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Estradiol up-regulates expression of the A + U-rich binding factor 1 (AUF1) gene in the sheep uterus

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ABSTRACT

The A + U-rich binding factor 1 (AUF1 or HNRPD) gene produces predominant RNA binding proteins. The AUF1 transcript is alternatively spliced to produce four protein isoforms that stabilize or destabilize hundreds of mRNAs. Previously, we discovered that estradiol (E2) treatment of ovariectomized sheep increased concentrations of AUF1p45 protein which stabilized estrogen receptor alpha (ER) mRNA in the uterus. This study examined E2 regulation of AUF1 mRNAs in the sheep uterus. Northern analysis determined that E2 treatment increased concentrations of total AUF1 mRNAs twofold in endometrial and myometrial tissue compartments. In situ hybridization indicated that the increase was most intense in the glandular epithelium of endometrium. In a well characterized *in vitro* RNA stability assay, AUF1 3′UTR sequences were much more stable in uterine extracts from E2-treated ewes compared to extracts from control ewes. AUF1 mRNAs with alternative splicing of exons 2 and 7 (in the coding sequence) and exon 9 (in the 3′UTR) were identified. The only effect of E2 treatment on alternative splicing was that it reduced the percentage of AUF1 mRNAs containing exon 9-derived sequences. These data indicate that E2 up-regulates AUF1 and ER genes coordinately by a post-transcriptional mechanism.

1. Introduction

Estrogens affect the expression of genes in many ways. While they are most studied for transcriptional activation of genes, estrogens also act post-transcriptionally to regulate splicing, stability and translation of specific mRNAs [1,2]. In the mammalian uterus, estrogens primarily act through the estrogen receptor alpha (ER) protein [3–5]. Our investigations of estrogen regulation of ER gene expression have determined that *in vivo* estradiol (E2) treatment strongly up-regulates ER mRNA and protein concentrations in the uteri of ovariectomized sheep by stabilizing ER mRNA [6,7]. Using discrete E2-stabilized sequences from the 3′ untranslated region (3′UTR) of ER mRNA, we identified AUF1p45 as the major protein induced by E2 treatment to bind and stabilize ER mRNA [8,9]. This positive autoregulation of ER gene expression by E2 serves to enhance estrogen responsiveness in the uterus during the follicular phases of the estrous and menstrual cycles in all mammals examined.

To determine the molecular mechanism by which estrogens increase ER gene expression, E2 regulation of the expression of the AUF1 (also known as heterogeneous nuclear ribonucleoprotein D or HNRPD) gene was examined. The AUF1 proteins shuttle between nuclear and cytoplasmic compartments and have several roles in the cell, including the post-transcriptional regulation of mRNAs from hundreds of genes [10,11]. The molecular biology of the products of the AUF1 gene is complex. The AUF1 mRNA sequence is remarkably conserved across mammalian species. This is true even within the 3′untranslated region (3′UTR), which is typically more variable and serves to regulate mRNA stability [12]. A single AUF1 transcript is alternatively spliced at exon 2 and exon 7 sequences to produce four protein isoforms: AUF1p37, -p40, -p42 and -p45 [13]. Exon 2 encodes a 19 amino acid long domain within AUF1p40 and -p45 isoforms that has two Ser residues with regulated phosphorylation [14]. With the exon 2 encoded domain, AUF1p40 and -p45 stabilize mRNAs in several model systems [9,15–18]. The AUF1p37 and p42 isoforms lack that domain. They bind RNA more strongly and are associated with rapidly degrading mRNAs [19]. Exon 7 encodes 49 amino acids that make the AUF1p42 and -p45 isoforms less susceptible to ubiquitination and rapid protein degradation than AUF1p37 and -p40 isoforms [20]. Exon 9-derived sequence in AUF1 mRNAs is also alternatively spliced, which is unusual because it is within the 3′UTR [12]. It is apparent that reg-

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ulation of the alternative splicing and stabilities of AUF1 mRNAs is critical to the effects of AUF1 proteins on the expression of other genes.

Numerous laboratories have demonstrated the importance of the regulated expression of the AUF1 gene to normal physiology and disease [21–24]. Cell stimuli including estrogen, angiotensin, and oxidative stress regulated AUF1 gene expression at the level of mRNA concentrations and alternative splicing, and protein concentrations, subcellular localization, and post-translational modifications [25,26]. In the uteri of ovariectomized rats, E2 treatment was reported to specifically up-regulate mRNAs encoding AUF1p40 and -p45 proteins by stabilizing the mRNAs [27]. The regulation of AUF1 gene expression, in turn, altered concentrations of AUF1 target mRNAs including those encoding the β-adrenergic receptor, c-myc, granulocyte-macrophage colony-stimulating factor and interleukins-1β and -6 [24,28,29]. In one example, angiotensin regulation of AUF1 gene expression destabilized the mRNAs encoding the β-adrenergic receptor and the Kv4.3 potassium ion channel as part of the pathophysiology of congestive heart failure [18,30]. When AUF1 gene expression was compromised experimentally in "knock-down" experiments, AUF1 target mRNAs were deregulated [29,31]. AUF1 knockout mice had severe inflammatory diseases resulting from failure to degrade mRNAs encoding proinflammatory cytokines [32,33]. In contrast, overexpression of AUF1p37 in transgenic mice lead to tumorigenesis [34]. Thus, it is important to study the regulation of the AUF1 gene to further understand post-transcriptional gene regulation. That knowledge could be applied to develop new therapeutic approaches for the prevention and/or treatment of diseases [35].

