Estradiol up-regulates estrogen receptor messenger ribonucleic acid in endometrial carcinoma (Ishikawa) cells by stabilizing the message

J A Robertson¹, Y Farnell¹, L S Lindahl¹ and N H Ing^{1,2}

¹Department of Animal Science, Texas A&M University, College Station, Texas 77843–2471, USA

²Department of Veterinary Anatomy and Public Health, Center for Environmental and Rural Health, and Center for Animal Biotechnology and Genomics, Institute of Biosciences and Technology, Texas A&M University System Health Sciences Center, Texas 77843-4458, USA

(Requests for offprints should be addressed to N H Ing, Department of Animal Science, Texas A&M University, MS 2471, College Station, TX 77843–2471, USA; Email: ning@cvm.tamu.edu)

Abstract

Estrogens up-regulate expression of the estrogen receptor alpha (ER) gene in most mammalian tissues studied. Using the ovariectomized ewe as a model, we determined that estradiol (E₂) acted post-transcriptionally to increase endometrial ER mRNA concentrations by enhancing the stability of the message. The purpose of this study was to determine whether a similar E₂ effect occurs in Ishikawa cells, a well-differentiated human endometrial adenocarcinoma cell line. The presence and function of ER protein in Ishikawa cells was demonstrated by transactivation of a transfected plasmid (ERE₂tkCAT) in response to 10^{–9} M E₂, resulting in a 550% increase in reporter gene RNA. Ishikawa cells also responded to E₂ by up-regulating their ER mRNA concentration an average of 100% between 7 and 24 h of treatment. The effect of E₂ on ER mRNA stability was measured after blocking transcription with actinomycin D to find that the half-life increased from 6 to 10 h in control and E₂-treated cells respectively. These results are consistent with cell-free studies which showed significant enhancement of the half-life of radiolabeled ER 3' untranslated region (3'UTR) RNA in extracts from E₂-treated cells versus those from control cells. Thus, Ishikawa cells provide a relevant model system for the study of E₂-regulated endometrial gene expression.

Journal of Molecular Endocrinology (2002) **29**, 125–135

Introduction

During times of natural estrogen dominance, both estrogen receptor alpha (ER) mRNA and protein levels increase in endometrium in species ranging from rodents to humans (Lessey *et al.* 1988, Brenner *et al.* 1991, Bergman *et al.* 1992, Geisert *et al.* 1993, Ott *et al.* 1993, Zheng *et al.* 1996). Similarly, physiological doses of estradiol (E₂) up-regulate endometrial ER gene expression in ovariectomized animals (Bergman *et al.* 1992, Okulicz *et al.* 1990, Ing *et al.* 1996). Our previous work showed that E₂ up-regulated ER mRNA levels 400% in 24 h by a post-transcriptional mechanism: by stabilizing the ER message without affecting the rate of ER gene transcription (Ing *et al.* 1996, Ing & Ott 1999). ER

mRNA (6500 bases long) is unstable because of its 4000 base long 3' untranslated region (3'UTR) which contains many AUUUA motifs, as do the typically unstable proto-oncogene mRNAs (Keaveney *et al.* 1993, Kenealy *et al.* 2000). Therefore, E₂ may stabilize endometrial ER mRNA similar to the way it stabilizes egg protein mRNAs in the livers of egg-laying animals: by inducing proteins that bind to specific sequences in the 3'UTR (Flouriot *et al.* 1996, Dodson & Shapiro 1997).

In order to define the molecular mechanisms by which E₂ up-regulates ER mRNA in endometrium, a physiologically relevant cell culture system is required to be able to mutate ER mRNA sequences and analyze resultant effects on stability in a living

system. Literature searches found no reports of estrogen up-regulation of ER gene expression in any cell line derived from endometrium. In fact, expression of ER and progesterone receptor (PR) genes is commonly lost when uterine cells are placed in culture (Helftenbein *et al.* 1991). The most common model for studying autoregulation of ER gene expression is the MCF7 breast cancer cell line. However, it shows down-regulation of ER mRNA and protein with E_2 treatment and the effect appears to be due to reduced stability of ER mRNA (Saceda *et al.* 1988, Berkenstam *et al.* 1989, Ree *et al.* 1989, Borrás *et al.* 1994, Saceda *et al.* 1998). This contrasts with the natural up-regulation of ER gene expression by estrogens in uterus and breast during estrous and menstrual cycles (Anderson *et al.* 1998).

