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Estrogen and phytoestrogen regulate the mRNA expression of adrenomedullin and adrenomedullin receptor components in the rat uterus

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Abstract

The serum estrogen surge in the uterus triggers precisely-timed physiological and biochemical responses required establishing and maintaining pregnancy. Previous reports have shown that consumption of phytoestrogen-containing plants may disrupt the precise control of pregnancy. To evaluate the effects of phytoestrogens in the uterus, we screened for estradiol (E2)-inducible genes in immature rat uteri. We identified the gene for receptor-activity-modifying protein 2 (Ramp2), known to be a component of the adrenomedullin (ADM) receptor, as responsive to both E2 and the phytoestrogen coumestrol (Cou). We further examined the expression of ADM and ADM signaling components Ramp2, Ramp3, and CRLR in the immature rat uterus and found that both E2 and Cou regulated these genes expression. In addition, treatment with ADM increased uterine weight and edema similar to that observed after Cou treatment. Our findings indicated that the phytoestrogen caused the abnormal induction of vasoactive factors in the uterus.

Keywords: Coumestrol; Estradiol; Adrenomedullin; Uterus; Gene expression

1. Introduction

Estrogen is produced mainly by the ovaries and has important roles in the growth, differentiation and maintenance of function of female reproductive organs. Various reproductive events, including the ovarian cycle, decidualization of the endometrium, maintenance of pregnancy, and growth of mammary glands, are under the strict control of estrogen (Korach, 1981). The uterus is considered to be the representative organ exhibiting estrogen control. During the menstrual cycle, the uterus undergoes drastic structural and functional changes in response to estrogen, with thickening of the endometrium and proliferation of uterine glands in preparation of embryo implantation (Rigsby, 2002). Exogenously administered estrogen rapidly increases the microvascular permeability, edema, and weight of the rodent uterus (Cullinan-Bove and Koos, 1993). As this uterotrophic response is very sensitive and rapid, it has been used as a bioassay for a variety of estrogenic compounds (Owens and Ashby, 2002), including plant-derived phytoestrogens (Yamasaki et al., 2003; Jefferson et al., 2002; Singh et al., 1988).

The activities of estrogen and phytoestrogens are mediated through the estrogen receptors ERα and ERβ, which act as ligand-dependent transcription factors (Glass and Rosenfeld, 2000). While it is known that both estrogen and phytoestrogens can bind to and activate ERs, and that ligand-bound ERs then stimulate the transcription of estrogen responsive genes (Barkhem et al., 1998; Kuiper et al., 1998), the full range of target genes has not yet been fully elucidated. Some estrogen actions are mediated by growth factors, such as insulin-like growth factor 1 (IGF1) (Klotz et al., 2002), epidermal growth factor (EGF) (Zhang et al., 1998), and vascular endothelial growth factor (VEGF)

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(Hyder et al., 2000). Although these factors can mediate estrogen-regulated endometrial cell growth and maintain uterine function, they cannot explain all estrogenic effects. To clarify the downstream genes that mediate estrogen effects on uterine growth and to evaluate the differences between the activity of endogenous estrogen and exogenous phytoestrogen, we screened for estradiol (E2)-inducible genes in the immature rat uterus.

Using the differential display method, we identified receptor-activity-modifying protein 2 (Ramp2) as an E2-responsive gene. Ramp2 is a member of a gene family (Ramp1, Ramp2, and Ramp3), with Ramp2 or Ramp3 forming a complex with calcitonin receptor-like receptor (CRLR) to function as an adrenomedullin (ADM) receptor. Ramp1 also forms a complex with CRLR, which then acts as a calcitonin gene-related peptide (CGRP) receptor (McLatchie et al., 1998; Kamitani et al., 1999; Nagae et al., 2000). It is well known that ADM has vasodilating and angiogenic functions (Iwasaki et al., 1998; Zhao et al., 1998; Nishimatsu et al., 2001). Also, ADM knockout mouse experiments have shown that ADM protects tissues from damage due to oxidative stress (Shimosawa et al., 2002). Recent global genomic expression studies revealed that Ramp3 gene expression is regulated by E2 in the rodent uterus (Watanabe et al., 2002; Hewitt et al., 2003). Furthermore, Thota et al. (2003) suggested that the expression of uterine ADM signaling-related genes was altered during pregnancy and labor. Based on these findings, it is reasonable to suppose that ADM signaling in the rat uterus may play an important role during pregnancy.

We observed that similar to E2 treatment, a phytoestrogen (coumestrol; Cou) treatment up-regulated Ramp2, Ramp3, and ADM mRNA expression in the immature rat uterus, while CRLR mRNA expression was down-regulated. However, compared with E2, Cou-mediated gene expression was transient. We also showed that 60 μg/kg ADM or 1 mg/kg Cou increased uterine weight and induced edema. We also found that in the rat uterus, expression of ADM-related genes was synchronized with the estrus cycle. Thus, our findings implied that phytoestrogens can affect the expression of ADM signaling components.

2. Materials and methods

2.1. Animals and treatments

All animals used in our experiments were female Wistar rats obtained from the SRC, Japan. To evaluate regulation of estrogen- and Cou-responsive genes, 3-week-old immature female rats were given single subcutaneous (s.c.) injections of 17β-estradiol (E2) (Sigma Chemical, USA) or Cou (Fluka Chemie AG) at 1.0 μg/kg or 1.0 mg/kg body weight (BW), respectively, in 50 μl propylene glycol (Wako Chemical, Japan) as a vehicle. Some animals were pre-treated with the synthetic estrogen receptor antagonist ICI 182,780 (Nakarai Tesque, Japan) at 1.0 mg/kg BW in propylene glycol 1 h prior to E2 or Cou treatment. Immature rats were also administered intravenously (i.v.) 60 μg/kg adrenomedullin (Sigma Chemical) dissolved in PBS. The animals were sacrificed at 6 h after single injection. Gene expression during the estrus cycle was analyzed in 8-week-old mature female rats. Proestrus, estrus, metestrus, and diestrus were confirmed by vaginal smears for 1 week and then uteri from normal estrus cycling animals used for RNA extraction. Three animals were used in each experimental group (Figs. 2–4). Single animal was used in each experimental group and experiments were repeated three times (Figs. 1 and 5). All animal experiments were approved by the Laboratory of Experimental Medicine JMS Animal Use and Care Committee.

2.2. RNA extraction and differential display

The animals were killed by decapitation. Excised tissues were frozen by liquid N2 immediately and stored at −80 °C. Total RNA was extracted from tissues using ISOGEN (Nippon Gene, Japan) according to the manufacturer's instructions. To identify E2-regulated genes in the rat uterus, differential display using a fluorescence differential display Kit (Rhodamine version; Takara, Japan) was employed according to the manufacturer's instructions with some modifications. Briefly, 1 μg total RNA from rat uteri treated with E2 or vehicle only for 6 h were reverse transcribed to cDNA with downstream primers that were a mixture of rhodamine labeled dT11VV (where V denotes dG, dC, or dA) primers supplied in the kit. One fiftieth of the cDNA was subsequently amplified by PCR with the downstream primer and each upstream primer supplied in the kit. PCR conditions were one cycle of 2 min at 94 °C, 5 min at 40 °C, and 5 min at 72 °C, then 40 cycles of 30 s at 94 °C, 5 min at 40 °C, 2 min at 72 °C, followed 5 min for 72 °C in a GeneAmp PCR system 9700 (Applied Biosystems, USA). Amplified DNA fragments were separated by denaturing polyacrylamide gel electrophoresis containing 8 M urea and visualized by scanning with FMBIO II multi-view fluoroimager (Takara). Differentially expressed bands were cut from the gel and the DNA eluted by boiling in 50 μl water. Isolated DNA fragments were then reamplified by PCR under the same conditions as described above and cloned into the pCRII-TOPO vector using the TA cloning system (Invitrogen, USA). DNA sequencing was performed on an ABI PRISM 310 (Applied Biosystems) using a BigDye terminator cycle sequencing reaction kit (Applied Biosystems).

2.3. Probes and Northern blot analysis

Differential display assay isolated the 3′ half of Ramp2 cDNA. Rat Ramp1 cDNA and Ramp3 cDNA were obtained from EST IMAGE clones UI-R-BJ1-ASZ-06-0-UI and UI-R-E0-D6-B-06-0-UI, respectively (Research Genetics, USA). cDNA for full-length rat ADM and CRLR were

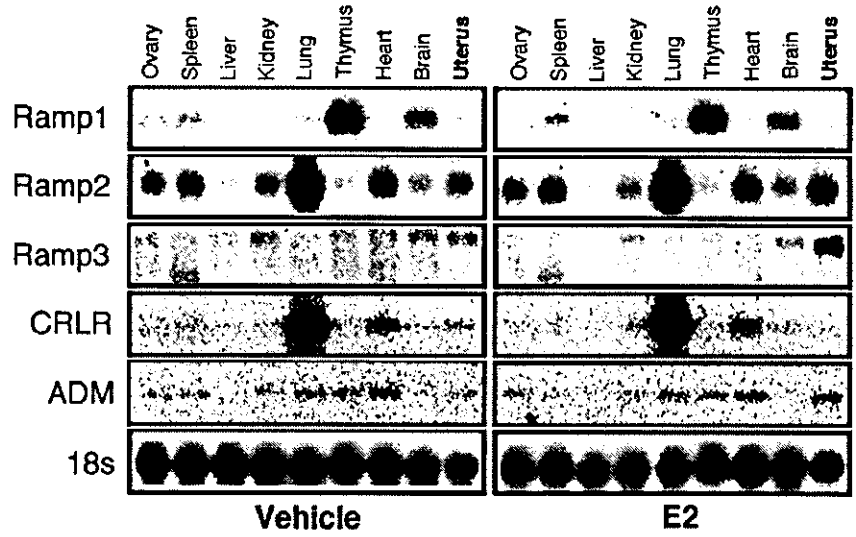

Fig. 1. Expression and estrogen regulation of Ramp1, Ramp2, Ramp3, CRLR, and ADM mRNA in immature rat tissues. Three-week-old female rats were administered vehicle or 1 μg/kg 17β-estradiol (E2) subcutaneously. After 6 h, total RNA was extracted from the indicated tissues. Northern blots were performed using 20 μg total RNA extracted from each tissue. Probes used for hybridization were rat Ramp1 and Ramp3 cDNA obtained from EST IMAGE clones, rat CRLR and ADM cDNA amplified by RT-PCR, and rat Ramp2 cDNA obtained by differential display screening. An antisense oligonucleotide for rat 18S ribosomal RNA was used as an internal control. Experiments (single rat in each group) were repeated three times and representative results are shown.

obtained by conventional RT-PCR using specific primers. cDNA clones were verified by sequencing, and then labeled with [α-^{32}P] dCTP using a random primer labeling kit (Takara) and used as probes for Northern blot analysis. An antisense oligonucleotide (5′-ACG GTA TCT GAT CGT CTT CGA ACC-3′) for rat 18S ribosomal RNA (Kim et al., 1995) was labeled with [γ-^{32}P] ATP using the T4 polynucleotide kinase (Takara) and hybridized as an internal control for the Northern blot analysis. Aliquots of 20 μg total RNA were separated in 1% formaldehyde denaturing agarose gels and RNA transferred onto Gene Screen nylon membranes (NEN, USA). Blots were hybridized with the ^{32}P-labeled probes in 0.1% SDS, 50% formamide, 5× SSC, 50 mM $NaPO_4$ (pH 6.8), 0.1% Sodium pyrophosphate, 5× Denhardt's solution and 50 μg/ml salmon sperm DNA at 42 °C overnight. After hybridization, blots were washed with 2× SSC and 0.1% SDS at 42 °C for 30 min and then 0.2× SSC and 0.1% SDS at 42 °C for 30 min. Hybridized signals were quantified using a Fuji BAS1000 phosphoimaging analyzer (Fuji film, Japan). The signal intensities of Ramp2, Ramp3, ADM, and CRLR mRNA were normalized with respect to 18S ribosomal RNA.