In this study, we investigated the AUF1 mRNAs produced by uterine tissues of control and E2-treated ovariectomized sheep. Analyses included E2 effects on the relative levels of alternatively spliced AUF1 mRNAs and the stabilities of the 3′UTR sequences of AUF1 mRNAs.

2. Materials and methods

2.1. Experimental animals

Adult cross-bred ewes (*Ovis aries*, weight 48–50 kg) with normal estrous cycles were ovariectomized during the breeding season and then rested 4 weeks. Ewes were split into three treatment groups ($n = 5$ ewes in each group) and received 0.5 ml charcoal stripped corn oil ("control ewes") or 0.5 ml oil containing 50 μg E2. Treated ewes were hysterectomized at 18 or 24 h after E2-injection, while controls were hysterectomized at 24 h post-injection. The 18 h time after E2-injection is the peak of ER mRNA stabilization while 24 h post-injection is the time of greatest ER mRNA accumulation [7,6]. Since we suspect coordinate regulation of AUF1 gene expression, the 18 h E2-treated ewe tissues were used for all experiments except the in situ hybridization. For that data, the 24 h treated ewes showed a greater increase in AUF1 mRNA than the 18 h treated ewes. Uterine cross-sections were taken at the external bifurcation of each horn and fixed with paraformaldehyde for in situ hybridization [36]. The lumina of the uterine horns were cut open and superficial endometrial and deeper myometrial tissue samples were dissected, snap frozen and stored at −80 °C for later RNA analyses. For *in vitro* stability assays, cytosolic extracts were made from fresh uterine tissue (deep endometrium and the adjacent inner layer of myometrium) as previously described [8]. The preparation the cytosolic extracts included a high speed centrifugation step that removed cellular organelles and polysomes. All animal procedures were approved by the Texas A&M University Laboratory Animal Care and Use Committee.

Table 1
Primer sequences used in reverse-transcription and PCR.

Primer set	Primer sequences (5′–3′)
1. AUF1 cds	AGGAGGCCTTAGCTGGGACA (sense)
	T7P-TTCCAATAGTTACTATATCC (antisense)
2. AUF1 3′UTR (exon 10)	T7P-TTGAAGGTGGCTCCTGCCACC (sense)
	AACCAGTAAGACACTACTACATCATGAC (antisense)
3. AUF1 cds (exons 1 and 8)	GCCAGTAAGAACGAGGAGGAT (sense)
	TTAGTATGGTTTGTAGCTATTTGATG (antisense)
4. RPS2 cds	TGGTCAAGGACATGAAGATCAAGTC (sense)
	GCCTTGGCGAAGTTGCCCAGGGTGGC (antisense)
5. AUF1 (exon 10 for RT)	TAGTTTGAACTGATATTAGC (antisense)
6. AUF1 3′UTR (exons 8 and 10)	ATGCAGGCGAGGCGGCCATC (sense)
	AGGTGGCAGGAGCCACCTTC (antisense)

Abbreviations: "cds" is coding sequence; "T7P" is the T7 promoter sequence (5′)TAATACGACTCACTATAGGG; "for RT" refers to use in reverse transcription.

2.2. Northern blot analysis

Total cellular RNA was isolated from samples of endometrium and myometrium from five control ewes and five E2-treated ewes. Absorbance at 260 nm assessed high RNA concentrations in all samples except one endometrial sample from a control ewe, which was not analyzed further. RNA samples (8 μg) were run on 1% agarose denaturing gels and blotted as previously described [6]. The ethidium bromide fluorescence on the blots was imaged with a Typhoon 8600 Variable Mode Imager (Molecular Dynamics; Sunnyvale, CA) to assess transfer efficiency and for later normalization of AUF1 mRNA hybridization signals to the fluorescence of 18S rRNA bands in each lane. The antisense cRNA for the AUF1 probe was transcribed from a PCR product generated from pcDNA3-p45AUF1, which contained the entire human AUF1p45 cDNA coding sequence. The plasmid (linearized with KpnI) was used as a PCR template with Primer Set 1 (Table 1). The PCR product was used in *in vitro* transcription with T7 RNA polymerase to generate a 596 base antisense cRNA. The majority of the cRNA (549 bases) was complementary to all of the differentially spliced mRNAs that generate the four AUF1 protein isoforms [13,37]. After hybridization and stringent washing of the Northern blots [6], AUF1 mRNA hybridization signals were captured with the Typhoon Imager. ImageQuant software (version 5.2) was used to quantitate the radioactive hybridization signals of AUF1 mRNA and fluorescent staining of the 18S rRNA bands in each lane.

