A + U-rich-element RNA-binding factor 1/heterogeneous nuclear ribonucleoprotein D gene expression is regulated by oestrogen in the rat uterus

Yukitomo ARAO*†[1], Atsumi KIKUCHI*†, Kazuhiro IKEDA*†, Satoshi NOMOTO*†, Hyogo HORIGUCHI*† and Fujio KAYAMA*†

*Department of Health Science, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Tochigi 329-0498, Japan, and †CREST, Japan Science and Technology Corporation, Kawaguchi, Saitama 332-0012, Japan

Oestrogen-mediated gene expression is regulated at both the transcriptional and post-transcriptional levels. The molecular mechanism of transcriptional regulation has been well characterized. On the other hand, there is little understanding of the mechanism of post-transcriptional regulation. To clarify the mechanism of oestrogen-mediated post-transcriptional regulation, we focused on A + U-rich-element RNA-binding factor 1/heterogeneous nuclear ribonucleoprotein D (AUF1/hnRNP D), which is known as a regulator of cytosolic mRNA degradation and nuclear pre-mRNA maturation. However, little is known about the expression levels and the regulation of AUF1/hnRNP D mRNA in tissues. We further investigated the expression levels of AUF1/hnRNP D isoform mRNAs to determine whether AUF1/hnRNP D gene expression is regulated by oestrogen in the ovariectomized adult female rat uterus. Uterine AUF1/hnRNP D mRNA was induced by a single subcutaneous injection (1 μg/kg) of 17β-oestradiol (E2), reaching a peak level within 6 h. Furthermore, we observed that the E2-induced AUF1/hnRNP D isoform mRNAs are p45 and p40 transcripts, and that E2-mediated induction is suppressed by the oestrogen receptor antagonist ICI 182,780. Finally, using the transcriptional inhibitor actinomycin D, we confirmed that the E2-mediated increase in AUF1/hnRNP D mRNA is caused by E2-dependent AUF1/hnRNP D mRNA stabilization.

Key words: isoform, mRNA stabilization, oestrogen receptor, post-transcriptional regulation.

INTRODUCTION

Post-transcriptional RNA modification is an important regulatory mechanism of gene expression. Several steps exist for the modification of RNA following transcription, such as the splicing of heterogeneous nuclear RNA to mature mRNA [1], adding a cap structure and poly(A)$^+$ tail [2], transportation to the cytosol [3] and cytosolic mRNA stabilization [4,5]. Many RNA-binding proteins involved in the regulation of RNA processing have been identified [1–3,6,7].

A + U-rich-element RNA-binding factor 1/heterogeneous nuclear ribonucleoprotein D (AUF1/hnRNP D) is one such RNA-binding protein, generating four protein isoforms (45, 42, 40 and 37 kDa) [8,9]. AUF1/hnRNP D p40 and p37 isoforms were first purified from cytoplasmic extracts of K562 human erythroleukaemia cells by monitoring protein-binding activity to the 3′-untranslated region (UTR) of c-Myc mRNA and the degradation activity of c-Myc mRNA in a cell-free mRNA-decay assay [10]. Many reports have demonstrated that AUF1/hnRNP D isoform proteins are components of some of the ribonucleoprotein complexes, associating with the 3′-UTR regulatory elements that control mRNA half-lives [11–14]. This suggests further that AUF1/hnRNP D acts as a regulator of mRNA half-life in the cytosol. Additionally, AUF1/hnRNP D was also purified from nuclear extract of HeLa cells as a protein binding to a pre-mRNA splice-site sequence ribo-(UUAG/G) (where / indicates the splice site) [15,16], suggesting that AUF1/hnRNP D also functions as a regulator of nuclear pre-mRNA maturation steps. From these observations, it appears that AUF1/hnRNP D is a multiple function factor in post-transcriptional regulation. Recently, several reports suggested that AUF1/hnRNP D protein expression is regulated in tissues [17,18]. However, little is known about the expression levels and regulation of AUF1/hnRNP D mRNA itself.

Oestrogen stimulates cell proliferation and differentiation in the female reproductive organs, with hormone activity mediated through gene expression. The molecular mechanism of oestrogen receptor (ER)-mediated transcriptional regulation of target genes has been well characterized [19,20]. On the other hand, it is also known that oestrogen controls the expression of several genes at the post-transcriptional level. More specifically, it is well known that transcripts of *Xenopus* and chicken genes, such as vitellogenin [21], albumin [22] and ovalbumin [23,24], are regulated through stabilization by oestrogen. Furthermore, there are several reports of oestrogen-mediated regulation of mRNA stabilization in mammalian cells. For instance, c-Myb mRNA accumulation in ER-positive tumour cell lines is regulated at the post-transcriptional level [25], and the stability of c-Myc mRNA is regulated by oestrogen in MCF7 cells [26]. Although the molecular mechanisms of oestrogen-mediated post-transcriptional regulation are poorly understood in mammalian cells, it is thought that this regulatory process is an important step in oestrogen-dependent gene expression.

The ovariectomized (OVX) adult female rat uterus has been used extensively as an experimental model to investigate the mechanisms by which oestrogen controls cellular proliferation in the endometrium. Several oestrogen-regulated genes have been identified, such as c-*fos* [27], c-*jun* [28], *zif268* [29], cyclin D [30] and cyclin A [30]. On the other hand, it has been reported that in other cells or tissues, these gene transcripts are regulated at the

Abbreviations used: AUF1/hnRNP D, A + U-rich-element RNA-binding factor 1/heterogeneous nuclear ribonucleoprotein D; UTR, untranslated region; ER, oestrogen receptor; E2, 17β-oestradiol; ActD, actinomycin D; CHX, cycloheximide; RPS2, ribosomal protein S2; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; RT-PCR, reverse transcriptase PCR; BW, body weight; OVX, ovariectomized.

[1] To whom correspondence should be addressed, at the Department of Health Science, Jichi Medical School (e-mail ya@jichi.ac.jp).