2.4. Histology

Uteri were removed from immature female rats at 6 h after single injection with ADM (60 μg/kg), E2 (1 μg/kg), Cou (1 mg/kg) or vehicle (50 μl). Rats were also treated with E2, Cou, or vehicle for up to 24 h. Removed uteri were immediately fixed in cold 10% buffered formalin (Wako Chemical), embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin according to standard protocols.

2.5. Statistics

Data are expressed as the mean ± S.E.M. Statistical significance of differences was evaluated by one-way ANOVA followed by Bonferroni's multiple range test. All data were initially analyzed to determine whether the assumptions for parametric testing (normal distributions and equal variances) had been met. In all instances, differences were considered significant at $P < 0.05$.

3. Results

3.1. Screening for estrogen-inducible genes in the rat uterus

To isolate estrogen-responsive genes in the rat uterus, immature female rats were administered 1 μg/kg 17β-estradiol (E2) or vehicle by subcutaneous injection, and after 6 h treatment total RNA isolated from uteri. The differential display method was then used to identify differentially-expressed transcripts between E2 and vehicle treated uteri. We obtained one clone that showed enhanced expression in E2 treated uterus, and it was found to correspond to the 3′ half of receptor-activity-modifying protein 2 (Ramp2) cDNA. Ramp2, along with Ramp1 and Ramp3, form a gene family. Both Ramp2 and Ramp3 can form a complex with calcitonin

receptor-like receptor (CRLR) to make the adrenomedullin (ADM) receptor.

3.2. Expression and estrogen responsiveness of ADM and its receptor components

To confirm the effects of estrogen on Ramp1, Ramp2, Ramp3, CRLR, and ADM mRNA expression, immature rats were treated with E2 for 6 h and total RNA from various tissues isolated for Northern blot analysis (Fig. 1). A high level of Ramp1 mRNA was expressed in the thymus, moderate levels in the brain, spleen, ovary, and lung, and low levels in the liver, kidney, heart, and uterus. E2-mediated up- or down-regulation of Ramp1 mRNA was not observed in these tissues. Ramp2 mRNA was expressed in various tissues, with high expression in the lung. In the uterus, Ramp2 mRNA level was slightly induced by E2 treatment. While expression levels of Ramp3 mRNA were quite low in all tissues examined, robust Ramp3 mRNA expression was observed in the uterus after E2 treatment. CRLR mRNA was expressed predominantly in the lung and only slightly in the heart, uterus, spleen and ovary. Uterine expression of CRLR mRNA was down-regulated by E2. While ADM mRNA was widely expressed, expression levels were lower than for Ramp mRNAs. The expression of uterine ADM mRNA was induced by E2 treatment. These results indicated that estrogen regulated the expression of ADM and its receptor components in the immature rat uterus.

3.3. The effects of phytoestrogen on the expression of ADM signaling components

We next examined the effect of the phytoestrogen coumestrol (Cou) on the gene expression of ADM signaling components. We performed a time course experiment on the E2- and Cou-responsiveness of Ramp2, Ramp3, CRLR, and ADM mRNA expression in the immature rat uterus (Fig. 2). Ramp2, Ramp3, and ADM mRNA levels were up-regulated by both E2 and Cou within 1 h, although peak and recovery times varied. In response to E2 treatment, Ramp2 mRNA levels peaked (2.0-fold) at 6 h after E2 administration and remained at high levels for up to 24 h. In contrast, Ramp2 mRNA levels reached a plateau level (1.7-fold) at 1 h after Cou treatment. Ramp3 mRNA reached peak levels (4.0-fold) after 3 h after both E2 and Cou treatments, but the recovery rate was faster after Cou treatment than after E2 treatment. ADM mRNA levels were transiently up-regulated by E2 and Cou treatments, but Cou-mediated expression was higher than E2-mediated expression (2.4-fold vs. 2.0-fold, respectively). In contrast, CRLR mRNA was decreased by both compounds, reaching a nadir at 3 h after treatment. Recovery of CRLR mRNA levels was faster after Cou treatment than after E2 treatment.

We also analyzed the effects of the pure anti-estrogen ICI 182,780 (ICI) on E2- and Cou-mediated regulation of Ramp2, Ramp3, ADM, and CRLR mRNA (Fig. 3). E2- and

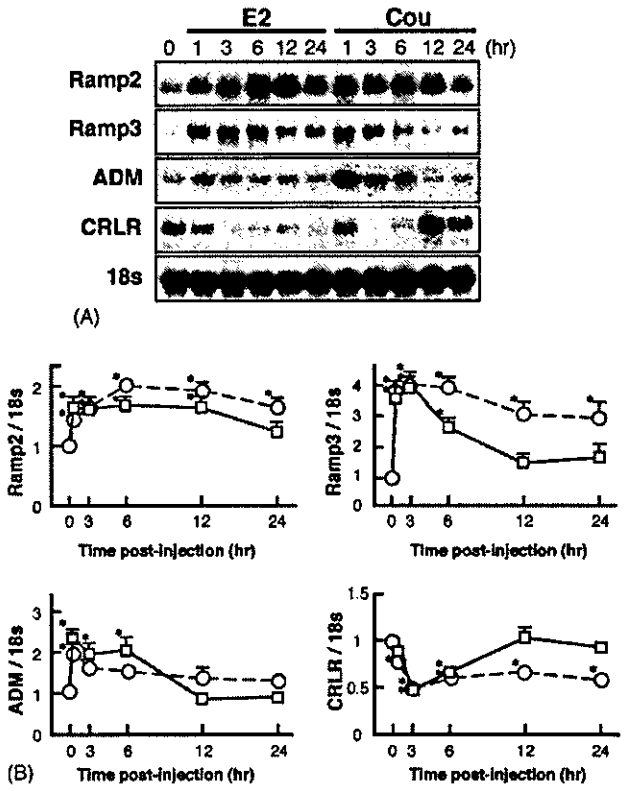

Fig. 2. Time course of Ramp2, Ramp3, CRLR, and ADM mRNA expression in immature rat uteri after treatment with E2 or Cou. (A) Total RNA was extracted from uteri 1, 3, 6, 12, or 24 h after treatment with 1 μg/kg 17β-estradiol (E2) or 1 mg/kg coumestrol (Cou). Northern blotting was performed as described in Fig. 1 and representative results are shown. (B) Experiments were repeated three times and signals normalized with the corresponding 18S ribosomal RNA. Results were shown as mean ± S.E.M. (three rats per point) and expressed as relative levels compared with time zero. $^*P < 0.05$ compared to the respective time zero. Rats were administered E2 (open circles, dotted lines) or Cou (open squares, solid lines).

Cou-mediated up-regulation of Ramp2, Ramp3, and ADM mRNA levels and down-regulation of CRLR mRNA were entirely blocked by ICI treatment. These results suggested that Cou-mediated Ramp2, Ramp3, ADM, and CRLR gene expression was regulated via the estrogen receptor.

3.4. ADM induces increases in uterus weight

We next examined whether ADM gene expression was important in the E2-induced early-response of the uterus. Immature female rats were given 60 μg/kg ADM i.v. for 6 h and then uteri excised and weighed. Uterine weights were assessed as g/kg body weight (Fig. 4A). The mean weight of the ADM-treated uteri was 0.7 g/kg, which was 1.4 times greater than the mean weight of vehicle treated uteri (0.5 g/kg), but only 75% that of E2-treated uteri (0.9 g/kg). Histological sections of representative vehicle-, ADM-, or E2-treated uteri are shown in Fig. 4B. Endometrial cells treated with ADM showed swelling at 6 h, but had returned

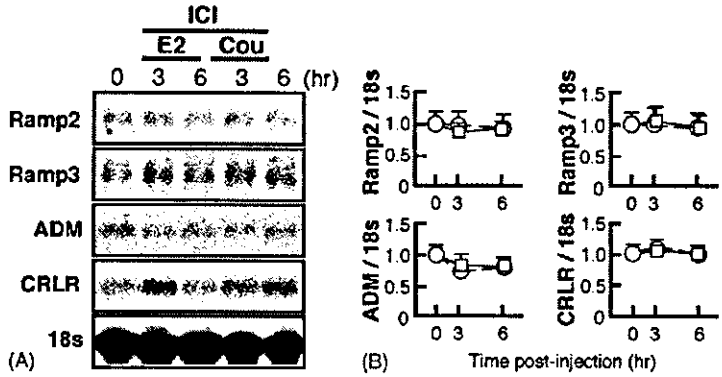

Fig. 3. Effects of ICI 182,780 (ICI) on E2- and Cou-regulated Ramp2, Ramp3, CRLR, and ADM mRNA expression levels. (A) Total RNA was extracted from uteri pretreated with 1 mg/kg ICI for 1 h followed by 17β-estradiol (E2) or coumestrol (Cou) treatment given for 3 or 6 h. Northern blotting was performed as described in Fig. 1 and representative results are shown. (B) Experiments were repeated three times and signals normalized to corresponding 18S ribosomal RNA. Results are shown as mean ± S.E.M. (three rats per point) and expressed as relative levels compared with time zero. Rats were administered E2 (open circles, dotted lines) or Cou (open squares, solid lines).

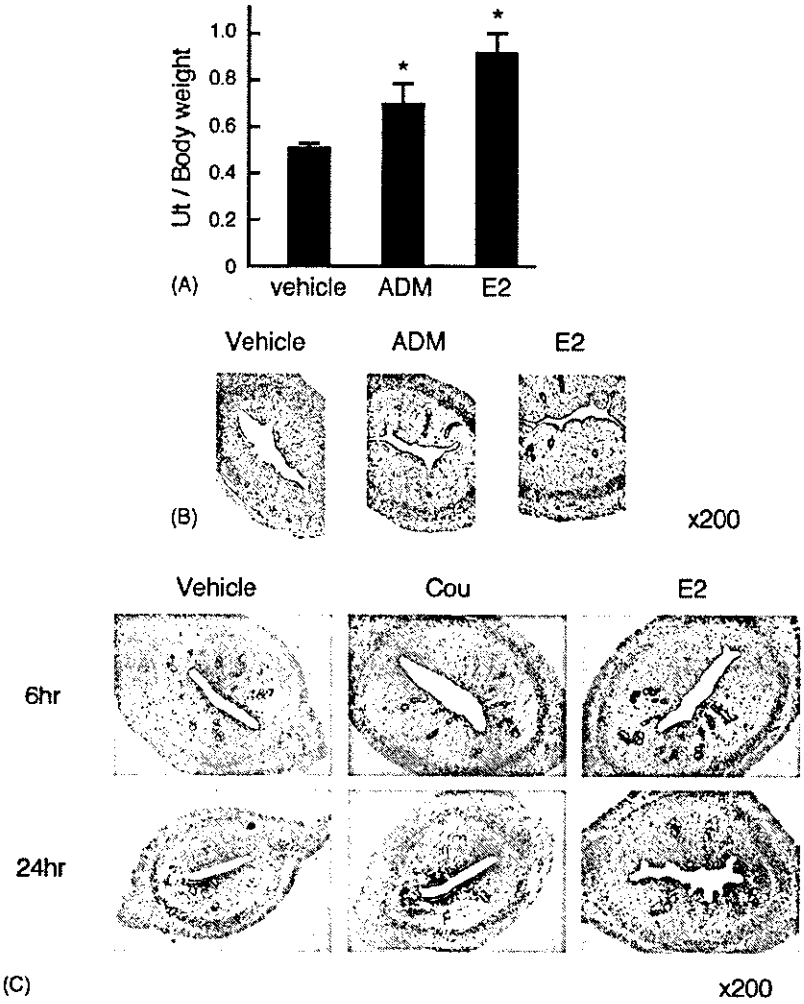

Fig. 4. ADM increases uterine weight and swelling of the endometrium in immature female rats. (A) Immature female rats were administered vehicle, 60 μg/kg ADM (ADM) or 1 μg/kg E2 (E2). After 6 h, uterine wet weights were measured and shown as mean ± S.E.M. (three rats per group). $^{*}P < 0.05$ compared to vehicle. Uterine wet weight is shown as g/kg body weight. (B) Uterine histological sections were examined and representative results are shown. (C) Immature female rats (three rats per group) were administered vehicle, 1 mg/kg Cou (Cou) or 1 μg/kg E2 (E2). After 6 or 24 h, uterine histological sections were prepared and examined. Representative results are shown.