2.3. In situ hybridization

In situ hybridization was performed as previously described for ER mRNA [36]. Similarly, antisense cRNA probes were synthesized *in vitro* with ^{35}S-UTP for AUF1 mRNA (as described above). The AUF1 cRNA probe was synthesized with three times higher specific activity than the ER cRNA probe [36]. A sense ER cRNA probe was synthesized and used as a negative control [36]. Adjacent uterine cross-sections from control and E2-treated ewes were hybridized, washed, and coated with autoradiographic emulsion. After development of the emulsion, nuclei were stained lightly with hematoxylin to visualize tissue architecture. Darkfield and brightfield images were captured with an Axioplan2 microscope equipped with an Axiovision camera and software (Zeiss, Thornwood, NY).

2.4. In vitro RNA stability assays

The procedures for the production radiolabeled sense ER mRNA and 18S rRNA by *in vitro* transcription and the *in vitro* RNA sta-

bility assay were described previously [8]. To synthesize a sense cRNA from AUF1 exon 10, an *in vitro* transcription template was generated by PCR using Primer Set 2 (Table 1) and cDNA reverse transcribed from RNA from sheep uterus (described below). Briefly, sense strand RNAs were produced with *in vitro* transcription reactions using T7 RNA polymerase and ribonucleotides including ^{32}P-UTP. Gel-purified RNAs (10,000 cpm) were combined with 5 μg of uterine cytosolic extract proteins in 10 μl reactions. After 15 min incubation at 37 °C, RNAs were purified with proteinase K, phenol-extraction and ethanol precipitation prior to analysis on denaturing 5% polyacrylamide/8 M urea gels alongside a sample of the input radiolabeled RNA. Time course experiments for AUF1 exon 10 and ER 3′UTR cRNAs (performed in duplicate) used 0, 2.5 and 10 min incubations for three control ewe extracts and 0, 1.5 and 3 h incubations for extracts from three E2-treated ewes. AUF1 3′UTR sequences with and without exon 9 (described below) were similarly analyzed for *in vitro* stability except that 0, 2, and 4 h time points were performed with extracts from the E2-treated ewes. Radioactive signals were recorded from the dried gels with the Typhoon Imager. Radioactive signals from the remaining intact cRNAs were quantitated with ImageQuant software.

2.5. Qualitative reverse transcription-polymerase chain reaction (RT-PCR)

Primers used in this work (Table 1) were designed to conserved sequences between the human and bovine AUF1 (GenBank accession nos. NM_031370 and XM_877236) and ribosomal subunit S2 (RPS2) mRNAs (GenBank accession nos. BC008329 and BC102227). Total cellular RNA samples (above) were analyzed for mRNAs encoding the four isoforms of AUF1 protein with RT-PCR, similar to a previous study of AUF1 mRNA in rat uteri [27]. AUF1 primers in exons 1 and 8 (flanking the alternatively spliced exons 2 and 7 sequences) were the same as in that study except the 13th nucleotide of the sense primer was changed from T to C to match human and bovine AUF1 sequences (Primer Set 3 in Table 1). Briefly, endometrial and myometrial RNA samples (3.0 μg) from control and E2-treated ewes were reverse transcribed in 25 μl reactions with Superscript II (Invitrogen; Carlsbad, CA) using oligo-dT primers. It is noteworthy that, in our experience with quantitative PCR, 100 ng of RNA is near the upper limit of quantitative reverse transcription in similar reactions, so results presented here are qualitative. From 1.0 μl reverse transcription, AUF1 cDNAs were amplified in 25 cycles of PCR [27] with ExTaq enzyme (Takara; Madison, WI). The 861, 804, 714 and 657 bp products originated from the mRNAs encoding AUF1p45, -p42, -p40 and -p37 isoforms, respectively [27]. Positive controls were KpnI-linearized plasmids pcDNA3-AUF1p37, -AUF1p40, -AUF1p42 and -AUF1p45, which encode the four human AUF1 isoforms. The PCR products were separated on a 3% NuSieve gel (BioWhittaker Molecular Applications, Rockland, ME) and visualized with ethidium bromide staining. All RT-PCR assays were repeated independently at least three times. Ribosomal protein S2 (RPS2) mRNA measurements were used to normalize amounts of uterine cDNA put into PCR, as in [27]. We determined that the concentrations of RPS2 mRNA in sheep uterine tissues were not affected by E2 treatment by Northern blot and real time PCR analyses (not shown). The RPS2 primers (Primer Set 4, Table 1) generated a 480 bp product in 20 cycles of PCR [27]. Detection of the product on 1% agarose gels was used to assess the amount of reverse transcribed cDNA added to each PCR reaction.

The 3′UTR sequences of AUF1 mRNAs were similarly investigated for differential splicing of exon 9-derived sequences in RNA samples from endometrium and myometrium from control and E2-treated ewes ($n = 3$ each). Reverse transcription and PCR used AUF1 primers (Primer Sets 5 and 6 in Table 1, respectively) designed to exons 8 and 10. PCR for 35 cycles of 94 °C for 30 s, 50 °C for 1 min, and 70 °C for 30 s generated products, which were visualized on ethidium bromide stained 1.8% agarose gels. Subcloning (TA Cloning Kit; Invitrogen; Carlsbad, CA) and sequencing of the 247 and 140 bp products confirmed that they contained or lacked the 107 bp exon 9 sequence, respectively. The 247 bp long sequence of the sheep AUF1 mRNA exons 8, 9 and 10 (combined) was submitted to GenBank (accession no. FJ197141).