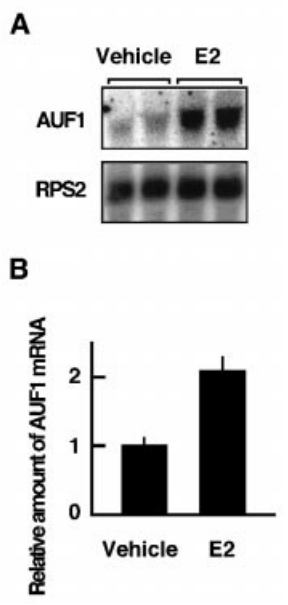

Figure 1 AUF1/hnRNP D mRNA is increased by oestrogen treatment in the OVX rat uterus

(**A**) Animals (6 rats/group) were injected with E2 (1.0 μg/kg) and, after 6 h, total RNA was isolated from OVX rat uteri. Samples were subjected to electrophoresis, blotted on to nylon membranes, and hybridized to probes specific for AUF1 p45 and RPS2. Autoradiograms from two examples of each treatment are shown. (**B**) mRNA levels were quantified by the quantification program of the Fuji BAS 1000 PhosphoImaging system. Results were normalized with respect to RPS2 hybridization in each lane. Means $\pm$ S.D. are shown, expressed as relative amounts of mRNA compared with the vehicle control.

post-transcriptional mRNA-degradation step [31–33]. To clarify the mechanisms of post-transcriptional oestrogen-mediated gene expression, we focused on the AUF1/hnRNP D gene. We therefore decided to investigate the expression levels of AUF1/hnRNP D isoform mRNAs in the OVX adult rat uterus, and sought to determine whether AUF1/hnRNP D gene expression was regulated by oestrogen. In this report, we demonstrate that uterine AUF1/hnRNP D mRNA was induced by a single subcutaneous injection of 17β-oestradiol (E2), reaching peak levels within 6 h. Furthermore, we observed that the E2-induced AUF1/hnRNP D mRNAs are p45 and p40 isoforms, and that the E2-mediated induction is suppressed by the ER antagonist ICI 182,780. Finally, using the transcriptional inhibitor actinomycin D (ActD), we confirmed that the E2-mediated increase of AUF1/hnRNP D mRNA is the result of E2-dependent mRNA stabilization. This report proposes that the oestrogen-mediated post-transcriptional regulation via AUF1/hnRNP D may contribute to the oestrogen-mediated gene expression in rat uterus.

EXPERIMENTAL

Animals and injection schedule for estimation of oestrogen responsiveness

Mature female rats (7 weeks old, 120–140 g), obtained from SRC Japan (Shizuoka, Japan), were ovariectomized at least 10 days before use in these studies. Animals received a single subcutaneous injection of E2 (Sigma, St Louis, MO, U.S.A.) at 1.0 μg/kg of body weight (BW) in propylene glycol (Wako Chemical Co., Osaka, Japan). Some animals were pre-treated with the synthetic ER antagonist ICI 182,780 (Nakarai Tesque, Kyoto, Japan) at 1.0 mg/kg of BW, with the transcription inhibitor ActD (Sigma) at 1.5 mg/kg of BW, or with the translational inhibitor cycloheximide (CHX; Sigma) at 1.0 mg/kg of BW in propylene glycol, 2 or 5 h prior to E2 treatment. Control animals were treated with 0.1 ml of propylene glycol (vehicle). All animal experiments were approved by the Laboratory of Experimental Medicine Jichi Medical School animal use and care committee.

Northern-blot analysis

Total RNA was prepared from OVX rat uteri using the guanidine thiocyanate/phenol/chloroform technique as described previously [23]. Samples were separated in 1.2 % formaldehyde denaturing agarose gels and RNAs were transferred to a Gene Screen nylon membrane (NEN). Blots were hybridized with α-^{32}P-labelled probes in ULTRAhyb (Ambion) at 42 °C for 16 h. After hybridization, the blots were washed with 1 × SSC/0.1 % SDS (where SSC is 0.15 M NaCl/0.015 M sodium citrate) at 65 °C for 30 min and then with 0.1 × SSC/1 % SDS at 65 °C for 30 min. The blots were visualized using the Fuji BAS 1000 PhosphoImaging analyser (Fuji Film, Tokyo, Japan).

α-^{32}P-Labelled DNA probes were prepared using the random-primer labelling method. The probes used were 0.9–1.2 kb fragments of rat AUF1 isoform cDNAs, encoding the full-length AUF1 proteins, and a 0.6 kb fragment of rat ribosomal protein S2 (RPS2/CHO B) cDNA as a constitutively expressed gene control [28]. The amount of sample to be loaded into each lane was decided by checking the amount of RPS2 mRNA in the samples (total RNA) by reverse transcriptase PCR (RT-PCR). Rat AUF1 isoform cDNAs were screened from a rat kidney cDNA library (p45, p42, p40 and p37; GenBank accession nos. AB046615, AB046616, AB046617 and AB046618 respectively). Rat RPS2 cDNA was amplified by PCR from a rat uterus cDNA library using specific primer sets (nucleotides 58–79 and 641–667; GenBank accession no. U92700).

RT-PCR

RT-PCR was performed to measure the relative AUF1 isoform mRNA expression levels. Total RNA (2 μg) was reverse-transcribed in 35 μl reaction mixes, containing 1 nmol of oligo-dT (18-mer), 400 units of Superscript II (Gibco BRL), 50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 units of human placental RNase inhibitor and 1.1 mM dNTPs, and incubated at 42 °C for 60 min. PCR was performed using specific primer sets for rat AUF1 (set A, nucleotides 250–271 and 1085–1111; set B, nucleotides 250–271 and 842–863; set C, nucleotides 413–438 and 1085–1111; these nucleotide positions are derived from GenBank accession no. AB046615) and for rat RPS2 (nucleotides 58–79 and 641–667; GenBank accession no. U92700). The PCR of AUF1 was run for 27 cycles (96 °C for 30 s, 66 °C for 50 s and 72 °C for 4 min) and the PCR of rat RPS2 was run for 15 cycles (96 °C for 30 s, 60 °C for 50 s and 72 °C for 4 min) using Takara *Taq* (Takara, Tokyo, Japan). The amounts of template cDNAs used in the AUF1 PCR samples were decided by the results of the RPS2 PCR. Aliquots (10 μl) of PCR products were resolved on 2 % (w/v) agarose gels stained with ethidium bromide and viewed using the FMBIO II fluoroimager (Takara).