The Ishikawa cell line, well-differentiated cells derived from a human endometrial adenocarcinoma (Nishida *et al.* 1985), appear to be an exception. Ishikawa cells express steroid hormone receptor genes including ER and PR. The ER α gene products, both mRNA and protein, predominate over those of estrogen receptor beta in Ishikawa cells as in the uterus (Kuiper *et al.* 1997, Paech *et al.* 1997, Nuttall *et al.* 2001, Wormke *et al.* 2000). In addition, Ishikawa cells maintain natural autoregulation of PR gene expression: up-regulation by E_2 and down-regulation by progesterone (Holinka *et al.* 1986, Anzai *et al.* 1989, Jamil *et al.* 1991, Hata & Kuramoto 1992, Lessey *et al.* 1996, Pinelli *et al.* 1998). E_2 affects Ishikawa cells similar to endometrium in altering expression of alkaline phosphatase, transforming growth factors-alpha and -beta, integrins, basic fibroblast growth factor, and androgen receptor genes (Anzai *et al.* 1989, 1992, Lessey *et al.* 1996, Fujimoto *et al.* 1997, Lovely *et al.* 2000 respectively).

The purpose of this study was to determine if Ishikawa cells would provide a relevant model for future studies of the E_2 -induced up-regulation of ER mRNA that occurs in endometrium. Criteria were that the model should express the ER gene and respond to physiological levels of E_2 by activating transcription of a reporter gene. In addition, the cultured cells should respond to E_2 by up-regulating ER mRNA within 24 h by stabilizing the message, as does endometrium. The cell cultures that fulfill these criteria should provide a useful model system for identifying the ER mRNA sequences and binding proteins

important to E_2 up-regulation of ER gene expression.

Materials and Methods

Cell culture

Ishikawa cells were grown and maintained in DME-F12 medium containing 5% (v/v) fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 U/ml penicillin G and 100 µg/ml streptomycin (GIBCO/BRL, Frederick, MD, USA). Passage numbers were between 20 and 40, with cells split consistently 1:5 every other day. Three days prior to experiments, medium was changed to 'steroid-depleted medium': DME-F12 without phenol red and with 5% dextran-charcoal-stripped FBS (Hyclone) and 100 U/ml sodium penicillin G and 100 µg/ml streptomycin sulfate. At treatment initiation, cells were exposed to the indicated concentration of E_2 or the drug vehicle (0.1% ethanol). Chemicals were of molecular biology or cell culture grade as appropriate and, unless otherwise indicated, were purchased from Sigma Chemical Co.

ER activation of transfected estrogen-responsive promoter constructs

On six-well cell culture plates, 6×10^4 Ishikawa cells plated the day before reached 60% confluence in steroid-depleted medium. In triplicate wells, the cells were transfected with no DNA ('mock transfection') or 1 µg each of plasmids pERE $_2$ tkCAT (Klein-Hitpass *et al.* 1989) and 1 µg pEGFP-C1 (Clontech) following the Superfect protocol (Qiagen). The first plasmid contained the chloramphenicol acetyltransferase (CAT) reporter gene under transcriptional control of estrogen-response elements (EREs) to measure ER transactivation. The second plasmid contained the enhanced green fluorescent protein (EGFP) reporter gene under the transcriptional control of the cytomegalovirus promoter and was used as a control for transfection efficiency. At 18 h post-transfection, cells were treated with 10^{-9} M E_2 or drug vehicle. Then 24 h after E_2 treatment, green fluorescent cells were visualized on an Eclipse TE 300 Microscope (Nikon, Melville, NY, USA) immediately prior to RNA isolation for analysis of CAT mRNA.

Synthesis of mRNA probes

To probe Northern blots for CAT and ER mRNAs, antisense cRNA probes were made from the pTRI-CAT DNA template (Ambion, Austin, TX, USA) and poER8 (Ing *et al.* 1996) respectively. For the cell-free RNA stability assays, a sense cRNA probe containing the entire 4000 base long 3'UTR of the ovine ER mRNA was synthesized from EcoR V-restricted poER-RACE2 plasmid (cDNA (GenBank accession AY033393) cloned into pCR 2.1 vector with the TA cloning kit (Invitrogen). As an E₂-insensitive control in the cell-free stability assay, a fragment of the 28S rRNA (encompassing the 989 base long HincII to BamHI fragment of the 28S rRNA cDNA; Gonzalez *et al.* 1985) was radiolabeled similarly. Briefly, cRNA probes were generated by *in vitro* transcription with T7 RNA polymerase with the Maxiscript kit (Ambion) using [³²P]UTP (3000 Ci/mmol, New England Nuclear, Boston, MA, USA). For Northern blots, unincorporated radionucleotides were removed from antisense probes with spin columns (Bio 101, La Jolla, CA, USA), while the sense RNAs for the cell-free stability assays were purified on 5% acrylamide/8 M urea denaturing gels to ensure that the latter were all full-length molecules (Ing *et al.* 1996).