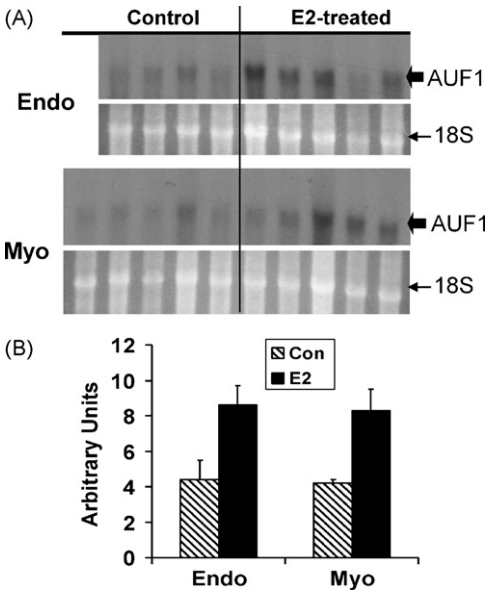

Fig. 1. E2 treatment doubled the concentration of AUF1 mRNAs in the uterus. (A) Northern blot analyses of AUF1 mRNA in the superficial endometrial and myometrial tissues from control and E2-treated ewes are shown in the upper "Endo" and "Myo" panels, respectively. Radioactive antisense cRNA probe bound to the diffuse AUF1 mRNA band (thick arrows). The lower "Endo" and "Myo" panels show the ethidium-bromide stained 18S rRNA bands (thin arrows) on the Northern gels. (B) Hybridization signals for AUF1 mRNAs were quantitated along with the ethidium bromide staining of the 18S RNA bands for normalization. Means for control and E2-treated ewes are shown graphed with standard errors.

2.6. Data analysis

Intensities of radioactive hybridization signals for AUF1 mRNA on Northern blots were quantitated along with ethidium bromide staining of the 18S rRNA band in each lane. The latter was used as a covariate to account for RNA loading differences in least squares analysis of variance using the General Linear Models procedures of the Statistical Analysis System [38]. Radioactivity of the intact RNA remaining after the *in vitro* stability assays was similarly analyzed except there was no covariate. The level of statistical significance was taken to be $P < 0.05$.

3. Results

3.1. E2 treatment increased concentrations of AUF1 mRNAs in endometrium and myometrium

The effects of E2 treatment on the concentration of total AUF1 mRNAs were examined by Northern blot analyses. Endometrial and myometrial tissues of the uteri were examined individually because of their functional and cellular composition differences. The antisense cRNA probe bound mRNAs encoding all four isoforms of AUF1. In both endometrium and myometrium, a relatively diffuse mRNA band of approximately 1700 ± 80 bases was detected (Fig. 1A). The diffuse nature was expected due to the varied sizes of the AUF1 mRNA splice variants, described above, and Northern blot results from rat uteri and a human erythroleukemia cell line [27,37].