Template plasmid pSP64poly(A)-rAUF1p45, containing the open reading frame of rat AUF1p45 cDNA, was used for *in vitro*-transcription assay. The plasmid (0.5 μg) was linearized with *Eco*RI, transcribed in 20 μl reaction mixes, containing 50 units of SP6 RNA polymerase (Takara), 40 mM Tris/HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 0.01 % BSA, 20 units of human placental RNase inhibitor and 0.5 mM NTPs, and incubated at 40 °C for 60 min. After that, 10

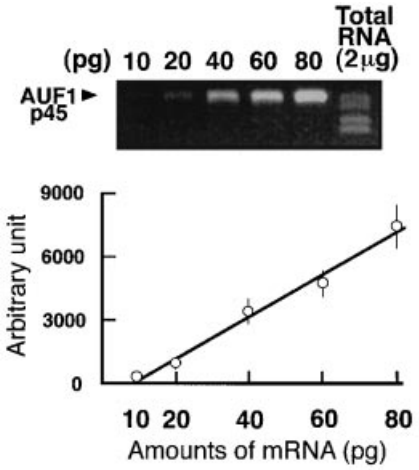

Figure 2 Standard curve from an RT-PCR assay of the AUF1/hnRNP D isoform

In vitro-transcribed AUF1 p45 mRNAs (10–80 pg) and 2 μg of total RNA from OVX rat uterus were reverse-transcribed and PCR was performed using the primer set A as shown in Figure 3(A). A typical result is shown in the upper panel. Amplifying fragments were quantified by the quantification program of the FMBIO II fluoroimaging system. The values in the lower panel are the means $\pm$ S.D. ($n = 3$), expressed as arbitrary units.

units of DNaseI (Takara) was added to the mixture and incubated for 10 min at 37 °C. Synthetic RNA was extracted and used for RT-PCR as described above.

RESULTS

The expression of AUF1/hnRNP D mRNA is induced by oestrogen in the OVX rat uterus

We first examined the effect of oestrogen on the AUF1/hnRNP D mRNA expression level in the OVX rat uterus. Total RNA was extracted from an OVX adult rat uterus 6 h after injection with E2 (1.0 μg/kg of BW) or vehicle. Northern blotting was then performed using radiolabelled cDNA probes for rat AUF1/hnRNP D and RPS2, the latter being a non-regulated and constitutively expressed gene, to quantify the relative amounts of AUF1/hnRNP D mRNA. In order to detect the expression level of total AUF1/hnRNP D mRNA in the OVX rat uterus, we used fragments of the full-length AUF1/hnRNP D isoform cDNAs as probes. Except for elements A and B, the cDNA sequences of the AUF1/hnRNP D isoforms are the same (see Figure 3A, below). No significant differences were observed between the hybridization results for each of the probes (results not shown), and so we concluded that Northern blots probed with an AUF1/hnRNP D p45 cDNA corresponded to total AUF1/hnRNP D mRNA. As shown in Figure 1, a single dose of E2 stimulated a 2-fold increase in total AUF1/hnRNP D mRNA in the OVX adult rat uterus.

Oestrogen modifies the expression levels of AUF1/hnRNP D isoforms

Next, we performed RT-PCR to examine the relative levels of each of the AUF1/hnRNP D isoform mRNAs. Specific primer sets (A, B and C) were synthesized to allow simultaneous

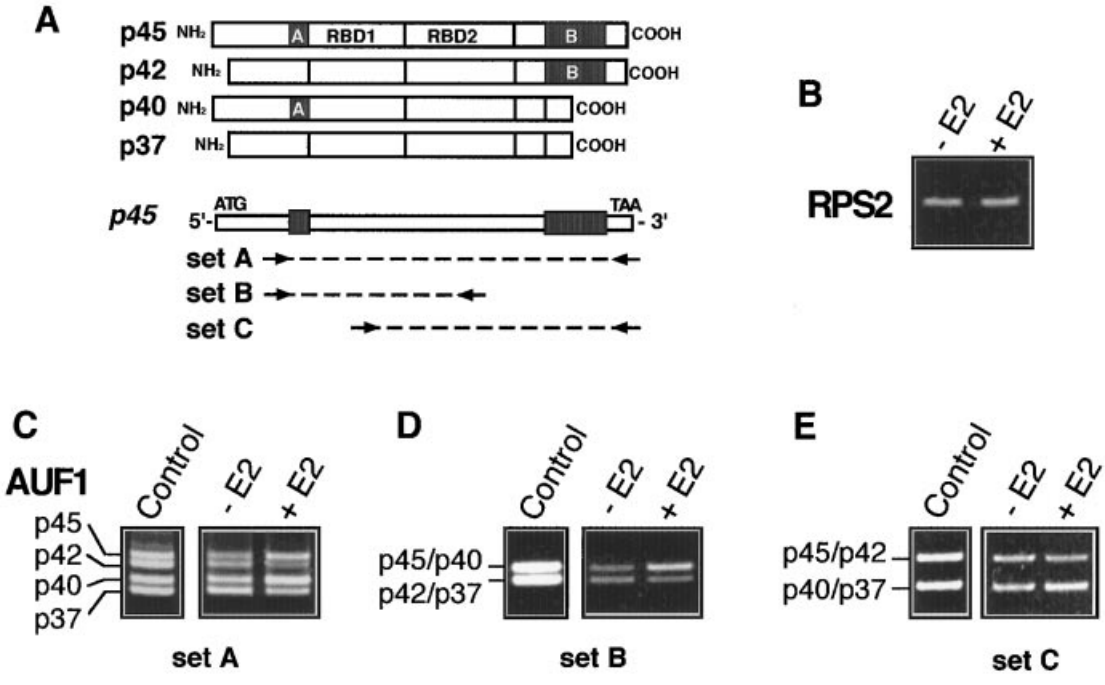

Figure 3 Expression of specific isoforms (p45/p40) of AUF1/hnRNP D mRNA are induced by oestrogen treatment in the OVX rat uterus

(**A**) The structures of the AUF1 isoform proteins (p45, p42, p40 and p37) and p45 cDNA are shown. Primer sets A, B and C for PCR are shown by arrows in the p45 cDNA and the PCR transcripts are indicated by broken lines. Hatched boxes (A and B) indicate isoform-specific inserted elements. RBD, RNA-binding domain. (**B**) Total RNAs from OVX rat uterus, with (+E2) or without (−E2) E2, were reverse-transcribed and PCR amplified using the primer set for RPS2. (**C**–**E**) PCR was performed using primer set A (**C**), B (**D**) or C (**E**). Equimolar amounts of AUF1 p45, p42, p40 and p37 cDNAs were mixed and PCR was performed using each primer set (Control).