Preparation of RNA samples and Northern analyses

Total cellular RNA samples were purified from live cell cultures with Tripure Reagent (Boehringer Mannheim). Extracted RNA was resuspended in TE buffer consisting of 50 mM Tris-Cl (pH 7.8) and 1 mM EDTA and stored at -80 °C pending analysis. Northern blot analyses of total cellular RNA samples (5–8 µg, consistent across the set of samples to be compared on one gel and blot) were performed as previously described (Ing *et al.* 1996). Stringent blot hybridization (55 °C) and washing (68 °C) in 0.1X SSC (1X=150 mM NaCl+15 mM Na citrate) containing 0.1% SDS were followed by treatment with 1 µg/ml RNase A (Boehringer Mannheim) in 2X SSC for 10 min at room temperature. After washing twice at 55 °C in 0.1X SSC containing 0.1% SDS for 15 min, blots were exposed to Fuji RX autoradiographic film for times ranging from 12 h to 7 days in order to record hybridization signals in the linear range of the film.

The signal intensities were measured by densitometry using Adobe Photoshop (Mountain View, CA, USA) scanning and Intelligent Quantifier (BioImage, Ann Arbor, MI, USA) quantitation software. ER and CAT message sizes were confirmed as 6500 and 1350 bases by comparing migration distances to those of 28S and 18S rRNAs (4800 and 1800 bases respectively) on gels stained with ethidium bromide (EtBr).

Inhibition of transcription by actinomycin D or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)

In order to find the most efficacious agent for blocking transcription in Ishikawa cells, inhibition of transcription by actinomycin D and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) were assessed. Triplicate cultures of cells in steroid-depleted medium were exposed to actinomycin D (5 µg/ml) or its vehicle (ethanol to a final concentration of 0.25%), while others were exposed to DRB (75 µg/ml) or its vehicle (dimethylsulfoxide to a final concentration of 0.1%). After 30 min of transcription inhibitor treatment, 5 mCi of [5,6-³H]uridine (40 Ci/mmol; New England Nuclear) was added to the medium for pulse-labeling of nascent RNA. Three hours later the medium was changed to 'chase' medium containing 2.5 mM unlabeled uridine and cytidine in addition to the transcription inhibitor. Cells were harvested 6 h after the addition of the [³H]uridine, and RNA was immediately extracted using the Tripure Reagent. Total cellular RNA (10 µg) from each sample was used to purify poly(A)⁺ RNA in duplicate preparations (Kingston 1997, Ing & Ott 2000). Incorporation of [³H]uridine into poly(A)⁺ RNA was determined by scintillation counts of 10 µl aliquots of the poly(A)⁺ preparations.

ER mRNA stability assays in Ishikawa cells

Triplicate cultures of cells were cultured in steroid-depleted medium with 10⁻⁹ M E₂ or drug vehicle for 24 h. At time 0 h, medium was exchanged for that containing 5 µg/ml actinomycin D plus E₂ or 0.1% ethanol. RNA samples were prepared from each of three triplicate cultures of

E₂-treated and untreated cells at time 0, 3, 6, 9 and 12 h. Northern analysis of ER mRNA was performed as described above.

Cell-free RNA stability analysis

High speed cytosolic extracts were prepared at 4 °C (Ratnasabapathy *et al.* 1990, Margot & Williams 1996) from Ishikawa cells treated for 24 h with 10⁻⁹ M E₂ or its vehicle, 0.1% ethanol. Briefly, 8 × 10⁶ cells were homogenized in 400 µl of buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and three protease inhibitors: 5 µg/ml aprotinin and 5 µg/ml leupeptin, 0.5 mM phenylmethylsulfonylfluoride. Large particles were removed by centrifugation at 800 *g* for 10 min. Then 0.11 volumes of buffer containing 30 mM HEPES (pH 7.9), 30 mM MgCl₂, 1.4 M KCl and protease inhibitors were added and the extracts were centrifuged at 100 000 *g* for 60 min to remove nuclei and cell debris. The cytosolic extracts were dialyzed against 80 volumes of buffer containing 20 mM HEPES (pH 7.9), 20% glycerol, 0.2 mM ethylenediaminetetraacetate, 100 mM KCl, and protease inhibitors. Protein concentrations of extracts were determined using the Bio-Rad Protein Assay (BioRad). Aliquots of extracts were stored at -80 °C until they were analyzed for the *in vitro* stability of the 3'UTR of the ER mRNA (Prokciak *et al.* 1994, Margot & Williams 1996).

Cell-free RNA stability assays consisted of incubating cytosolic extracts (2.5 µg protein) from E₂-treated or untreated Ishikawa cells with a radiolabeled ER 3'UTR or 28S RNA (1000 c.p.m.) in 10 mM Tris (pH 7.5), 60 mM KCl, 2 mM Mg acetate, 2 mM DTT, 10% glycerol, 2.5 µg/µl yeast tRNA, and 0.4 mM spermine for 30 min at 37 °C. After reactions were treated with proteinase K, proteins were denatured in urea buffer and RNA was extracted with phenol-chloroform and precipitated with ethanol (Klein-Hitpass *et al.* 1990). Intact and degraded RNAs were separated on 1.5 mm thick denaturing 5% polyacrylamide gels (Bernstein *et al.* 1992). Gels were dried and directly quantitated for the remaining intact ER RNA (migrating at the position of the full length RNA probe) on an InstantImager (Packard, Meriden, CT, USA).