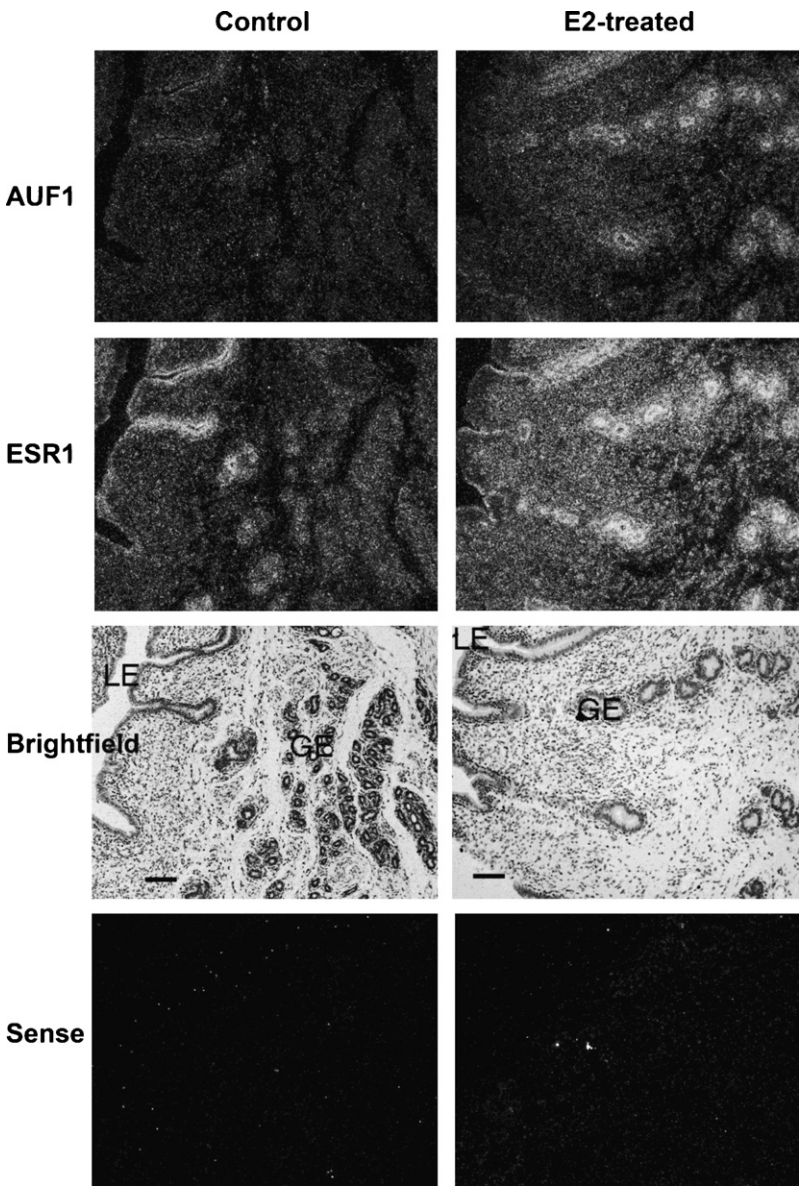

Fig. 2. E2 treatment coordinately up-regulated concentrations of AUF1 and ER ("ESR1") mRNAs in uterine cells. Representative in situ hybridization results on adjacent uterine cross-sections from a control ewe (left panels) and a ewe treated with E2 (right panels) are shown. In each panel, the uterine lumen is at the upper left and the deep endometrium is at lower right. Hybridization signals in the top four panels are white silver grains in the darkfield views. The "Brightfield" views demonstrate the histoarchitecture of the endometrium stained with hematoxylin. Luminal ("LE") and glandular epithelia ("GE") are labeled and the bar indicates 100 μm. The lowest panels are darkfield views of the negative controls ("Sense"), which were hybridized with sense cRNA and demonstrate very little non-specific hybridization.

AUF1 mRNA band intensities appeared greater in E2-treated ewes. Note that the order of samples is matched between top and bottom panels for control ewes but not E2-treated ewes. Quantitation of the AUF1 mRNA hybridization signals and normalization to ethidium bromide stained 18S rRNA (to account for loading differences between samples) made the up-regulation clear (Fig. 1B). Concentrations of AUF1 mRNAs were similar in endometrium and myometrium. E2 treatment increased the concentrations of AUF1 mRNAs twofold in each tissue ($P < 0.05$).

3.2. *E2 treatment up-regulated the concentrations of AUF1 and ER mRNAs, especially in glandular epithelium of the endometrium*

To determine which cell types in the uterus participate in up-regulating AUF1 mRNA in response to E2 treatment, in situ hybridization was performed. Adjacent uterine cross-sections were hybridized with antisense AUF1 and ER cRNAs. Fig. 2 shows representative results, with white silver grains indicating AUF1 and ER mRNAs on the darkfield images in the upper two rows. The bottom darkfield images demonstrate the very low level of non-specific hybridization of a negative control cRNA on uterine cross-sections. For orientation, the brightfield image shows the hematoxylin-stained tissue. The luminal epithelium ("LE") is in the left upper corner and the tubular glands lined with glandular epithelium ("GE") wind through the endometrium toward the myometrium (out of view to the right). In uteri from control ewes, hybridization signals for AUF1 mRNA were low to moderate in intensity over most endometrial cells (Fig. 2, top left panel). This was also true for the caruncles (endometrial evaginations that are unique to ruminant animals and function as sites of vascular attachment in the placenta) and myometrial cells. In the panel below, hybridization signals for ER mRNA in uteri from control ewes also appeared in most uterine cells. However, the signals were strong in the GE of superficial glands and moderately strong in the deeper glandular

epithelia (Fig. 2). E2 treatment increased AUF1 mRNA hybridization signals to very high levels in the GE of the middle and deep endometrial glands (upper right panel in Fig. 2). AUF1 mRNA also increased in the stroma surrounding the GE, as well as the caruncular stroma and myometrium (not shown). The panel below demonstrates the E2 induced increase in ER mRNA signals to extremely high levels in the GE and stroma. These data demonstrate that E2 treatment up-regulated the expression of AUF1 and ER genes concurrently within similar cell compartments of the uterus.