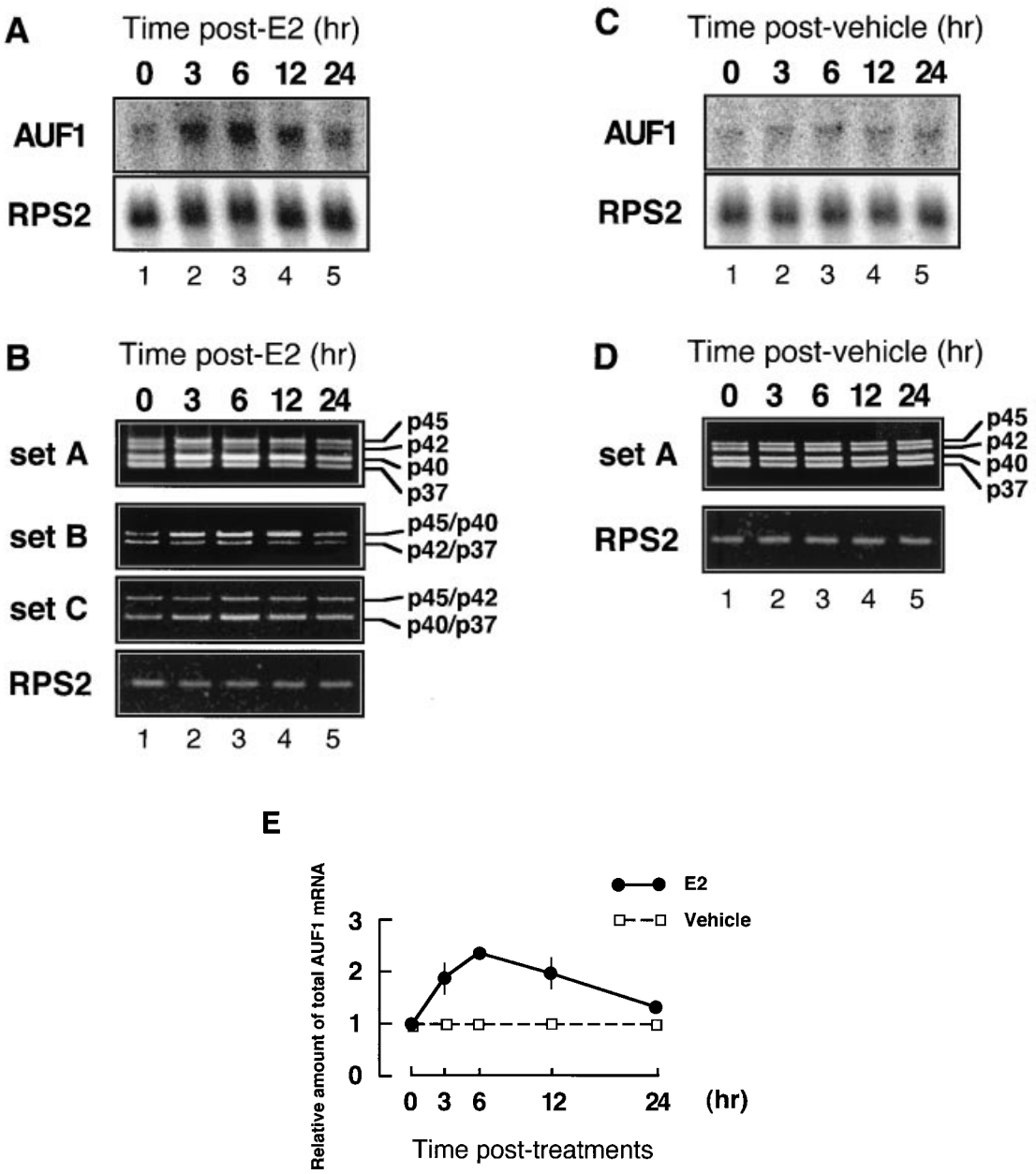

Figure 4 Time course for accumulation of AUF1/hnRNP D mRNA after treatment with oestrogen

(**A**) Animals (6 rats/group) were treated with 1.0 μg/kg E2 and uterine total RNA was prepared at the times indicated. Northern-blot analysis was performed as described in Figure 1. Typical autoradiograms are shown. (**B**) The primer sets for RT-PCR were as described in Figure 3 and typical results are shown. (**C**) Animals (3 rats/group) were treated with 100 μl of solvent (vehicle) and uterine total RNA was prepared at the times indicated. Northern-blot analysis was performed and typical autoradiograms are shown. (**D**) RT-PCR was performed using the primer set A and RPS2. Typical results are shown. (**E**) Total AUF1 mRNA levels were quantified as described in Figure 1. Results were normalized to RPS2 hybridization in each lane. The values are the means $\pm$ S.D. and expressed as relative amounts compared with time zero; rats were administered solvent (□; vehicle) or 1.0 μg/kg E2 (●).

amplification of each fragment. The PCR products were observed by ethidium bromide staining following agarose-gel electrophoresis. At first, to confirm that this assay was quantitative, we made a standard curve using *in vitro*-transcribed AUF1/hnRNP D p45 mRNA as an RT-PCR template. As shown in Figure 2, quantitative results were obtained from 10–80 pg of mRNA in this assay. In each experiment, equimolar amounts of AUF1/hnRNP D isoform cDNA plasmid template were used to confirm that simultaneous amplification had occurred (Figures 3C–3E; Control). RNAs isolated for Northern-blot analysis (Figure 1) were reverse-transcribed and amplified by PCR using each primer set. The results using primer set A are shown in Figure 3(C). All four AUF1/hnRNP D isoform mRNAs were expressed in equal amounts in untreated OVX adult rat uterus ($-$E2). Alternatively, in the E2-treated OVX adult rat uterus, the relative levels of p45 and p40 transcripts were increased ($+$E2). To confirm the increase of p45 and p40 transcripts, we used the other primer sets to detect the p45/p40 and p42/p37 transcripts (set B) and to