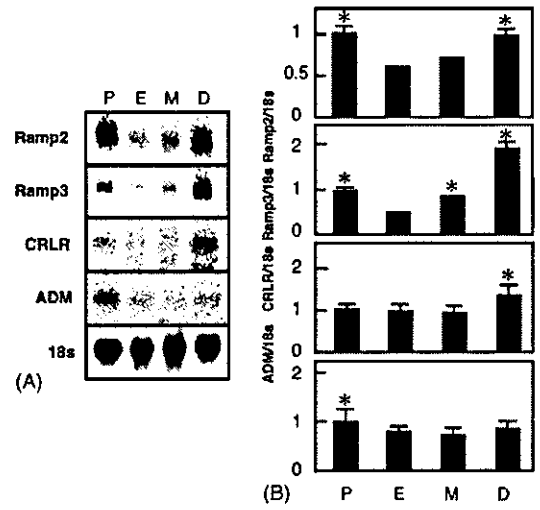

Fig. 5. Expression of Ramp2, Ramp3, CRLR, and ADM mRNA in the adult uterus during the estrus cycle. (A) Total RNA was isolated from uteri of 8-week-old female rats. Proestrus (P), estrus (E), metestrus (M), and diestrus (D) were confirmed by vaginal smear. Northern blotting was performed as described in Fig. 1 and a representative result is shown. (B) Experiments (single rat in each point) were repeated three times and signals normalized to corresponding 18S ribosomal RNA. Results are shown as mean ± S.E.M. and expressed as relative levels compared with the proestrus stage. $^*P < 0.05$ compared to the respective estrus stage.

to normal by 12 h (data not shown). As shown in Fig. 4C, 1 mg/kg Cou treated immature rat uterus also showed endometrial cell swelling at 6 h, with recovery by 24 h. In contrast, 1 μg/kg E2-treated uterus showed increased cell proliferation at 24 h. Thus, the thickening of the endometrium seen after ADM or Cou treatment was due to hyperplasia and edema rather than cell division.

3.5. Expression of ADM and ADM receptor components during the estrus cycle

Our results indicated that the mRNA expression of ADM and its receptor components in the immature rat uterus could be altered by exogenously administered E2 or Cou. Therefore, we hypothesized that estrogen-mediated regulation of Ramp2, Ramp3, CRLR, and ADM mRNA expression occurred during the estrus cycle, as it is well known that during the estrus cycle the uterus is precisely controlled by E2. Gene expression was examined in adult female rats during the four phases of the estrus cycle, proestrus (P), estrus (E), metestrus (M), and diestrus (D) (Fig. 5). Each stage was determined by vaginal smear. The uterus in proestrus was obviously larger than the uteri from other stages. We considered that the diestrus phase of this study corresponded to the early-proestrus stage. Northern blotting results indicated that the expression levels of ADM and ADM receptor component genes were higher during the diestrus and proestrus stages but lower during the estrus and metestrus stages. This result suggested that expression of these genes correlated with E2 level during the estrus cycle.

4. Discussion

Previous reports have suggested that ADM is expressed in the epithelial and stromal cells of the rodent endometrium and that ADM expression level correlates with serum estrogen concentration (Cameron and Fleming, 1998; Cameron et al., 2002). Furthermore, recent global genomic expression studies have shown that expression of the ADM receptor component Ramp3 is regulated by E2 in the rodent uterus (Watanabe et al., 2002; Hewitt et al., 2003). These findings suggested that ADM signaling in the rodent uterus was important to maintain function. In this study, we investigated the effect of the phytoestrogen coumestrol (Cou) on the gene expression of ADM signaling components (Ramp2, Ramp3, CRLR, and ADM) in the rat uterus.

Northern blot analyses demonstrated that mRNA for Ramp2, Ramp3, CRLR, and ADM were expressed in both the immature and adult female rat uterus (Figs. 2 and 5). Ramp2, Ramp3, ADM, and CRLR mRNA expression levels were regulated by both E2 and Cou (Fig. 2), with both E2- and Cou-mediated regulation in the immature rat uterus completely blocked by the addition of the anti-estrogen ICI (Fig. 3). Our results suggested that the expression of ADM and ADM receptor components (Ramp2, Ramp3, and CRLR) could be altered by both endogenous estrogen level and the presence of exogenous phytoestrogen via ER signaling.

In the adult female rat uterus, the highest levels of Ramp2 mRNA were expressed during the diestrus and proestrus stages, while Ramp3 mRNA was expressed prominently during the diestrus stage and declined during the proestrus stage (Fig. 5). This result implied that Ramp2 mRNA was more stable than Ramp3 mRNA. This is supported by the time course of expression in the immature rat uterus, such that the maximum accumulation of Ramp2 mRNA occurred 6–12 h after treatment with E2, whereas Ramp3 mRNA reached maximum levels 1–3 h after treatment and declined between 6 and 12 h (Fig. 2). These results suggested that Ramp2 and Ramp3 mRNA expression was regulated differently during the estrus cycle and that the regulation of mRNA turnover might be an important mechanism in the strict control of gene expression in the rat uterus. However, further studies are necessary to resolve the molecular mechanisms underlying the E2-mediated expression of these genes and to allow greater understanding of the regulation of ADM signaling in the rat uterus. While CRLR gene expression was down-regulated by both E2 and Cou in the immature rat uterus, expression in the adult female rat uterus was highest during the diestrus and proestrus stages. Thus, it appears that CRLR gene expression is differently regulated in the adult and immature rat uterus.

Cameron et al. (2002) suggested that ADM gene expression was synchronized with the estrus cycle in the adult female rat uterus and showed that ADM mRNA levels peaked during the proestrus stage, which correlated well with plasma E2 levels. We obtained similar results for

ADM. However, we found that Ramp3 and CRLR genes transcripts were expressed at their highest levels during diestrus (early-proestrus) rather than proestrus. This may mean that the expression of ADM receptor components is more to sensitive than ADM to the presence of E2, or that expression from those genes is regulated by other hormones in addition to E2 in the adult female rat uterus. In either case, that ADM receptor components are expressed prior to ADM may reflect the strict regulation of ADM signaling during the estrus cycle in the rat uterus.

As shown in the Fig. 4, administration of ADM to immature rats led to increased uterine weight and endometrial cell swelling. As it is thought that ADM is unstable in circulation, this may explain the relatively weak effect of ADM on the uterus observed in our study. We propose that ADM promotes endometrial cell growth during the estrus cycle. It is also known that vascular endothelial growth factor is rapidly induced by E2 in the uterus (Hyder et al., 2000). VEGF is a mitogen for vascular endothelial cells, stimulates neovascularization, and induces a rapid increase in microvascular permeability. As ADM also exhibits these effects on the vascular system, ADM and VEGF may cooperatively contribute to estrogen-induced early changes in the uterus, particularly edema following neovascularization.

It is well known that eating very high amounts of phytoestrogen-rich plants can induce reproductive problems in laboratory animals and farm animals (Oldfield et al., 1966; Elias and Kincaid, 1984; Whitten et al., 1995). Cou is a compound produced by various legumes and exerts an estrogen-like activity in immature animals, as shown in this report, and can suppress the estrus cycle in adult females. We observed that Cou regulated the expression of ADM signaling components, which suggests that food-derived Cou may induce the abnormal induction of vasoactive factors in the uterus and lead to Cou-mediated disruption of pregnancy.

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Stability of A+U-Rich Element Binding Factor 1 (AUF1)-Binding Messenger Ribonucleic Acid Correlates with the Subcellular Relocalization of AUF1 in the Rat Uterus upon Estrogen Treatment

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The nucleocytoplasmic shuttling protein, A+U-rich element binding factor 1 (AUF1), is one of the RNA-binding proteins that specifically bind adenylate-uridylate rich elements (AREs) in mRNA 3′-untranslated regions (UTRs), and acts as a regulator of ARE-mediated mRNA degradation in the cytoplasm. We previously reported that in the female rat uterus, the levels of specific AUF1 isoform mRNAs (p40/p45) were increased by 17β-estradiol (E2) treatment. Therefore, we examined the role of AUF1 in the regulation of E2-mediated mRNA turnover in the rat uterus. We identified ABIN2 and Ier2/pip92 mRNAs as candidate targets of AUF1 in the rat uterus. We found that AUF1-binding elements were present in the 3′-UTR of both mRNAs and that the 3′-UTRs functioned as mRNA turnover regulatory elements. In the ovariectomized rat uterus, the nucleocytoplasmic localization of AUF1p40/p37 isoform proteins was regulated by E2. We also found that cytoplasmic AUF1-bound mRNA levels changed coincidentally with the cytoplasmic levels of AUF1p40/p37. Finally, we confirmed that the subcellular localization of AUF1p40 controlled the stability of target mRNAs *in vitro*, such that cytoplasmically localized AUF1p40 led to marked mRNA stabilization, whereas nuclear-localized AUF1p40 stabilized target mRNA only slightly. These results suggested that E2-inducible ARE-containing gene transcripts are regulated, at least in part, via mRNA stabilization through the nucleocytoplasmic relocalization of AUF1. (*Molecular Endocrinology* 18: 2255–2267, 2004)

THE RODENT REPRODUCTIVE tract responds properly to precisely timed physiological signals in order for pregnancy to be established and maintained. Before ovulation, serum estrogen levels surge and cause the well-characterized physiological and biochemical effects in uterine tissue. This acute and rapid response of the uterus to estrogen appears to be biphasic in nature (1–3). Early events, occurring within the first hour of estrogen exposure, include estrogen receptor (ER) occupancy, transcription of early phase genes such as c-*fos*, fluid uptake, and hyperemia. Later-phase responses, occurring up to 24 h after estrogen exposure, include the transcription of late-phase genes such as *lactoferrin*, increase in uterine wet weight, development of the epithelial layer into columnar secretory epithelial cells, and subsequent mitosis in the epithelial layer. Recent global genomic expression studies have revealed that the expression patterns of distinct gene clusters reflect the early and late physiological responses and that the timing of expression of these gene clusters is strictly regulated (4). It is well accepted that estrogen-mediated gene expression is regulated at the transcriptional level (5, 6). To regulate the precisely timed gene expression, it is highly likely that transcript expression is also regulated at the posttranscriptional level, especially via the control of mRNA turnover (7). However, little is known about the molecular mechanism of estrogen-mediated control of mRNA turnover in the rodent uterus.