3.3. E2 treatment stabilized AUF1 and ER mRNAs in uterine extracts

The predominant mechanism of E2 up-regulation of ER mRNA in the uterus is by increased stability of the ER mRNA [7,8]. The *in vitro* stability assay used recapitulates the magnitude of ER mRNA stabilization by E2 treatment measured by *in vivo* labeling and in explants cultured with transcription inhibitor [7]. It is based on assays that are widely used for studying regulated mRNA stability [39,40]. The assay demonstrates appropriate specificity because globin mRNA and 18S rRNA are not stabilized *in vitro*, as *in vivo* [8]. To determine whether E2 treatment up-regulates AUF1 mRNA by stabilizing it, we assessed the stability of radiolabeled sense AUF1 cRNA *in vitro* with uterine cytosolic extracts from the control and E2-treated ewes. Because 3′ UTR sequences generally regulate mRNA stabilities, we tested the *in vitro* stability of a sense cRNA containing exon 10 of AUF1 mRNA ("AUF1ex10") which composes the majority of the 3′ UTR of all AUF1 mRNAs. Control reactions were performed with sense cRNAs for ER 3′UTR and 18S rRNA, which are and are not stabilized by E2 treatment, respectively [8]. Representative results are shown in Fig. 3A. Lanes indicated by "-" show the intact cRNAs, that were not incubated with uterine extracts. In the initial stability assays (15 min incubation), the AUF1ex10 and ER cRNAs were degraded into smaller fragments by uterine extract proteins from a control ewe. However, AUF1ex10 and ER cRNAs survived incubation with the extract proteins from the E2 treated ewe with very little degradation. The 18S rRNA was stable in extracts from both control and E2 treated ewes. This indicates that the 3′UTR of AUF1 mRNA is stabilized by E2 treatment, similar to that of ER mRNA.

To measure half-lives of the AUF1 and ER cRNAs, time courses were performed with extracts from three control and three E2-treated ewes. Results are graphed in Fig. 3B. Both AUF1 and ER cRNAs were equally unstable in control ewe extracts, with half-lives around 4 min. They were also similarly stabilized by E2 treatment, with half-lives of approximately 56 min. Thus, it appears that E2-treatment up-regulates expression of AUF1 and ER genes post-transcriptionally, by stabilizing their mRNAs.

3.4. AUF1 mRNA splice variants produced by the sheep uterus and the effect of E2-treatment

Several splice variants of AUF1 mRNA have been reported. The cartoon in Fig. 4A shows the positions of the alternatively spliced exons. Within the coding sequence, exons 2 and 7 are alternatively spliced to yield mRNAs encoding four AUF1 protein isoforms. To determine which AUF1 protein isoforms were produced in the sheep uterus, we performed qualitative RT-PCR with primers flanking exons 2 and 7. Controls for the expected sizes of the AUF1 cDNA were performed with plasmids containing the appropriate human cDNAs (Fig. 4B, left lanes). Myometrial RNA samples from all control ("Con") and E2-treated ("E2") ewes generated cDNAs from all four AUF1 mRNA splice variants that encode AUF1p37, -p40, -p47 and -p45 (representative results in Fig. 4B, right lanes), as did endometrial samples (not shown). In all samples, the cDNAs

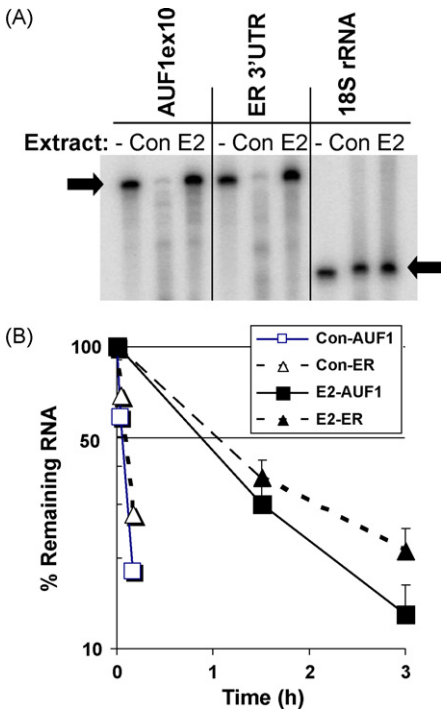

Fig. 3. The 3′UTR of AUF1 mRNA was more stable in extracts from E2-treated ewes. (A) Representative results of *in vitro* RNA stability assays performed with uterine cytosolic extracts from control and E2-treated ewes are shown. Radiolabeled sense AUF1 3′UTR cRNA (from exon 10) was incubated in the absence ("-") or presence of extracts from control ("Con") or E2 treated ("E2") ewes. Concurrent reactions were performed with sense ER 3′UTR and 18S cRNAs. On the phosphorimage of the gel after RNA electrophoresis, positions of the intact cRNAs are indicated by the arrows. (B) Intact RNA from replicate assays with extracts from three control and three E2-treated ewes was quantitated over a time course. The graph shows the percent of input RNA remaining on the *y* axis (logarithmic scale) over time on the *x* axis for AUF1 (squares and solid line) and ER (triangles and dashed line) 3′UTR cRNAs.

from the mRNAs encoding AUF1p40 and -p45 isoforms appeared to be more predominant than the others. Amplification of ribosomal protein S2 (RPS2) cDNA was similar from the Con and E2 reverse transcription reactions, indicating that similar levels of cDNA were synthesized and used for PCR (Fig. 4B, lower panel). Over all ewe samples, myometrial and endometrial, E2-treatment did not affect levels of splice variants for AUF1 mRNA exons 2 and 7.