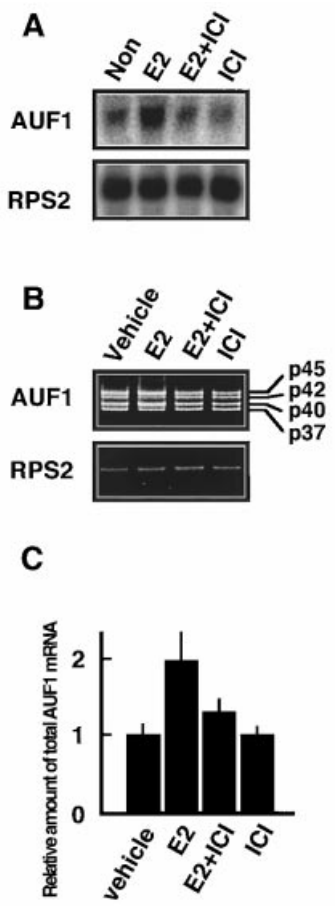

Figure 5 Oestrogen-mediated mRNA accumulation is prevented by the ER antagonist ICI 182,780

(**A**) Uterine total RNA was prepared from animals (6 rats/group) treated with 1.0 mg/kg ICI 182,780 (ICI) with or without E2 (1.0 μg/kg). ICI 182,780 was given 2 h before E2 administration. Control animals were treated with 100 μl of solvent vehicle and uterine total RNA was prepared (Non). Northern-blot analysis was performed as described in Figure 1 and typical autoradiograms are shown. (**B**) RT-PCR was performed using primer set A as described in Figure 3 and typical results are shown. (**C**) Total AUF1 mRNA levels were quantified as described in Figure 1. Results were normalized to RPS2 hybridization in each lane. The values are the means $\pm$ S.D. and expressed as relative amounts compared with the vehicle.

detect the p45/p42 and p40/p37 transcripts (set C; Figure 3A). Figure 3(D) shows the result of using primer set B. In the vehicle-treated uterus, the amounts of p45/p40 and p42/p37 transcripts were the same ($-$E2). As expected, in the E2-treated uterus, the level of p45/p40 transcripts was 2.1-fold higher than that of the p42/p37 transcripts. Figure 3(E) shows the result of using primer set C. When the level of p40/p37 transcripts in the E2-treated uterus was compared with the level of p45/p42 transcripts, the former was 1.3-fold higher. These results suggest that the increase in AUF1/hnRNP D mRNA, as observed by Northern blotting, was mainly the result of an increase in p40 and p45 mRNA isoforms, with the transcript of p40 being slightly more abundant than that of p45. To confirm whether the PCR-amplified fragments from each primer set corresponded with the rat AUF1/hnRNP D mRNA isoforms, we subcloned and sequenced each fragment and determined that all fragments coincided with the rat AUF1/hnRNP D sequence (results not shown).

The effect of oestrogen on steady-state levels of AUF1/hnRNP D mRNA

To observe the time course of AUF1/hnRNP D isoform gene expression, OVX adult rats were injected with E2 (1.0 μg/kg) at time zero, and total RNA was isolated at regular intervals from 3 to 24 h. Relative amounts of total AUF1/hnRNP D mRNA isoforms were quantified by Northern blotting using the probe described in Figure 1 and compared with RPS2 transcript expression used as an internal control. As shown in Figures 4(A) and 4(E), the addition of E2 stimulated a 1.9-fold increase in total AUF1/hnRNP D mRNA following a 3 h exposure, and reached peak levels (2.4-fold) at 6 h, after which a decrease in expression occurred by 24 h. Figure 4(B) shows the changes in AUF1/hnRNP D mRNA isoform levels using each primer set described in Figure 3. These results suggest that the relative amounts of p45 and p40 transcripts increased within 3 h of E2 treatment. Additionally, injection of solvent alone (vehicle) had no effect on the expression levels of AUF1/hnRNP D mRNA isoforms (Figures 4C–4E).

Increase of specific AUF1/hnRNP D mRNA isoforms (p45/p40) by E2 is inhibited by the ER antagonist ICI 182,780

To determine whether the accumulation of specific isoforms of AUF1/hnRNP D mRNA is mediated by the ER, we used the ER antagonist ICI 182,780, reported to be devoid of agonist activity [34]. ICI 182,780 (1.0 mg/kg of BW) was injected subcutaneously 2 h prior to E2 treatment. As shown in Figures 5(A) and 5(C), ICI 182,780 inhibited the E2-mediated induction of AUF1/hnRNP D mRNA, but retained basal expression levels. Furthermore, we confirmed by RT-PCR that ICI 182,780 inhibited the E2-mediated induction of p45 and p40 isoform transcripts (Figure 5B). These results suggest that the AUF1/hnRNP D gene is constitutively expressed in the OVX adult rat uterus and that E2 enhances the accumulation of p45 and p40 transcripts via ER-mediated signalling.

Oestrogen stabilizes AUF1/hnRNP D mRNA

To determine whether the E2-induced accumulation of AUF1/hnRNP D mRNA occurs by mRNA stabilization, the effect of E2 on AUF1/hnRNP D mRNA accumulation following transcription inhibition was estimated. When the rats were treated with the transcription inhibitor, ActD, only, the level of total AUF1/hnRNP D mRNA declined by about 50 % between 5 and 10 h following ActD treatment ($-$E2). On the other hand, when E2 was injected 5 h after ActD treatment, the decline in total AUF1/hnRNP D mRNA did not occur (compare ActD 5 h and $+$E2 5 h in Figures 6A and 6C). Furthermore, we again examined the relative levels of AUF1/hnRNP D mRNA isoforms following ActD treatment. As shown in Figure 6(B), p45 and p40 isoforms were accumulated by E2.

Several reports have shown that treatment with the translation inhibitor CHX increases mRNA stability [4,35]. To confirm further the stabilization of p45 and p40 mRNAs, we analysed the effect of CHX on the accumulation of AUF1/hnRNP D mRNA isoforms. As shown in Figure 6(D), when the rats were treated with CHX (1.0 mg/kg) and ActD (1.5 mg/kg) for 5 h, the relative levels of p45 and p40 transcripts increased, analogous with E2 treatment in the uterus. This result suggests that the stabilization of p45 and p40 transcripts is related to translational regulation. Therefore, these results imply that E2's effects on the translational machinery stabilize the AUF1/hnRNP D p40 and p45 mRNAs.