Adenylate-uridylate rich elements (AREs) in the 3′-untranslated regions (UTRs) of short half-life mRNAs have been well characterized as *cis*-acting elements

Abbreviations: ABIN2, A20 binding inhibitor of NF-κB activation-2; Akt/PKB, protein kinase B; ARE, adenylate-uridylate rich element; AUF1, A+U-rich element RNA binding factor 1; CAT, chloramphenicol acetyltransferase; dNTP, deoxynucleotide triphosphate; Dox, doxycycline; DTT, dithiothreitol; E2, 17β-estradiol; FITC, fluorescein isothiocyanate; GST, glutathione-*S*-transferase; HA, hemagglutinin; HB, homogenization buffer; Ier2/pip92, immediate early response-2; Luc, luciferase; NF, nuclear factor; OVX, ovariectomized; REMSA, RNA EMSA; RPS2, ribosomal protein S2; SDS, sodium dodecyl sulfate; TRE, tetracycline responsive element; UTR, untranslated region.

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involved in mRNA turnover regulation (8). Recently, several RNA-binding proteins thought to be involved in the regulation of mRNA turnover have been identified, including A+U-rich element binding factor 1 (AUF1), that specifically bind to the AREs of unstable mRNAs (9–11). Several lines of evidence suggest that AUF1 acts as a regulator of ARE-mediated mRNA degradation in the cytoplasm. First, AUF1 purified from cytoplasmic extracts of K562 human erythroleukemia was shown to lead to enhanced degradation of c-*myc* mRNA after binding to an ARE in the 3′-UTR of c-*myc* (11). Second, many reports have demonstrated that AUF1 is present in some ribonucleoprotein complexes that associate with 3′-UTR-regulatory elements and control mRNA turnover (12–16).

The cloning and characterization of human and mouse genomic clones showed that the transcribed AUF1 gene can undergo alternate splicing to give rise to four protein isoforms with apparent molecular masses of 45, 42, 40, and 37 kDa (17, 18). We have previously reported that in the female rat uterus, the mRNA levels of specific AUF1 isoforms (p45 and p40) were increased by 17β-estradiol (E2) treatment (19). Furthermore, we also demonstrated different nuclear-cytoplasmic localization patterns and functions of AUF1 that were isoform dependent, such that the p45 and p42 isoforms were predominantly located in the nucleus whereas the p40 and p37 isoforms shuttled between the nucleus and cytoplasm (20). Therefore, we focused on the AUF1p40 isoform to investigate the role of AUF1 in the regulation of E2-mediated mRNA turnover in the rat uterus.

Here, we identified A20 binding inhibitor of nuclear factor (NF)-κB activation-2 (ABIN2) and immediate early response-2 (Ier2/pip92) mRNAs from the rat uterus as candidate targets for AUF1 *in vivo*. The 3′-UTRs of both mRNAs include AUF1-binding element and function as mRNA turnover regulation. In *in vivo* experiments, the mRNA expression of ABIN2 and Ier2/pip92 was transiently up-regulated by E2. Furthermore, the nuclear or cytoplasmic accumulation of AUF1p40/p37 isoform proteins was regulated by E2, and cytoplasmic AUF1-bound mRNA levels changed coincidentally with the cytoplasmic levels of AUF1p40/p37. Finally, we confirmed that the subcellular localization of AUF1p40 controlled the stability of target mRNAs *in vitro*, *i.e.* cytoplasmically localized AUF1p40 led to marked stabilization, whereas nuclear-localized AUF1p40 only slightly stabilized the target mRNA. Our present study provides the possibility that E2-mediated nucleocytoplasmic relocalization of AUF1 isoforms contributes to the E2-mediated regulation of mRNA stability in the rat uterus.

RESULTS

Screening of AUF1p40-Binding mRNAs in the Rat Uterus

A modified differential display method was used to detect mRNAs that bound AUF1p40. Glutathione sepharose 4B resin bound with glutathione-*S*-transferase (GST)-AUF1p40 fusion protein or GST protein-only was mixed with poly(A)$^+$ RNA extracted from E2-treated rat uteri. Complexes of poly(A)$^+$ RNA and GST-AUF1p40- or GST-bound resin were collected using oligo dT magnetic beads. Recovered poly(A)$^+$ RNAs were reverse transcribed and subjected to differential display, and comparisons were made between the different capture proteins. As shown in Fig. 1, several bands were specifically observed in GST-AUF1p40-derived samples (lanes 6, 8, 10, and 12). The screening was performed in duplicate, and bands that exhibited good reproducibility were selected. We finally obtained two cDNA clones derived from GST-AUF1p40-bound mRNA.

ABIN2 and Ier2/pip92 3′-UTR Sequences and AUF1-Binding Elements

As shown in Fig. 2, two short PCR fragments (*underlined*) were obtained and sequenced. One of these clones was the rat homolog of A20-binding inhibitor of NF-κ B activation-2 (ABIN2) gene (clone 3) (21). The

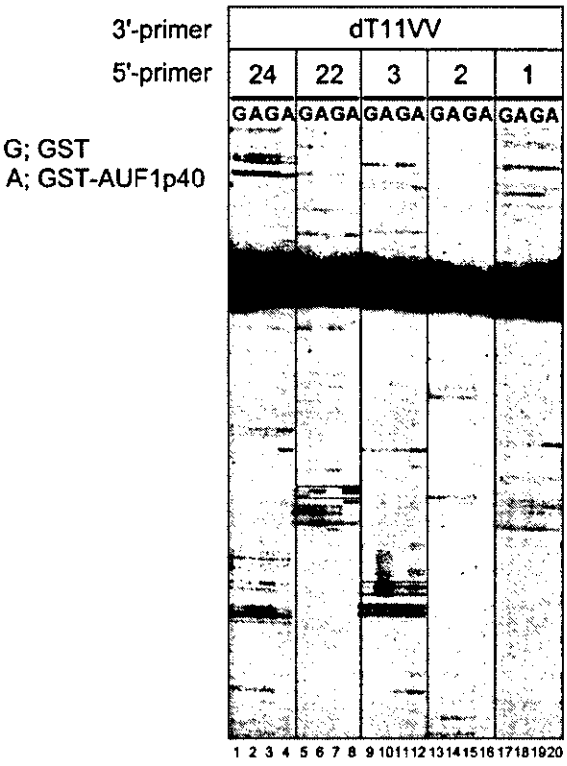

Fig. 1. Differential Display of GST Protein- and GST-AUF1p40 Fusion Protein-Bound mRNAs

Example of a differential display gel for mRNAs isolated from GST (G) and GST-AUF1p40 fusion (A) protein binding. Results were obtained using five different 5′-primers (1, 2, 3, 22, and 24) and a 3′-primer (dT11VV). After PCR amplification, rhodamine-labeled products were separated on 6% polyacrylamide-8 M urea DNA sequencing gels and scanned using a FMBIO II multiview fluoro imager.

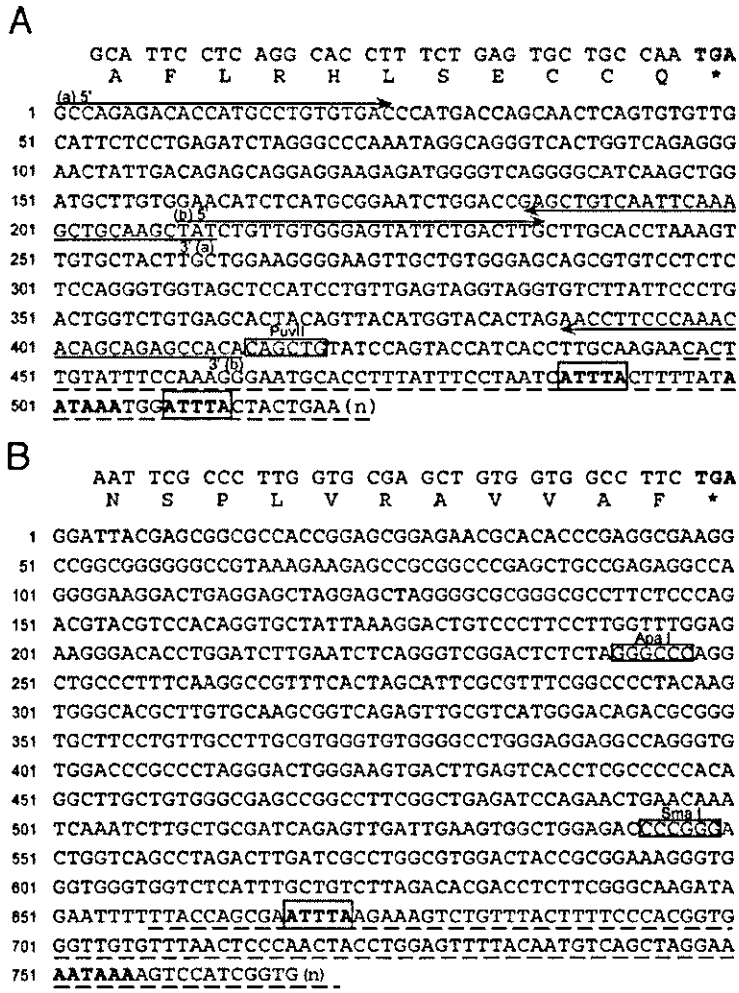

Fig. 2. 3′-UTR Sequences of AUF1-Binding mRNAs

A, 3′-UTR of rat ABIN2 mRNA (clone 3). B, 3′-UTR of rat Ier2/pip92 mRNA (clone 22). The TGA translation termination codon is in *bold* and the next nucleotide designated as 1. Locations of the pentanucleotide AUUUA motifs are *boxed and in bold.* Locations of poly(A) signals are in *bold. Dotted lines* indicate the cDNA fragments obtained from the differential display screening. *Arrows* in (A) indicate the PCR primers used to make the deletion constructs. The restriction enzyme sites (*Pvu*II, *Apa*I, and *Sma*I) in panels A and B indicate the boundaries of the deletion constructs.

other clone was the rat homolog of immediate early response-2 (Ier2/pip92) gene (clone 22), known to be a growth factor-induced immediate-early response gene with a short mRNA half-life (22, 23). We performed further screening and obtained cDNAs for both these genes that included the full-length 3′-UTRs. The cDNA sequences of rat ABIN2 and Ier2/pip92 3′-UTRs were 521 and 768 bases in length, respectively, as shown in Fig. 2. Notably, both UTRs contained at least one copy of the ATTTA pentanucleotide sequence, known to function as an mRNA degradation regulation sequence, in close proximity to the poly(A) signal (Fig. 2, A and B). To investigate the AUF1-binding element(s) in the 3′-UTRs, we performed an RNA EMSA (REMSA) using *in vitro* synthesized AUF1p40 protein. ^{32}P-labeled c-*fos* ARE was used as the REMSA probe, as the c-*fos* ARE has been well characterized as an AUF1-binding element (24). The 3′-UTRs of ABIN2 (clone 3) and Ier2/pip92 (clone 22) were divided into three regions (a, b, and c) and used as equimolar cold competitors in the REMSA (sequences as shown in Fig. 2, A and B). As shown in Fig. 3, regions containing the AUUUA-pentanucleotide (3c and 22c) exhibited stronger competitive activity than the other regions (3a, 3b, 22a, or 22b). These results suggested that ABIN2 and Ier2/pip92 mRNAs were candidate targets of AUF1 binding in the rat uterus.

