapid but brief increase in all four AUF1 proteins isoforms in crude cytosolic extracts. All four AUF1 isoforms are probably present in sheep uterus; however, the S100 extracts used here and elsewhere as a source of cytosolic RNA-binding proteins (70–72) had polysomes and nuclear remnants removed by high speed centrifugation. Thus, it is likely that AUF1p42 and AUF1p40 proteins were lacking in our S100 extracts because of their preferential association with nuclei and polysomes (33, 73, 74). Use of the P1b and P3a antisera developed to isoform-specific peptides was key to our detection of a strong E2-induced increase in AUF1p45 concentrations. Because the P1b antisera was developed to a synthetic peptide, it is likely that the increased AUF1p45 was nonphosphorylated.

The phosphorylation state of AUF1 proteins is important to their function in binding and regulating the stabilities of ARE-bearing mRNAs (75, 76). For example, phorbol ester treatment of monocytes triggers dephosphorylation of AUF1p40 protein on Ser83 and Ser87, which are central to the 19-amino acid mRNA stabilizing domain (77). (The peptide used to generate P1a antisera contained both Ser83 and Ser87 in nonphosphorylated states (44).) In monocytes, the dephosphorylated AUF1p40 binds and stabilizes interleukin 1β and tumor necrosis factor-α mRNAs, leading to the conclusion that dephosphorylation may be the physiological switch for activation of AUF1 proteins (77). In rat parathyroid gland, hypocalcemia induced dephosphorylation of AUF1p45, which binds the regulatory region of parathyroid hormone receptor mRNA during its stabilization (75). Dephosphorylation of Ser83 and Ser87 increases AUF1p45 protein binding to AREs, which induces a condensed conformation of the mRNA that lowers the affinity of AUF1p37 for the ARE (61). Thus, cell stimuli that dephosphorylate Ser83 and Ser87 in the mRNA stabilizing domains of AUF1p45 and AUF1p40 may not only enhance their mRNA binding but also prevent binding of the destabilizing AUF1p37 isoform.

E2 action is also intimately linked with phosphorylation and dephosphorylation of critical proteins (1, 78–81). ERα gene expression and function is dependent upon phosphoryrases (82–85). In breast cancer cell lines, ERα gene expression is coordinately regulated with that of protein phosphatase 2A, and inhibition of the phosphatase destabilizes ERα mRNA via its 3′-UTR sequence (83). With the data presented here, one could speculate that protein phosphatase 2A may dephosphorylate AUF1p45, which stabilizes ERα mRNA. Further investigations are required to reveal how E2 affects the phosphorylation state of specific AUF1 protein isoforms and their subsequent functions in regulating the stabilities of ARE-bearing mRNAs, including ERα mRNA.

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