AUF1 mRNAs are unusual in that they have either two or three exons contributing to the 3′UTR while most mRNAs have only one [12]. Intriguingly, the exon 9 sequence of AUF1 mRNA is alternatively spliced in human K562 cells [12]. The exon 9 sequence is also unique in that it is remarkably conserved across species: the sheep AUF1 exon 9 sequence is 99% identical to human, bovine, canine and rat sequence. The rest of the AUF1 3′UTR is also highly conserved, with the sheep sequence being 97% identical to that of the human. For these reasons, we used RT-PCR to determine if E2 treatment affected the levels of AUF1 mRNAs with alternately spliced exon 9 sequences. PCR products from AUF1 mRNA lacking exon 9-derived sequence were predominant in uterine samples from control and E2-treated ewes (Fig. 4C), similar to results with K562 cells [12]. However, the proportion of the AUF1 mRNAs bearing exon 9 derived sequence was decreased by E2 treatment, from 10% in control ewes to 1% of the total AUF1 mRNA products in E2 treated ewes. Results with myometrial and endometrial RNA samples from control and E2-treated ewes were similar. This E2-induced decrease in the percentage of AUF1 mRNAs bearing exon 9-derived sequence was the only effect on AUF1 mRNA splicing detected.

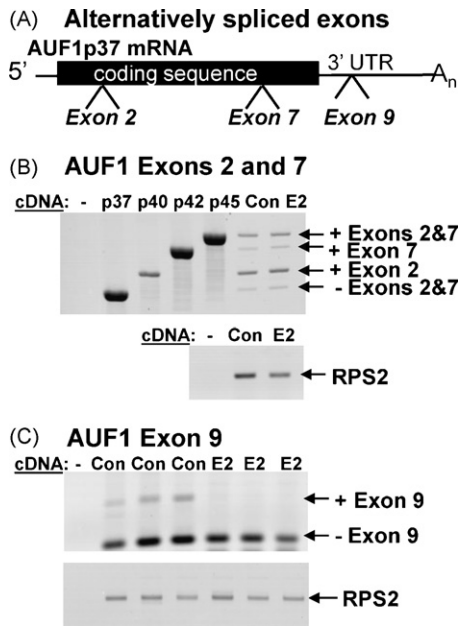

Fig. 4. E2 treatment decreased the prevalence of AUF1 mRNAs bearing exon 9 derived sequences. (A) The cartoon of AUF1p37 mRNA shows positions of the missing exons 2, 7 and 9. (B) Representative PCR results using primers that flank the alternatively spliced exons 2 and 7 are shown for myometrial RNA from one control ("Con") and one E2-treated ("E2") ewe (right lanes) demonstrate no E2 effects on exons 2 and 7 splice variants. The left lane shows results from the negative PCR control ("-"), which received no cDNA. Positive controls were plasmids containing the individual AUF1 isoform coding sequences ("p37","p40", "p42", and "p45"). The gel migration positions of cDNAs with or without exon 2 and/or 7-derived sequences are indicated at right. Ribosomal protein S2 ("RPS2," lower panel) cDNA was amplified by PCR to demonstrate that similar amounts of reverse transcribed cDNA were used for the two ewe samples. (C) PCR primers flanking the exon 9 sequence amplified cDNAs from the 3′UTR of AUF1 mRNAs in myometrial RNA samples from three control and three E2-treated ewes. RPS2 cDNA was amplified as in panel B (above).

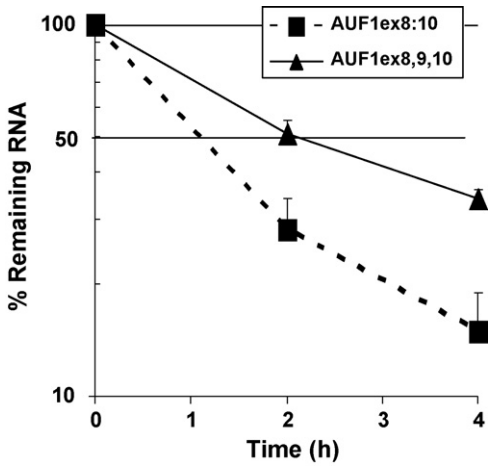

Fig. 5. Exon 9-derived sequences stabilized the 3′UTR of AUF1 mRNA in extracts from E2-treated ewes. Radiolabeled AUF1 3′UTR cRNAs with ("AUF1ex8,9,10") and without ("AUF1ex8:10") exon 9 sequence were analyzed for stability *in vitro* as in Fig. 3. Replicate assays with uterine extracts from three E2-treated ewes were performed over a time course. Quantitative data are presented graphically as described in Fig. 3B.