The 3′-UTRs Function as Regulatory Elements of mRNA Turnover

To determine whether the 3′-UTRs of ABIN2 (clone 3) and Ier2/pip92 (clone 22) regulate mRNA stability, we developed a transient transfection system in HeLa cells. Plasmids containing the tetracycline-responsive element (TRE) with the cytomegalovirus (CMV) pro-

moter inserted upstream of the luciferase (Luc) gene (pTRE-Luc) were used (Fig. 4A), such that Luc gene expression was suppressed by the addition of synthetic tetracycline [doxycycline (Dox)]. Luc mRNA levels in response to Dox treatment were quantified by real-time RT-PCR and compared with chloramphenicol acetyltransferase (CAT) transcript expression, used as an internal control. As shown in Fig. 4, when full-length 3′-UTRs of ABIN2 (3UTR) or Ier2/pip92 (22UTR) were inserted downstream of the Luc coding

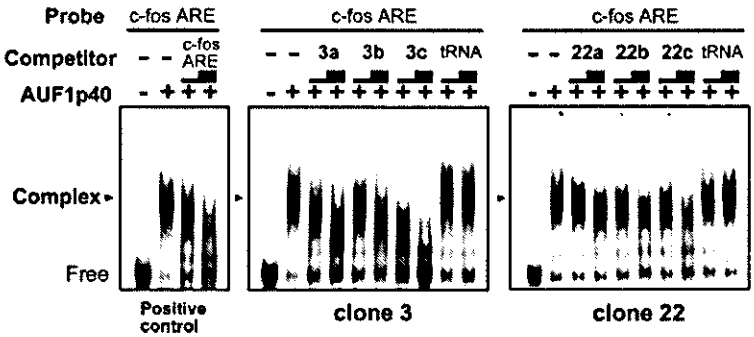

Fig. 3. AUF1 Binds to AUUUA-Containing Sequences

Binding reactions containing ^{32}P-labeled c-*fos* ARE RNA (1 × 10^5 cpm, 3 pmol), AUF1p40 protein (20 ng), and competitor fragments (c-*fos* ARE, 3a, 3b, 3c, 22a, 22b, 22c, or tRNA; 6 and 18 pmol) were fractionated by 4% acrylamide-native gel electrophoresis. tRNA was used as a nonspecific competitor. The competitor fragments used were as follows: 3a, 1–212; 3b, 210–414; 3c, 418–521 (nucleotide numbers originate from Fig. 2A); 22a, 1–246; 22b, 243–546; 22c, 547–769 (nucleotide numbers originate from Fig. 2B). "Complex" indicates complexes between AUF1p40 and c-*fos* ARE RNA, "Free" indicates unbound c-*fos* ARE RNA.

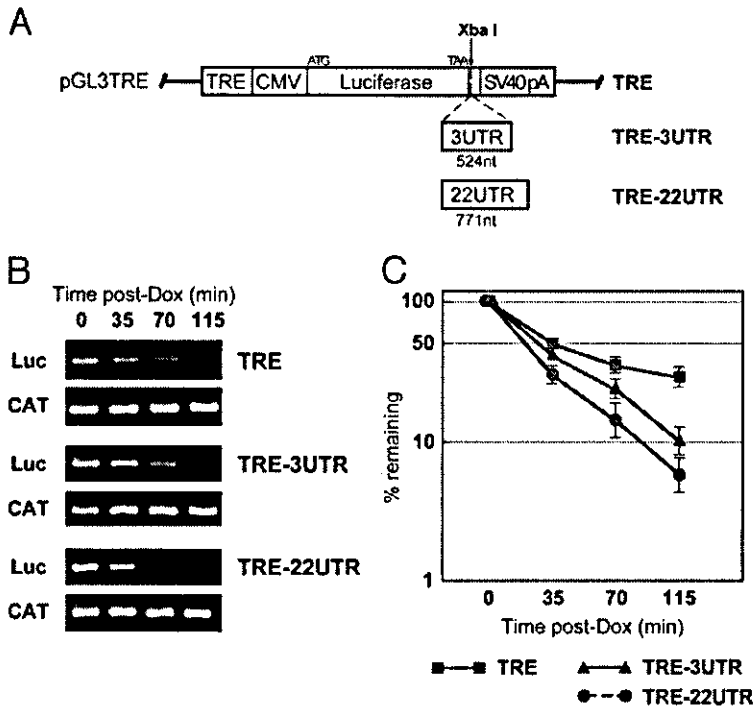

Fig. 4. The 3′-UTRs Function as Regulatory Elements of mRNA Turnover

A, Construction of chimeric luciferase reporter genes. DNA fragments corresponding to the entire 3′-UTRs of clone 3 and clone 22 were inserted into the *Xba*I site of the pGL3TRE expression vector. The functional elements of the vector are indicated in the schema. TRE, Tetracycline response element; CMV, cytomegalovirus promoter; SV40pA, SV40 late poly(A) signal. B, pTRE-Luc plasmids containing the full-length 3′-UTRs (3UTR or 22UTR) and pTet-off plasmid were transiently transfected into HeLa cells. Cytoplasmic RNA was isolated at time intervals after 1 μg/ml Dox treatment. Chase began at 45-min after Dox treatment and shown as time zero. Typical PCR results are shown. C, Quantitative real-time PCR was performed to calculate RNA levels with values normalized with respect to amount of cotransfected constitutively expressed CAT mRNA. Data are shown as the mean ± SD of remaining RNA compared with time zero in three separate assays.

region in the pTRE-Luc vector, the half-lives of Luc+3UTR (ca. $t_{1/2}$ = 30 min) and Luc+22UTR mRNA (ca. $t_{1/2}$ = 30 min) were shorter than that of Luc mRNA (ca. $t_{1/2}$ = 80 min). This suggested that the 3′-UTRs functioned as mRNA turnover-regulatory elements.

E2 Regulates the Uterine Expression of AUF1-Binding mRNAs

As there was no available information regarding the E2-mediated regulation of ABIN2 and Ier2/pip92 gene expression in the rat uterus, we profiled the time course of ABIN2 and Ier2/pip92 gene expression after exposure to E2. Ovariectomized (OVX) adult female rats were injected with 5.0 μg/kg E2 at time zero, and total RNA was isolated at the times indicated. Relative amounts of ABIN2 and Ier2/pip92 mRNA were quantified by Northern blotting and compared with the expression of rat ribosomal protein S2 (RPS2) transcripts, used as an internal control. As shown in Fig. 5, A and C (*upper panel*), ABIN2 mRNA levels increased 1.5-fold at 3 h after E2 treatment and recovered to basal levels within 6 h. For Ier2/pip92, the addition of E2 induced a 4-fold increase in Ier2/pip92 mRNA levels 3 h after E2 exposure, which then subsequently decreased over the next 3 h (Fig. 5, A and C, *lower panel*). As shown in Fig. 5B, ABIN2 and Ier2/pip92 gene expression was unchanged by vehicle treatment. The E2-mediated responses of these genes were highly reproducible and indicated that the expression of the AUF1-binding mRNAs was transiently up-regulated by E2 in the OVX rat uterus.

Nucleocytoplasmic Localization of AUF1 Protein Is Regulated by E2 in the Rat Uterus

Previously, we reported that AUF1 mRNA levels were increased by E2 in the rat uterus (19). We identified this finding at the protein level in the OVX rat uterus. Relative levels of AUF1 protein were quantified by Western blotting using anti-AUF1 antibody that reacts with all four isoforms of AUF1. Total AUF1 levels increased 1.5-fold in the OVX rat uterus after the addition of E2 (data not shown). We then investigated the effect of E2 on the nucleocytoplasmic localization of AUF1. OVX rats were injected with vehicle or 5.0 μg/kg E2 at time zero and killed at 3, 6, and 12 h. The excised uteri were homogenized and fractionated to obtain nuclear and cytoplasmic fractions. As shown in Fig. 6, AUF1 protein isoforms p40 and p37 were localized to the nuclear and cytoplasmic fractions in untreated animals (−E2). After 3 h exposure to E2, p40/p37 isoforms transiently accumulated in the cytoplasm with a concurrent decrease in nuclear p40/p37 isoform levels.

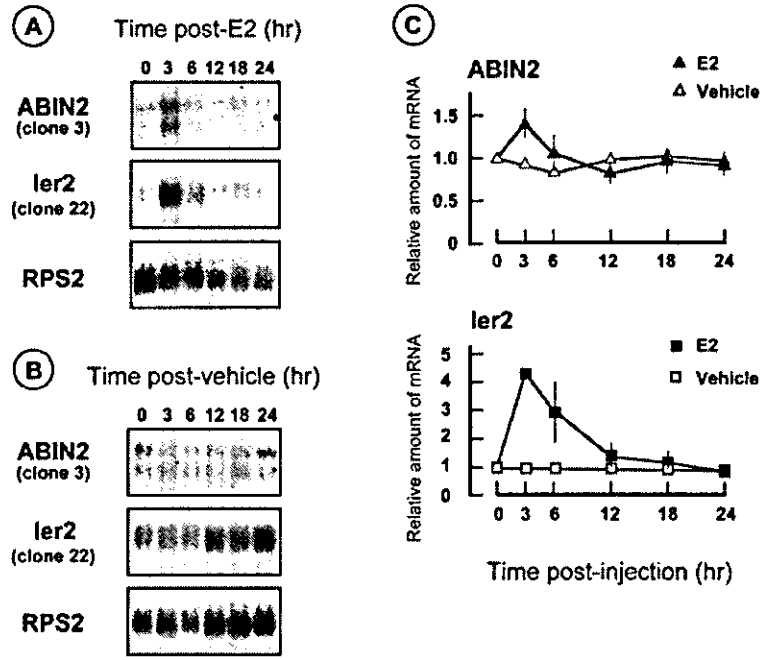

Fig. 5. Estrogen Regulates the Expression of AUF1 Target mRNAs in the Rat Uterus

A, Animals (six rats per group) were treated with 5.0 μg/kg E2 and uterine RNA prepared at the times indicated. B, Animals (four rats per group) were treated with 100 μl solvent (vehicle) and uterine RNA prepared at the times indicated. Samples were subjected to electrophoresis, blotted onto nylon membranes, and hybridized to probes specific for ABIN2, Ier2/pip92, or RPS2. Typical autoradiograms are shown. C, mRNA levels were quantified using the quantification program of the Fuji BAS1000 phospho imaging system. Results were normalized with respect to RPS2 hybridization in each lane. Data are shown as mean ± SD and expressed as relative levels compared with time zero; rats were administered solvent (*open triangle and square*; Vehicle) or 5.0 μg/kg E2 (*closed triangle and square*; E2).

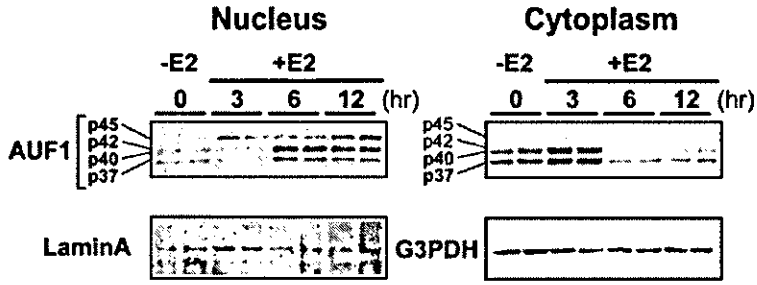

Fig. 6. Nucleocytoplasmic Localization of AUF1 Protein Is Regulated by E2 in the Rat Uterus

Animals were treated with E2 (5.0 μg/kg) and the uteri were excised at the times indicated. Nuclear and cytoplasmic fractions were prepared as described in *Materials and Methods*, and proteins were separated by electrophoresis on 4–20% gradient SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. AUF1, G3PDH, and Lamin A proteins were detected with specific antibodies. Two examples of each treatment are shown.

Over the next 3-h time period, these levels were reversed with p40/p37 isoform levels increasing in the nucleus and decreasing in the cytoplasm. In contrast, p45 isoform level increased after 3 h of E2 treatment and remained within the nucleus even after 12 h of E2 exposure. Injection of vehicle had no effect on the nuclear-cytoplasmic localization of AUF1 proteins (data not shown). Thus, subcellular AUF1p40/p37 isoform levels were regulated by E2.