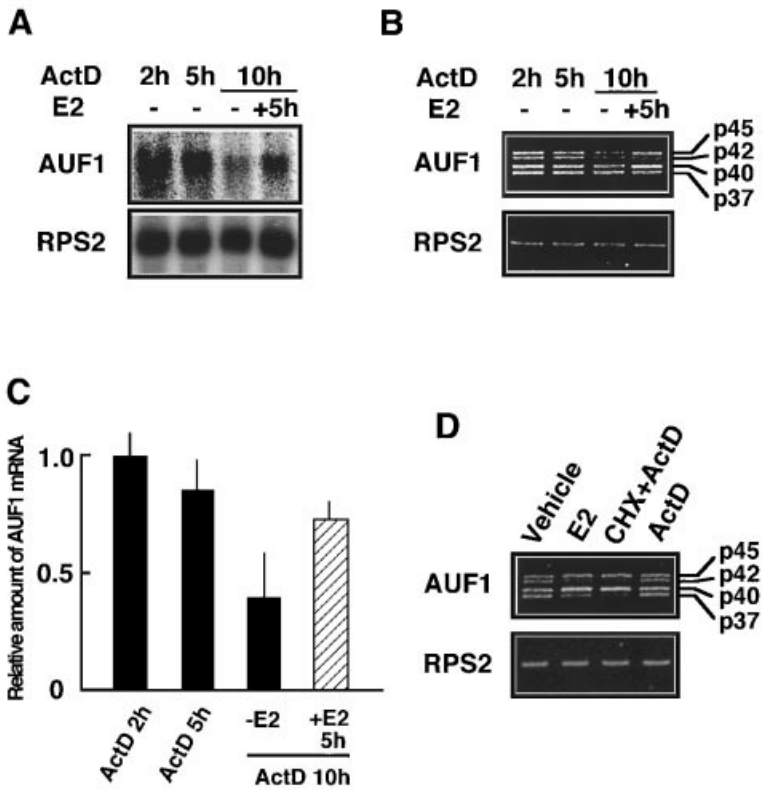

Figure 6 Oestrogen stabilizes AUF1/hnRNP D p45 and p40 mRNAs

(**A**) Uterine total RNA was prepared from animals (4 rats/group) treated with ActD (1.5 mg/kg) with or without E2 (1.0 μg/kg) at the times indicated. E2 was given 5 h after ActD administration. Northern-blot analysis was performed as described in Figure 1. Typical autoradiograms are shown. (**B**) RT-PCR was performed using primer set A as described in Figure 3 and typical results are shown. (**C**) Results were normalized with respect to RPS2 hybridization in each lane. The values are the means $\pm$ S.D. and expressed as relative amounts compared with 2 h after ActD treatment (ActD 2 h). (**D**) Uterine total RNA was prepared from animals (3 rats/group) treated with ActD (1.5 mg/kg) with or without CHX (1.0 mg/kg) for 5 h. RT-PCR was performed using primer set A and typical results are shown.

DISCUSSION

Oestrogen initiates a sequence of metabolic and morphological changes in the uterus. In adult OVX rats, the major effect of stimulation with oestrogen is the onset of DNA synthesis and cell proliferation in the endometrium, preceded by specific changes in cell-cycle-related gene expression. Previously, several oestrogen-regulated genes have been identified, such as c-*fos* [27], c-*jun* [28], *zif268* [29] and the cell-cycle-regulating factors cyclin D [30] and cyclin A [30]. It has been recognized that these genes are regulated by oestrogen-mediated transcription. On the other hand, it is also known that the transcripts of these genes are regulated at the mRNA-degradation step in other cells or tissues [31–33]. Thus these observations imply that the oestrogen-dependent regulation of mRNA degradation might be a major post-transcriptional regulatory mechanism in the rat uterus. However, little is known about oestrogen-mediated mRNA turnover in the rat uterus.

In order to clarify oestrogen-mediated post-transcriptional regulation events in the rat uterus, we focused on AUF1/hnRNP D, which is an RNA-binding protein that participates in many post-transcriptional regulation events [10–14]. We found that oestrogen stimulates the accumulation of AUF1/hnRNP D mRNA in OVX adult rat uterus and that it appears to be regulated by post-transcriptional mRNA stabilization. These results suggest that oestrogen-mediated regulation of mRNA turnover via AUF1/hnRNP D may contribute to oestrogen-mediated gene expression in the rat uterus.

The results from the experiments using the ER antagonist ICI 182,780 suggest that AUF1/hnRNP D gene expression is partially regulated by ER-mediated signalling (Figure 5). Recent reports have suggested that oestrogen activates the protein kinase-mediated signalling pathways through non-genomic action of the ER [36,37]. This oestrogen-dependent kinase activation is also blocked by the ER antagonist ICI 182,780 [36]. Simoncini et al. [36] showed that stimulation with oestrogen increases the association of phosphoinositide 3-kinase (PI 3-kinase) with the ERα, leading to the activation of protein kinase B (Akt/PKB) and endothelial nitric oxide synthetase in human vascular endothelial cells. Akt/PKB is a downstream effector of PI 3-kinase and regulates gene transcription by directly or indirectly modifying phosphorylation of transcription factors [38,39]. Recently, several groups have suggested that mRNAs are stabilized upon activation of the PI 3-kinase signalling pathway [40,41]. For instance, Sheng et al. [40] showed that K-Ras-inducible cyclo-oxygenase 2 expression involves both transcription and mRNA-stabilization regulation in rat intestinal epithelial cells, and that K-Ras-mediated activation of the PI 3-kinase/Akt/PKB pathway is important for the stabilization of cyclo-oxygenase 2 mRNA. Furthermore, Dufourny et al. [41] showed that in MCF7 cells the insulin-like growth factor-I-mediated activation of the PI 3-kinase pathway stabilizes the

cyclin D1 mRNA. These observations suggest that the oestrogen-mediated stabilization of AUF1/hnRNP D mRNA in the rat uterus may be controlled by protein kinase signalling, such as through the PI 3-kinase-mediated pathway.