3.5. *AUF1 exon 9 sequences stabilized the 3′UTR in uterine extracts from E2-treated ewes*

Since exon 9 is alternatively spliced within the 3′UTR, we next assessed the effects of exon 9 derived sequences on AUF1 mRNA stability *in vitro*. When structures of 272 base long sequences were modeled [41], exon 9 derived sequences altered the secondary structure of the AUF1 mRNA 3′UTR (predicted free energy decreased from −57.1 to −81.4 kcal/mole). It was expected that this structure destabilized AUF1 mRNAs bearing exon 9 derived sequence in E2 treated ewes to explain why they were less abundant. Therefore, the stabilities of two sense cRNAs, bearing the entire AUF1 3′UTR with or without exon 9, were compared *in vitro*. Both were equivalently unstable in extracts from three control ewes, with half-lives of about 7 min. Unexpectedly, in extracts from three E2 treated ewes examined over a time course, the half-life of the AUF1 RNA bearing exon 9 derived sequence was twice that of the cognate lacking exon 9 sequence (2.2 h vs. 1.1 h; Fig. 5). The latter was comparable in stability to the AUF1ex10 and ER 3′UTR cRNAs (Fig. 3 and Ref. [9]). Because AUF1 RNAs bearing exon 9 derived sequence were not less stable *in vitro*, two alternate explanations of how E2 treatment decreased them *in vivo* are discussed below.

4. Discussion

This report expands our knowledge of the regulation of AUF1 gene expression by estrogens. The data demonstrate that E2 treatment up-regulates the concentrations of total AUF1 mRNAs in the two major tissue compartments of the uterus: the endometrium and myometrium. The data are consistent with those from a study of ovariectomized rats treated with the same dose of estradiol which induced a 2–3-fold increase in AUF1 mRNA concentrations within 6 h of treatment [27]. ICI 182,780 blocked the effect in the rats, indicating that ER protein was required for the response. In the sheep uteri, the up-regulation of AUF1 mRNA was concurrent with that of ER mRNA (this report and Ref. [36]). In situ hybridization results illustrated that the greatest up-regulation of AUF1 and ER mRNA concentrations occurred in similar uterine compartments, especially in the glandular epithelium of the endometrium.

This is the first report revealing that the mammalian endometrium and myometrium produce AUF1 mRNAs encoding all four AUF1 protein isoforms. Among them, the mRNAs encoding AUF1p40 and -p45 appeared predominant in uteri from all ewes. During the E2 induced up-regulation of AUF1 mRNA, treatment did not affect the proportions of splice variants with exons 2 or 7. This is in contrast to data from nearly identical PCR assays of rat uteri [27]. In that study, AUF1 mRNAs encoding all four variants were expressed at equivalent levels in the uteri of control ovariectomized rats and E2 treatment specifically increased the percentages of the mRNAs encoding AUF1p40 and p45. These isoforms contain the domain encoded by exon 2 which is known to stabilize mRNAs [9,15–18]. In both rat and sheep uteri, E2 induced increased concentrations of AUF1p45 protein [9,15].

To determine how E2 treatment up-regulated AUF1 mRNA, the stability of AUF1 mRNA 3′UTR sequences was assessed using a validated *in vitro* assay [8]. This assay, employing uterine extracts from control and E2-treated ewes, demonstrated that AUF1 mRNAs were greatly stabilized by E2 treatment, as was ER mRNA. These data are consistent with data from rats pretreated with the transcription inhibitor actinomycin D, which indicated that E2 treatment stabilized AUF1 mRNA in uterine tissues [27]. A possible mechanism for E2 up-regulation of AUF1 mRNA in the rat and sheep uterus is by E2 enhancing AUF1 gene expression so that stabilizing AUF1 proteins act on AUF1 mRNAs (this work and [27]).

This is also the first report of E2 effects on alternative splicing of exon 9 derived sequences of AUF1 mRNAs. E2 treatment decreased the percentage of AUF1 transcripts bearing exon 9 sequences. Since exon 9 is within the 3′UTR, we tested effects on mRNA stability with the *in vitro* assay. Surprisingly, the highly conserved exon 9 derived sequence stabilized AUF1 mRNA in extracts from E2 treated ewes. There are two possible explanations for how E2 decreased

levels of exon 9-bearing AUF1 mRNAs in the sheep uterus. One is that E2 treatment altered splicing to increase "skipping" of exon 9 in AUF1 mRNAs. Others have reported similar estrogen effects on several mRNAs in various mammalian tissues including the uterus [42–44]. A second viable explanation is that E2 treatment increased nonsense mediated RNA decay (NMD). NMD is studied in living cells because of its dependence upon nuclear and cytoplasmic proteins and cell functions that include translation [45]. One trigger of NMD is splicing within ultraconserved 3′UTR sequences to generate an exon:exon junction that is more than 55 bases downstream from a translation stop site [46–48]. This description fits the exon 9:10 junction in the 3′UTR of exon 9 bearing AUF1 mRNAs [12]. In HeLa cells, exon 9 derived sequence destabilized AUF1 mRNA from a 3.1 h half-life to one of 1.5 h via NMD [49]. How E2 treatment affects NMD is unknown and no other reports exist. Together, the data indicate that specific 3′UTR sequences of AUF1 mRNAs are important to the regulation of AUF1 gene expression.

In conclusion, this report describes the coordinate up-regulation of AUF1 and ER gene products in the uterus. Since both AUF1 and ER proteins individually regulate the expression of hundreds of genes [10,11,35,50], their up-regulation by estrogens is likely to directly and indirectly impact the expression levels of a large population of genes.

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