Binding Activity of Uterine AUF1 to Target mRNAs in the Rat Uterus

To determine whether AUF1 binds to ABIN2 and Ier2/pip92 mRNA *in vivo*, we performed immunoprecipitation and RT-PCR. AUF1-binding mRNA species were immunoprecipitated with anti-AUF1 from rat uteri cytoplasmic extracts treated with or without E2. Immunoprecipitated mRNAs were then extracted and reverse transcribed using oligo dT. PCR was performed with specific primer sets for ABIN2 or Ier2/pip92 cDNA. As shown in Fig. 7B, ABIN2 and Ier2/pip92 mRNA bound to AUF1 present in the rat uterus cytoplasm.

Nucleocytoplasmic Localization of AUF1p40 Correlates with Target mRNA Stability

Together, our results suggested that there was a correlation between the E2-mediated regulation of AUF1-target mRNA stability and E2-mediated changes in the subcellular localization of AUF1 isoforms. To determine whether nuclear- or cytoplasmically localized AUF1 played a causal role in regulating the stability of target mRNA, we ectopically overexpressed wild-type (p40wt) and mutant (GALp40, p40S) AUF1 proteins in HeLa cells with the TRE-22UTR reporter expression vector. GALp40 consisted of the GAL4 DNA binding domain (DBD; amino acids 1–147 of GAL4) fused to the N-terminal domain of AUF1p40. GALp40 was strictly localized in nucleus due to the presence of a nuclear localization signal within the GAL4 DBD (25). In contrast, the p40S protein [previously designated as p45Cdel.(285) (20) as it excludes the p45-specific domain so making it equivalent to p40] was localized to the cytoplasmic fraction predominantly as the unclassified nuclear retention signal was removed by a C-terminal deletion (26). As shown in Fig. 8A, overexpressed p40wt and GALp40 localized to the nucleus, whereas p40S localized to the cytoplasm. We then measured the stability of Luc+22UTR mRNA in HeLa cells that ectopically overexpressed p40wt, GALp40, or p40S. As shown in Fig. 8B, cytoplasmically localized AUF1 (p40S) led to marked Luc+22UTR mRNA stabilization, whereas nuclear-localized AUF1 (GALp40) only slightly stabilized the target mRNA. Moreover, ectopically expressed p40wt, able to shuttle between the nucleus and cytoplasm (20), stabilized Luc+22UTR mRNA at a level intermediate between p40S and GALp40. These results suggested that the nucleocytoplasmic localization of AUF1p40 regulated the stability of target mRNA.

DISCUSSION

In this study, we identified two AUF1-binding mRNAs, ABIN2 (clone3) and Ier2/pip92 (clone22), expressed in the rat uterus and showed that the level of both transcripts was regulated by E2. To our knowledge, this is the first report that E2 regulates ABIN2 and Ier2/pip92 gene expression. ABIN2 is a recently discovered binding partner for the zinc finger protein A20 that functions as a feedback inhibitor of the NF-κB pathway, and inhibits NF-κB-mediated inflammatory gene expression (21). Several recent reports have shown that ABIN2 interacts with Tie2, a receptor tyrosine kinase expressed predominantly in endothelial cells that is essential for blood vessel formation and maintenance (27). Tadros *et al.* (28) suggested that ABIN2 is involved in the Tie2 ligand (Ang1)-mediated inhibition of endothelial apoptosis. Previously, Wong *et al.* (29) reported that Tie2 is expressed in the endothelium of the developing endometrial vasculature in the ovarian hormone-stimulated uterus. Based on this finding, it is

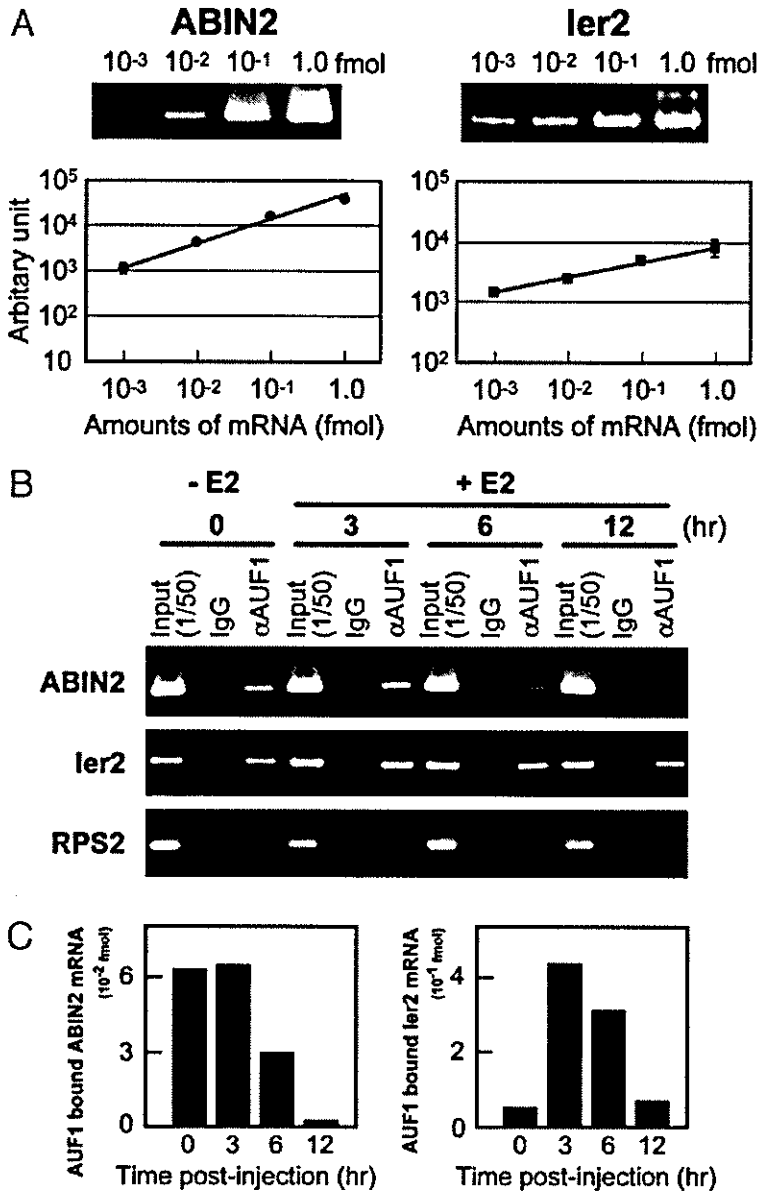

Fig. 7. *In Vivo* Binding Activity of Uterine Cytoplasmic AUF1 to Target mRNAs

A, *In vitro*-transcribed ABIN2 and Ier2 mRNAs (10^{-3} to 1.0 fmol) were reverse transcribed, and PCR was performed using the specific primer sets as described in *Materials and Methods*. PCR products were resolved on 2% (wt/vol) agarose gels and stained with ethidium bromide. A typical result is shown in the *upper panel*. Amplified fragments were quantified by the quantification program of the FMBIO II fluoroimaging system. The values in the *lower panel* are the mean ± SD (n = 3), expressed in arbitrary units. B, AUF1-binding mRNAs were immunoprecipitated with anti-AUF1 antibody (αAUF1) from rat uterine cytoplasmic fractions in the presence or absence of E2 as described in *Materials and Methods*. Antirabbit IgG antibody (IgG) was used as a negative control. Total RNA was extracted from the cytoplasmic fraction used for immunoprecipitation (input). Immunoprecipitated mRNAs and 1/50 volume of cytoplasmic mRNAs were reverse transcribed using oligo dT. PCR was performed as described in panel A. C, Amplified fragments were quantified visually using standard curves (A).

possible that ABIN2 may contribute to blood vessel formation in the E2-stimulated uterus.

Ier2/pip92 (also known as Chx1 or ETR101) is rapidly and transiently induced by stimulation with serum, growth factors, or 12-O-tetradecanoylphorbol-13-acetate in fibroblasts and with nerve growth factor in PC12 cells (22). Ier2/pip92 encodes a short-lived, proline-rich protein with no significant sequence similarity to any other known protein and little is known about Ier2/pip92 protein function. The Ier2/pip92 promoter region has been characterized and is induced by serum via a serum response element (SRE) (30, 31). Our present study suggests that expression of Ier2/pip92 is regulated by E2 in the rat uterus and shows

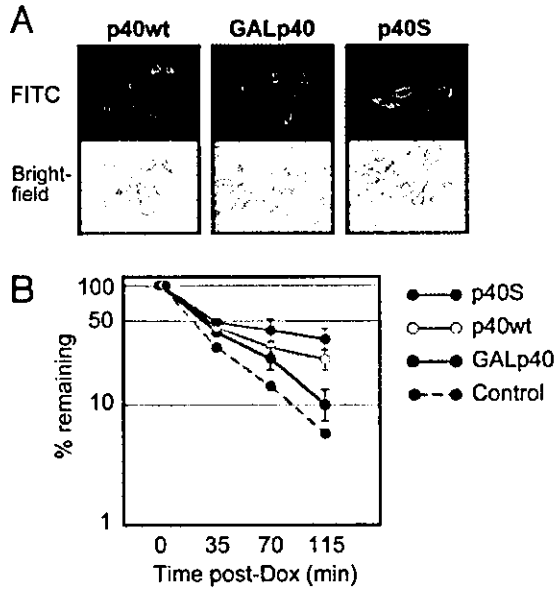

Fig. 8. Subcellular Localization of AUF1p40 Controls Target mRNA Turnover

A, HeLa cells were transiently transfected with the expression plasmids for HA-AUF1p40 (p40wt), GAL-AUF1p40 (GALp40), and HA-AUF1p45Cdel.(285) (p40S). Transfected cells grown on glass coverslips were incubated for 16 h in DMEM with 5% fetal calf serum before fixation. Cells were fixed and immunostained with the anti-HA tag antibody (p40wt and p40S) or anti-GAL4BD antibody (GALp40). Cells were photographed under immunofluoressence (FITC) and bright field. B, HeLa cells were transiently cotransfected with p40wt, GALp40, or p40S expression plasmids and reporter plasmid containing full-length ler2/pip92 (clone 22) 3′-UTR (pTRE-22UTR). Cytoplasmic RNA was isolated at time intervals after 1 μg/ml doxycycline (Dox) treatment. Chase began at 45-min after Dox treatment and shown as time zero. mRNA levels were calculated by quantitative real-time PCR. The data from three separate assays are shown as described in Fig. 4.

that its expression may be at least partly regulated at the level of mRNA turnover. The role of ler2/pip92 in the physiological response of the uterus to E2 remains to be explored.

AUF1 protein isoforms p40 and p37 localized to both the nuclear and cytoplasmic fractions in untreated OVX rat uteri, and the target mRNAs exhibited constitutive, low-level expression in the cytoplasm. In contrast, cytoplasmic levels of the p40/p37 isoforms were transiently increased, and nuclear levels were concurrently decreased 3 h after treatment with E2. After a further 3 h, this effect was reversed, with levels of the protein isoforms decreasing in the cytoplasm and increasing in the nucleus. Interestingly, levels of the target mRNAs were altered coincidentally with cytoplasmic p40/p37 isoform levels (Figs. 5 and 6). Several reports have suggested that signals that induce changes in the subcellular localization of AUF1 protein isoforms also appear to affect the stability of ARE-containing mRNAs (32–34). These findings led us to the hypothesis that the nucleocytoplasmic localization of AUF1 was coupled with the regulation of target mRNA stability. To test this hypothesis, we investigated the contribution of nuclear or cytoplasmically located AUF1p40 to the regulation of mRNA turnover using an *in vitro* assay. We found that the stability of target mRNA was dependent on the subcellular localization of AUF1, such that cytoplasmically localized AUF1 protein stabilized a target mRNA, whereas nuclear-localized AUF1 protein had only a limited stabilizing effect (Fig. 8). Thus, the cytoplasmic AUF1p40 isoform appeared to function as a stabilizer of target mRNAs.