Using specific primer sets, RT-PCR analysis revealed that the specific AUF1/hnRNP D mRNA isoforms p45 and p40 were increased after a single subcutaneous injection of E2. The structural differences between the p45/p40 isoforms and the p42/p37 isoforms existed with or without insert A, which is derived from an alternatively spliced exon (Figure 3A). When transcription was blocked by ActD, p45 and p40 mRNAs accumulated following E2 treatment (Figure 6B). It is unlikely that oestrogen induces the accumulation of p45/p40 isoform transcripts through alternative splicing. Several reports have shown that treatment with the translation inhibitor CHX increases mRNA stability [4,35]. Thus we examined the effect of CHX on the stability of AUF1/hnRNP D isoform transcripts. We found that in rats treated with CHX the levels of uterine AUF1/hnRNP D p45/p40 transcripts were induced in a similar manner to E2 treatment (Figure 6D). These results suggest that the transcripts of p45 and p40 are more stable than the p42 and p37 transcripts in the rat uterus. Previously, Wilson et al. [42] investigated the regulation of AUF1/hnRNP D gene expression in K562 human erythroleukaemia cells. Their work indicated that the levels of AUF1/hnRNP D mRNA 3′-UTR splice variants were based on the action of regulatory pathways during mRNA degradation. In our case the results suggested that the alternatively spliced element in the open reading frame of AUF1/hnRNP D mRNA might contribute to the regulation of its stability. From these results, it is likely that the expression of AUF1/hnRNP D is regulated during the mRNA-degradation step through the alternatively spliced elements in the AUF1/hnRNP D mRNA.

Our results provide evidence that AUF1/hnRNP D gene expression is regulated by oestrogen in the rat uterus. Interestingly, our results suggest that AUF1/hnRNP D mRNA itself is regulated at the mRNA-degradation step by oestrogen. It is possible that the AUF1/hnRNP D protein, in turn, controls the oestrogen-mediated stabilization of AUF1/hnRNP D mRNA; however, this possibility calls for further investigation into AUF1/hnRNP D protein expression in the rat uterus and binding activity to the AUF1/hnRNP D mRNA.

This work was supported by a grant-in-aid for the Encouragement of Young Scientists (A) from the Japan Society for the Promotion of Science (to Y.A.), and the Jichi Medical School Young Investigator Award (to Y.A.).

REFERENCES

1 Valcarcel, J. and Green, M. R. (1996) The SR protein family: pleiotropic functions in pre-mRNA splicing. Trends Biochem. Sci. **21**, 296–301

2 Shatkin, A. J. and Manley, J. L. (2000) The ends of the affair: capping and polyadenylation. Nat. Struct. Biol. **7**, 838–842

3 Siomi, M. C. (2000) The molecular mechanisms of messenger RNA nuclear export. Cell Struct. Funct. **25**, 227–235

4 Williams, D. L., Sensel, M., McTigue, M. and Binder, R. (1993) Hormonal and developmental regulation of mRNA turnover. In Control of Messenger RNA Stability (Belasco, J. and Brawerman, G., eds.), pp. 161–197, Academic Press, San Diego

5 Chan, C.-Y. A. and Shyu, A. B. (1995) AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem. Sci. **20**, 465–470

6 Loflin, P., Chan, C.-Y. A. and Shyu, A. B. (1999) Unraveling a cytoplasmic role for hnRNP D in the *in vivo* mRNA destabilization directed by the AU-rich element. Genes Dev. **13**, 1884–1897

7 Fan, X. C. and Steitz, J. A. (1998) Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the *in vivo* stability of ARE-containing mRNAs. EMBO J. **17**, 3448–3460

8 Dempsey, L. A., Li, M., Depace, A., Bray-Ward, P. and Maizels, N. (1998) The human HNRPD locus maps to 4q21 and encodes a highly conserved protein. Genomics **49**, 378–384

9 Wagner, B. J., DeMaria, C. T., Sun, Y., Wilson, G. M. and Brewer, G. (1998) Structure and genomic organization of the human AUF1 gene: alternative pre-mRNA splicing generates four protein isoforms. Genomics **48**, 195–202

10 Brewer, G. (1991) An A + U-rich element RNA-binding factor regulates c-myc mRNA stability *in vitro*. Mol. Cell. Biol. **11**, 2460–2466

11 Kiledjian, M., DeMaria, C., Brewer, G. and Novick, K. (1997) Identification of AUF1 (heterogeneous nuclear ribonucleoprotein D) as a component of the β-globin mRNA stability complexes. Mol. Cell. Biol. **17**, 4870–4876

12 Pende, A., Tremmel, K. D., DeMaria, C. T., Blaxall, B. C., Minobe, W. A., Sherman, J. A., Bisognano, J. D., Bristow, M. R., Brewer, G. and Port, J. D. (1996) Regulation of the mRNA-binding protein AUF1 by activation of the β-adrenergic receptor signal transduction pathway. J. Biol. Chem. **271**, 8493–8501

13 Sirenko, O. I., Lofquist, A. K., DeMaria, C. T., Morris, J. S., Brewer, G. and Haskill, J. S. (1997) Adhesion-dependent regulation of an A + U-rich element-binding activity associated with AUF1. Mol. Cell. Biol. **17**, 3894–3906

14 Sela-Brown, A., Silver, J., Brewer, G. and Naveh-Many, T. (2000) Identification of AUF1 as a parathyroid hormone mRNA 3′-untranslated region-binding protein that determines parathyroid hormone mRNA stability. J. Biol. Chem. **275**, 7424–7429

15 Ishikawa, F., Matunis, M. J., Dreyfuss, G. and Cech, T. R. (1993) Nuclear proteins that bind the pre-mRNA 3′ splice site sequence r(UUAG/G) and the human telomeric DNA sequence d(TTAGGG)n. Mol. Cell. Biol. **13**, 4301–4310

16 Kajita, Y., Nakayama, J.-I., Aizawa, M. and Ishikawa, F. (1995) The UUAG-specific RNA binding protein, heterogeneous nuclear ribonucleoprotein D0. Common modular structure and binding properties of the 2xRBD-Gly family. J. Biol. Chem. **270**, 22167–22175

17 Sheflin, L. G. and Spaulding, S. W. (2000) Testosterone and dihydrotestosterone regulate AUF1 isoforms in a tissue-specific fashion in the mouse. Am. J. Physiol. Endocrinol. Metab. **278**, 50–57