The nucleocytoplasmic shuttling protein HuR is another well-characterized ARE-dependent stabilizer of mRNA (35–37). HuR protein is predominantly localized in the nucleus although certain signal(s) enhance its accumulation in the cytoplasm. Yaman *et al.* (37) suggested that the nutrient-dependent stabilization of ARE-containing mRNAs was caused by the transient accumulation of HuR in the cytoplasm. We thus checked the nucleocytoplasmic localization of HuR in rat uterus during E2 treatment. We could not find any difference in the subcellular localization of HuR in response to treatment with E2 (Arao, Y., A. Kikuchi, and F. Kayama, unpublished results). Moreover, preliminary *in vitro* experiments have suggested that the HuR protein does not stabilize the AUF1 target mRNA (our unpublished results). Taken together, we believe that AUF1 is a major factor in the regulation of ARE-mediated mRNA turnover in E2-treated rat uterus.

ARE motifs have been classified into three categories according to the distribution of the AUUUA pentamers (8). Class I AREs contain multiple isolated AUUUA motifs, class II AREs contain at least two overlapping UUAUUUA(A/U)(A/U) nonamers, whereas class III AREs have U-rich or AU-rich regions instead of AUUUA motifs. A recent report has defined the ARE motif consensus as WWWUAUUUAUWWW (38). Based on this definition, and the presence of two- and one-AUUUA sequence elements in the 3′-UTRs of ABIN2 and ler2/pip92 respectively, the AREs in these 3′-UTRs likely fall into class III. Recent genomic expression profiling has revealed a cluster of genes that are expressed within 30 min of exposure of rodent uteri to E2. Expression of these genes peaks after 2 h and then steeply declines over the next 4–10 h (4). Hewitt *et al.* (4) categorized c-*fos* (NM_010234), cyr61 (M32490), casein kinase 1 (BC048081), axin2 (NM_015732), and fz-1 (BC053010) as E2-mediated immediate-early response genes whose pattern of mRNA expression is similar to that of ABIN2 and ler2/pip92. Interestingly, each of these transcripts contains ARE(s) in their 3′-UTR that are proximal to the poly(A) signal (supplemental Fig. 1 published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). The stability of p21/WAF1 mRNA has been reported to be regulated through AREs (39, 40). The expression of p21/WAF1 is induced by E2 within 30 min in the rodent uterus and

is maintained at an elevated level for 12 h (4). The AREs of p21/WAF1 mRNA are located proximal to the stop codon, rather than the poly(A) signal (supplemental Fig. 1). Thus, it is likely that the stability of E2-mediated immediate-early response gene mRNAs in the rodent uterus is regulated by the subcellular relocalization of AUF1 p40/p37 isoforms that bind the element proximal to the poly(A) signal.

The RNA binding activity and/or subcellular distribution of some ARE-binding proteins are regulated by posttranslational modification (41–43). Recently, Wilson *et al.* suggested that AUF1p40 is phosphorylated at Ser^{83} by glycogen synthase kinase 3β and at Ser^{87} by protein kinase A. The phosphorylation of Ser^{83} in AUF1p40 in particular inhibits the initial step of ARE binding (44). Glycogen synthase kinase 3β is a downstream effector of protein kinase B (Akt/PKB), and several reports have suggested that estrogen activates Akt/PKB-mediated pathways via a nongenomic action of the estrogen receptor (45, 46). These findings imply that E2 treatment may regulate the binding affinity of AUF1p40 for the target mRNA.

Previous reports have suggested that both phosphorylated and nonphosphorylated AUF1p40 are found in cytoplasmic polysomes, suggesting that phosphorylation of AUF1p40 does not influence its subcellular localization (47). Further research of the E2-regulated nucleocytoplasmic AUF1 shuttling mechanism will be important to clarify the precise regulation of estrogen-mediated gene expression in the rodent uterus.

MATERIALS AND METHODS

Plasmids

The plasmids pGEX-AUF1p40, pGAL4-AUF1p40, pSG5-hemagglutinin (HA)-AUF1p40, and pSG5-HA-AUF1p45Cdel.(285). have been described previously (20). pSG5-c-fos ARE was constructed as follows: pSPT-fos cDNA purchased from the Japan Health Science Foundation was amplified by PCR using the primers 5′-TGT GAG ATC TAT GTC AAA AGA CCT CAA GGT-3′ and 5′-TGT GGG ATC CCC ATG AAA ACG TTT TAT TGT-3′, and the amplified fragments were digested by *Bam*HI and *Bgl*II and inserted into the *Bam*HI site of the pSG5 vector (Stratagene, La Jolla, CA). To construct the tetracycline-responsive element (TRE)-containing luciferase reporter vector pGL3-TRE, the CMV promoter-fused TRE fragment was inserted into the *Sma*I site of the pGL3-Basic vector (Promega Corp., Madison, WI). DNA fragments containing the 3′-UTR were digested with *Eco*RI from pCR2.1–3UTR and pCR2.1–22UTR vectors and blunt-ended using a blunting kit (Takara, Shiga, Japan), and the fragments were inserted into the *Xba*I site (blunted) of the pGL3-TRE vector to give pGL3-TRE-3UTR and pGL3-TRE-22UTR, respectively. The plasmids pSP64poly(A)-3UTR and pSP64poly(A)-22UTR were constructed as follows: blunt-ended fragments of 3UTR and 22UTR were inserted into the *Sma*I site of the pSP64 poly(A) vector (Promega), respectively.

Generation of the Recombinant AUF1p40 Protein

Plasmids for pGEX-AUF1p40 (20) or pGEX4T-1 (Amersham Biosciences, Piscataway, NJ) were transformed into *E. coli* DH5α and induced with 0.5 mM isopropyl-β-thiogalactopyranoside for 1.5 h at 30 C. GST fusion proteins were extracted using glutathione sepharose 4B resin (Amersham Biosciences). GST-fusion protein-binding resins were used to screen for AUF1p40-binding mRNAs. For the RNA EMSA, GST-AUF1p40 fusion protein was digested with thrombin and purified by affinity column chromatography.

Screening of AUF1p40-Binding mRNAs

Total RNA was extracted from 12-h E2-treated rat uterus using ISOGEN (Nippon Gene, Tokyo, Japan) and poly(A)$^+$ RNA purified using Oligotex-dT30 beads (Takara), as previously described (48). Purified poly(A)$^+$ RNA (2.5 μg) was incubated with binding resin bound with GST-AUF1p40 fusion protein or GST protein in 50 μl buffer A [5% glycerol, 10 mM HEPES (pH 7.6), 3 mM $MgCl_2$, 50 mM KCl, 50 mM dithiothreitol (DTT)], and 2.5 U/μl RNasin (Takara) on ice for 2 h. Protein-RNA-conjugated resins were UV cross-linked (1.2 × 10^5 μJ/cm^2) and washed using buffer A. Resins were resuspended in 300 μl of buffer B [10 mM Tris/HCl (pH 7.5), 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), and 100 mM NaCl] and 25 μl Oligotex-MAG (Takara) and incubated at 70 C for 3 min and then at 37 C for 10 min. Protein-RNA conjugated magnetic resins were collected using a magnet and washed twice with buffer B. Pellets were resuspended in diethylpyrocarbonate-treated H_2O and the supernatants were used for differential display analysis.

Differential Display Analysis

Candidate AUF1-binding mRNAs were identified using a differential display technique. Single-stranded cDNA was synthesized from recovered poly(A)$^+$ RNA by incubation with 100 U ReverTra Ace (Toyobo, Osaka, Japan) at 42 C for 60 min in a 20-μl reaction volume containing 1 mM deoxynucleotide triphosphates (dNTPs), 50 μmol dT18VN primers (where V is dA, dC, or dG and N is dA, dC, dG, or dT) and 60 U RNasin (Takara). After the reaction, 80 μl H_2O were added to the reaction mixtures and 4 μl reverse-transcribed cDNA were used for PCR. PCR amplification was performed in a 20-μl mixture containing 0.3 μM rhodamine-labeled dT11VV, 0.5 μM of one of 24 different arbitrary sequence 10-mer primers, 0.1 mM dNTPs, and 0.025 U/μl Ex-Taq DNA polymerase (Takara). PCR conditions used were 96 C for 5 min, 40 C for 5 min, 72 C for 5 min for one cycle, and then 96 C for 30 sec, 40 C for 5 min, and 72 C for 2 min for 40 cycles, followed by 72 C for 5 min, in a PerkinElmer GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). Aliquots (3.5 μl) of amplified cDNA were mixed with 2 μl loading dye, denatured at 80 C for 10 min, and electrophoresed on 6% polyacrylamide 8 M urea DNA sequencing gels. After electrophoresis, the gels were scanned by FMBIO II multi-view fluoro-imager (Takara). Screening reactions were repeated at least twice, and only the bands that showed good reproducibility were excised from the gels for further analysis.

Cloning and Sequencing of PCR Products

PCR products were excised from the sequencing gels, eluted by soaking in water, and reamplified using conditions as described in the previous section. After reamplification, PCR products were excised from 1.5% agarose gels stained with ethidium bromide and cloned into the pCR2.1-TOPO vector using the TA cloning system (Invitrogen, San Diego, CA). Plasmid DNA sequencing was performed on an ABI PRISM 310 (Applied Biosystems) using the BigDye terminator cycle sequencing reaction kit (Applied Biosystems).

Screening of mRNA 3′-UTR Coding cDNAs

3′-UTR fragments of rat ABIN2 (clone 3) and ler2/pip92 (clone 22) mRNA were obtained from rat uterus poly(A)$^+$ RNA by RT-PCR using specific primer sets (5′-GAA TTC AGG GAG ATG CAA CAG CTG ATG AGC CAG C-3′ and 5′-GGT GAT GGT ACT GGA TAC AGC TGT GTG GCT-3′ for clone 3, 5′-GGT GCG AGC TGT GGT GGC CTT CTG A-3′and 5′-CAC CGA TGG ACT TTT ATT TTC CTA GCT GAC-3′ for clone 22). PCR was performed using Ex-Taq (Takara). After amplification, rat ABIN2 and ler2/pip92 PCR products were cloned into the pCR2.1-TOPO vector using the TA cloning system to give pCR2.1-3UTR and pCR2.1-22UTR, respectively, and sequenced as described in the previous section. Sequences were checked by comparison with the rat genome database (NCBI).

In vitro Transcription

Linearized template (pSG5-c-fos ARE; 0.2 μg) was transcribed *in vitro* using T7 RNA polymerase (Toyobo) in the presence of 24 μM UTP, 500 μM each ATP, CTP, and GTP, and 50 μCi [α-^{32}P]UTP (800 Ci/mmol). Linearized templates (pCRII-3UTRa, pCRII-3UTRb, pCRII-22UTRc, pCDNA3-22UTRa, pCRII-22UTRb, and pCRII-22UTRc; 0.2 μg) were transcribed to make unlabeled RNAs. These were transcribed in a similar fashion except that the radiolabeled nucleotide was omitted and 500 μM UTP was added.

RNA-Protein Binding Reaction and Electrophoresis of Complexes

In vitro synthesized AUF1p40 (20 ng/reaction) was incubated in 10 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 50 mM KCl, 10% glycerol, 1 mM DTT, and α-^{32}P-labeled c-*fos* ARE RNA (1 × 10^5 cpm/reaction), with or without cold competitors (6 or 18 pmol/reaction) at 4 C for 1 h. Heparin sulfate to a final concentration of 5 mg/ml was then added, and reaction mixtures were incubated for 5 min at 4 C. Electrophoresis of RNA-protein complexes was carried out on 4% native polyacrylamide gels (acrylamide-methylene bis acrylamide ratio, 19:1) with 0.5× Tris-borate-EDTA buffer at 4 C. Gels were preelectrophoresed for 1 h at 100 V and then RNA-protein complexes were electrophoresed for 3 h at 150 V. Gels were dried and visualized using the Fuji BAS1000 phospho imaging analyzer (Fuji Film, Tokyo, Japan).

Measurement of mRNA Half-Life

HeLa cells at 90–95% confluence were transfected with a total of 1.5 μg DNA in 35-mm petri dishes using LipofectAMINE2000 (Invitrogen) according to the manufacturer's protocol. Cells were transfected with 0.25 μg pGL3-TRE, pGL3-TRE-3UTR, or pGL3-TRE-22UTR plasmids, 0.4 μg pTet-off (CLONTECH, Palo Alto, CA) as a tetracycline-mediated transcription suppressor expression plasmid, and 0.1 μg pCAT3 control plasmid (Promega) for normalization of transfection efficiency. pBluescript (Stratagene) was used as a carrier to adjust the total amount of DNA (1.5 μg/well). After 14 h, cells were treated with 1 μg/ml Dox to suppress *de novo* transcription of the luciferase reporter gene. Cells were harvested at 45, 80, 115, and 150 min after Dox treatment and resuspended in 50 μl ice-cold homogenization buffer (HB) containing 50 mM HEPES (pH 7.9), 200 mM sucrose, 70 mM KCl, 10 mM $MgCl_2$, 5 mM NaCl, 1 mM EGTA, 1 mM DTT, 100 U/ml RNasin (Takara), and 1× Compleat (protease inhibitor cocktail; Roche, Switzerland). After 15 min, 6.25 μl 5% Nonidet P-40 was added and the cells were vortexed for 10 sec and centrifuged at 10,000 × *g* for 5 min at 4 C, and the supernatants were collected as the cytoplasmic fraction. To extract cytoplasmic RNA, cytoplasmic fractions (50 μl) were suspended in 150 μl ISOGEN-LS (Nippon Gene), and RNA extraction was performed according to the manufacturer's instructions. To eliminate plasmid DNA, extracted RNA was incubated with 0.05 U/μl DNase I at 37 C for 60 min. Cytosolic RNA was reverse transcribed in 20-μl reaction mixes containing 1 nmol oligo dT (18mer), 400 U ReverTra Ace (Toyobo), 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 U human placental RNase inhibitor, and 1.1 mM dNTPs, incubated at 42 C for 60 min, and then used for quantitative real-time PCR.

Quantitative Real-Time PCR

Quantification of luciferase (Luc) and CAT mRNA was performed using an ABI Prism 7700 sequence detector (Applied Biosystems). For real-time PCR, amplification reactions were performed in 25 μl containing specific primer sets and CYBR-green master mix (Applied Biosystems). PCR conditions consisted of 45 cycles of 95 C for 15 sec and 62 C for 1.5 min. PCR were performed in triplicate for each sample and CAT mRNA was coamplified in each reaction as an internal control. For CAT, forward and reverse primers were 5′-GGA TAT ACC ACC GTT GAT ATA TCC CAA TGG-3′ and 5′-TGC CAC TCA TCG CAG TAC TGT TGT AAT TC-3′, respectively, to give a 432-bp amplicon. Luc mRNA was amplified using the forward primer 5′-AAG GCT ATG AAG AGA TAC GCC CTG G-3′ and reverse primer 5′-TGT CAA TCA AGG CGT TGG TCG CTT C-3′ to give a 629-bp amplicon. Concentrations of Luc and CAT mRNA were calculated in comparison with the standard. Levels of Luc mRNA were normalized according to the amount of CAT mRNA and expressed as percent remaining against time zero.

Animals and Injection Schedule for Estimation of Estrogen Responsiveness

Mature female rats (7 wk old, 120–140 g) obtained from SRC Japan were ovariectomized (OVX) at least 14 d before use. Animals received a single sc injection of E2 (Sigma Chemical Co., St. Louis, MO) at 5.0 μg/kg body weight in propylene glycol (Wako Chemical, Osaka, Japan). Vehicle animals were treated with 0.1 ml propylene glycol. All animal experiments were approved by the Laboratory of Experimental Medicine JMS Animal Use and Care Committee.

Northern Blot Analysis

Total RNA was extracted from OVX rat uteri using ISOGEN (Nippon Gene) according to the manufacturer's instructions. Samples were separated on 1.2% formaldehyde denaturing agarose gels, and RNA was transferred to Gene Screen nylon membranes (New England Nuclear, Boston, MA). Blots were hybridized with α-^{32}P-labeled probes in ULTRAhyb (Ambion, Inc., Austin, TX) at 42 C for 18 h. After hybridization, blots were washed with 1× standard saline citrate and 0.1% SDS at 65 C for 30 min and then 0.1× standard sodium citrate and 1% SDS at 65 C for 30 min. Blots were visualized using a Fuji BAS1000 phospho imaging analyzer (Fuji Film). α-^{32}P-labeled DNA probes were prepared using the random primer labeling method. The probes used were a 1.2-kb fragment of rat ABIN2 cDNA that encoded the coding region, a 0.8-kb fragment of rat ler2/pip92 cDNA that encoded the 3′-UTR, and a 0.6-kb fragment of RPS2 cDNA as a constitutively expressed internal control (19). mRNA levels were quantified using the quantification program of the Fuji BAS1000 system. Results were normalized to RPS2 hybridization in each lane.

Preparation of Nuclear and Cytoplasmic Fractions from Rat Uteri

Excised uteri were homogenized in 10 vol ice-cold HB containing 50 mM HEPES (pH 7.9), 200 mM sucrose, 70 mM KCl,

10 mM $MgCl_2$, 5 mM NaCl, 1 mM EGTA, 1 mM DTT, 100 U/ml RNasin (Takara), and 1× Compleat (protease inhibitor cocktail) using a polytron homogenizer for 45 sec. Homogenates were treated with 10% Triton X-100 with gentle mixing to obtain a final concentration of 1% and then stored on ice for 20 min. Homogenates were filtered through two-layered nylon gauze to remove tissue debris, and then centrifuged at 800 × *g* at 4 C for 5 min. Precipitates were washed twice with HB and used as the nuclear fraction. Supernatants were further centrifuged at 10,000 × *g* at 4 C for 5 min with the resultant supernatants used as the cytoplasmic fraction. Precipitates (nuclear fractions) were homogenized in ISOGEN (Nippon Gene), whereas the supernatants (cytoplasmic fractions; 200 μg) were suspended in ISOGEN-LS (Nippon Gene), and proteins were extracted according to the manufacturer's instructions.

Western Blot Analysis

ISOGEN-extracted proteins were separated by electrophoresis on 4–20% gradient SDS-polyacrylamide gels (Dai-ichi Chemical, Tokyo, Japan) and transferred onto nitrocellulose membranes in buffer containing 125 mM Tris, 960 mM glycine, and 20% methanol, pH 9.0. After blocking with PBS containing 10% skim milk (Life Science Technologies, Gaithersburg, MD), AUF1 protein was detected with anti-AUF1 rabbit polyclonal-antibody (Upstate Biotechnology, Inc., Lake Placid, NY) using peroxidase-conjugated antirabbit IgG (Amersham Biosciences), G3PDH protein was detected with anti-G3PDH mouse monoclonal-antibody (Chemicon International, Temecula, CA) using peroxidase-conjugated antimouse IgG (Amersham Biosciences), and Lamin A protein was detected with anti-Lamin A goat polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) using peroxidase-conjugated antigoat IgG (MBL, Nagoya, Japan). Blots were washed three times in PBS containing 0.1% Tween 20 and developed using the ECL system (Amersham Biosciences).

Immunoprecipitation and Detection of AUF1-Binding mRNAs

Cytoplasmic extracts were treated with protein G magnetic beads (New England Nuclear) to deplete nonspecific binding. After removal, anti-AUF1 antibody was added to the cytoplasmic extracts and incubated overnight at 4 C. The magnet beads were recovered and washed five times in washing buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 7.9), 0.5% NP-40, and 1× Compleat (protease inhibitor cocktail). To extract the AUF1 binding mRNAs, ISOGEN was added to the beads and isolated RNA was used for RT-PCR. Reverse-transcribed products were amplified by PCR using specific primer sets for rat ABIN2 (5′-GCC AGA GAC ACC ATG CCT GTG TGA C-3′ and 5′-TGT GGC TCT GCT GTG TTT GGG AAG GTT-3′), rat ler2/pip92 (5′-GGT GCG AGC TGT GGT GGC CTT CTG A-3′and 5′-CAC CGA TGG ACT TTT ATT TTC CTA GCT GAC-3′) and rat RPS2 (nucleotides 58–79 and 641–667, GenBank accession no. U92700). Conditions for ABIN2 PCR were 30 cycles of 96 C for 30 sec, 62 C for 50 sec, and 72 C for 4 min. Conditions for ler2/pip92 PCR were 40 cycles of 96 C for 30 sec, 69 C for 50 sec, and 72 C for 4 min. Conditions for RPS2 PCR were 22 cycles of 96 C for 30 sec, 60 C for 50 sec, and 72 C for 4 min. All PCRs used TAKARA *Taq* (Takara). PCR products were resolved on 2% (wt/vol) agarose gels stained with ethidium bromide and viewed using the FMBIO II fluoro-imager (Takara). To quantify the AUF1-binding mRNAs, standard curves were prepared. Template plasmids pSP64poly(A)-3UTR and pSP64poly(A)-22UTR, containing the entire 3′-UTRs from rat ABIN2 (clone 3) and ler2/pip92 (clone 22), were used for the *in vitro* transcription assay. Plasmid (0.5 μg) was linearized with *Eco*RI and transcribed in 100-μl reaction mixtures containing 50 U SP6 RNA polymerase (Takara), 40 mM Tris/HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 0.01% BSA, 40 U human placental RNase inhibitor, and 0.5 mM NTPs and incubated at 40 C for 60 min. Subsequently, 10 U DNaseI (Takara) were added to the mixture, which was then incubated for a further 10 min at 37 C to digest the template plasmid. Synthetic RNA was extracted and then quantified by A260. RT-PCR was performed as described above.

Immunofluorescence Microscopy

Cells were grown on coverslips and transfected with 1.5 μg pSG5-HA-AUF1p40, pSG5-HA-AUF1p45Cdel.(285) or pGAL4-AUF1p40 plasmids. Cells were washed with PBS and treated for 30 min with 4% paraformaldehyde, followed by 5 min in methanol. After washing with PBS, coverslips were incubated at 4 C in PBS containing 10% goat serum, and then with anti-HA tag rabbit-polyclonal antibody (MBL) or anti-GAL4DB mouse-monoclonal antibody (Santa Cruz Biotechnology) in 10% goat serum/PBS at room temperature for 1 h. After several washes with PBS, coverslips were incubated with antirabbit IgG-coupled fluorescein isothiocyanate (FITC) (MBL) or antimouse IgG-coupled FITC (Wako Chemical) in 10% goat serum/PBS at room temperature for 1 h. The samples were then washed with PBS several times, mounted, and examined using a fluorescence microscope (Olympus, Tokyo, Japan).

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