18 Blaxall, B. C., Dwyer-Nield, L. D., Bauer, A. K., Bohlmeyer, T. J., Malkinson, A. M. and Port, J. D. (2000) Differential expression and localization of the mRNA binding proteins, AU-rich element mRNA binding protein (AUF1) and Hu antigen R (HuR), in neoplastic lung tissue. Mol. Carcinogen **28**, 76–83

19 Glass, C. K. and Rosenfeld, M. G. (2000) The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev. **14**, 121–141

20 Beato, M., Herrlich, P. and Schutz, G. (1995) Steroid hormone receptors: many actors in search of a plot. Cell **83**, 851–857

21 Brock, M. I. and Shapiro, D. J. (1983) Estrogen stabilizes vitellogenin mRNA against cytoplasmic degradation. Cell **34**, 207–214

22 Pastori, R. L., Moskaitis, J. E., Buzek, S. W. and Schoenberg, D. R. (1991) Coordinate estrogen-regulated instability of serum protein-coding messenger RNAs in *Xenopus laevis*. Mol. Endocrinol. **5**, 461–468

23 Arao, Y., Yamamoto, E., Miyatake, N., Ninomiya, Y., Umehara, T., Kawashima, H., Masushige, S., Hasegawa, T. and Kato, S. (1996) A synthetic oestrogen antagonist inhibits oestrogen-induced transcriptional, but not post-transcriptional regulation of gene expression. Biochem. J. **313**, 269–274

24 Palmiter, R. D. and Carey, N. H. (1974) Rapid inactivation of ovalbumin messenger ribonucleic acid after acute withdrawal of estrogen. Proc. Natl. Acad. Sci. U.S.A. **71**, 2357–2361

25 Gudas, J. M., Klein, R. C., Oka, M. and Cowan, K. H. (1995) Posttranscriptional regulation of the c-myb proto-oncogene in estrogen receptor-positive breast cancer cells. Clin. Cancer Res. **1**, 235–243

26 Santos, G. F., Scott, G. K., Lee, W. M., Liu, E. and Benz, C. (1988) Estrogen-induced post-transcriptional modulation of c-myc proto-oncogene expression in human breast cancer cells. J. Biol. Chem. **263**, 9565–9568

27 Weisz, A. and Bresciani, F. (1988) Estrogen induces expression of c-fos and c-myc protooncogenes in rat uterus. Mol. Endocrinol. **2**, 816–824

28 Bigsby, R. M. and Li, A. (1994) Differentially regulated immediate early genes in the rat uterus. Endocrinology **134**, 1820–1826

29 Suva, L. J., Harm, S. C., Gardner, R. M. and Thiede, M. A. (1991) *In vivo* regulation of Zif268 messenger RNA expression by 17 beta-estradiol in the rat uterus. Mol. Endocrinol. **5**, 829–835

30 Altucci, L., Addeo, R., Cicatiello, L., Germano, D., Pacilio, C., Battista, T., Cancemi, M., Petrizzi, V. B., Bresciani, F. and Weisz, A. (1997) Estrogen induces early and timed activation of cyclin-dependent kinases 4, 5, and 6 and increases cyclin messenger ribonucleic acid expression in rat uterus. Endocrinology **138**, 978–984

31 Chan, C.-Y. A. and Shyu, A. B. (1994) Selective degradation of early-response-gene mRNAs: functional analyses of sequence features of the AU-rich elements. Mol. Cell. Biol. **14**, 8471–8482

32 Lin, S., Wang, W., Wilson, G. M., Yang, X., Brewer, G., Holbrook, N. J. and Gorospe, M. (2000) Down-regulation of cyclin D1 expression by prostaglandin A(2) is mediated by enhanced cyclin D1 mRNA turnover. Mol. Cell. Biol. **20**, 7903–7913

33 Maity, A., McKenna, W. G. and Muschel, R. J. (1997) Cyclin A message stability varies with the cell cycle. Cell Growth Differ. **8**, 311–318
34 Hyder, S. M., Chiappetta, C., Murthy, L. and Stancel, G. M. (1997) Selective inhibition of estrogen-regulated gene expression *in vivo* by the pure antiestrogen ICI 182,780. Cancer Res. **57**, 2547–2549
35 Ragheb, J. A., Deen, M. and Schwartz, R. H. (1999) The destabilization of IL-2 mRNA by a premature stop codon and its differential stabilization by trans-acting inhibitors of protein synthesis do not support a role for active translation in mRNA stability. J. Immunol. **163**, 3321–3330
36 Simoncini, T., Hafezi-Moghadam, A., Brazil, D. P., Ley, K., Chin, W. W. and Liao, J. K. (2000) Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature (London) **407**, 538–541
37 Kousteni, S., Bellido, T., Plotkin, L. I., O'Brien, C. A., Bodenner, D. L., Han, L., Han, K., DiGregorio, G. B., Katzenellenbogen, J. A., Katzenellenbogen, B. S. et al. (2001) Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell **104**, 719–730
38 Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R. and Tsichlis, P. N. (1995) The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell **81**, 727–736
39 Kane, L. P., Shapiro, V. S., Stokoe, D. and Weiss, A. (1999) Induction of NF-kappaB by the Akt/PKB kinase. Curr. Biol. **9**, 601–604
40 Sheng, H., Shao, J. and Dubois, R. N. (2001) K-Ras-mediated increase in cyclooxygenase 2 mRNA stability involves activation of the protein kinase B1. Cancer Res. **61**, 2670–2675
41 Dufourny, B., van Teeffelen, H. A. A. M., Hamelers, I. H. L., Sussenbach, J. S. and Steenbergh, P. H. (2000) Stabilization of cyclin D1 mRNA via the phosphatidylinositol 3-kinase pathway in MCF-7 human breast cancer cells. J. Endocrinol. **166**, 329–338
42 Wilson, G. M., Sun, Y., Sellers, J., Lu, H., Penkar, N., Dillard, G. and Brewer, G. (1999) Regulation of AUF1 expression via conserved alternatively spliced elements in the 3′ untranslated region. Mol. Cell. Biol. **19**, 4056–4064

Received 8 July 2001/8 October 2001; accepted 29 October 2001