Statistical analyses

Quantitative data were analyzed by Student's *t*-test (transfection assay and *in vitro* stability data), one-way ANOVA (E₂ dose and time-course data), and least squares ANOVA (transcription inhibitor trials) using SAS software (version 6.12 from the SAS Institute, Cary, NC, USA). Data are presented as means ± S.E. In Northern blot analyses, the hybridization signal of the ER mRNA band was divided by the intensity of the EtBr staining of the 18S rRNA in each lane (determined by densitometry) to normalize for the amount of RNA loaded. ER RNA half-lives (times to 50% of original concentrations) were estimated from semilogarithmic plots (Figs 4 and 6) of relative ER RNA levels on the *y*-axis (logarithmic) graphed against times on the *x*-axis (linear). The assumption that the RNA degrades with first-order kinetics was made (Hargrove *et al.* 1991). Throughout the paper, *P* < 0.05 was considered as the level of significance.

Results

ER protein is functional in Ishikawa cells

Because some reports in the literature have indicated a low or absent E₂ response in Ishikawa cells (Lee *et al.* 2000, Mueller *et al.* 2000), transfections were performed with an estrogen-responsive reporter plasmid, pERE₂tkCAT, to assess the presence and function of ER protein in our cell cultures. Transfection efficiency was high and consistent across cultures, with about 25% of the cells demonstrating green fluorescence resulting from EGFP produced from the cotransfected pEGFP-C1 plasmid. RNA samples were analyzed for CAT RNA on Northern gels and subsequent blots. Representative analyses from mock transfections (no plasmid) and transfected cells treated with drug vehicle or E₂ are shown in lanes 1–3, respectively, of Fig. 1. The EtBr-stained gel in the left panel confirmed equivalent loading across the lanes by the consistent brightnesses of the 18S rRNA bands in each lane. The autoradiographic results of the blot after hybridization to CAT cRNA probe (right panel) showed strong signals in transfected cells treated with E₂ (lane 3). Hybridization occurred on regions relating to RNA molecules of 1350 bases ('CAT RNA') and 1800 bases in length. CAT RNA hybridization signals

Figure 1 Transient transfection of pERE₂tkCAT demonstrates ER function. Ishikawa cells cultured in medium containing 5% FBS were plated in phenol red-free medium containing 5% charcoal–dextran–stripped FBS ('steroid-depleted medium') in six-well plates. After 48 h in the latter, triplicate cultures of cells were transfected with 1 µg each of pERE₂tkCAT and pEGFP-C1 using the Superfect transfection reagent protocol. Mock transfection controls were treated similarly but lacked plasmid DNAs. Eighteen hours after transfection, cells were treated with 10⁻⁹ M E₂ or vehicle (0.1% ethanol) for 24 h. Representative data are shown for three RNA samples: from mock-transfected cells (lane 1), and pERE₂tkCAT-transfected cells without and with E₂ treatment (lanes 2 and 3 respectively). The EtBr-stained gel shows equivalent brightnesses of 18S rRNA bands, indicating equal loading of RNA in each lane ('EtBr Gel', left panel). The results from Northern blot hybridization to CAT mRNA probe (right panel) show major signals in the RNA from E₂-treated transfected cells (lane 3), with much lower signals in lanes 1 and 2. The migration positions of the two hybridization signals correlate to 1350 bases ('CAT RNA') and 1800 bases ('18S rRNA'). Results from replicate experiments were averaged to show that E₂ increased CAT mRNA by 550% in transfected Ishikawa cells.

were much weaker in RNA samples from transfected cells lacking E₂ treatment (lane 2) and were absent in those from mock-transfected cells (lane 1). Since the signals at 1800 bases comigrated with 18S rRNA and were present even in mock-transfected cells, it was designated '18S rRNA' and presumed to be non-specific. Densitometric analyses of the CAT RNA bands in Northern blots from two experiments showed that E₂ treatment of Ishikawa cells transfected with pERE₂tkCAT induced a 550% increase in CAT mRNA in transfected cells ($P < 0.05$). This indicated that ER protein was both present and functional in

our Ishikawa cells: it bound E₂ and activated transcription of the CAT reporter gene through its upstream pair of ERE sites.

E₂ up-regulates steady-state levels of ER mRNA in Ishikawa cells

To determine if E₂ up-regulates ER mRNA in Ishikawa cells as it does in the endometrium of sheep, Ishikawa cells were grown in steroid-depleted medium for 3 days. Triplicate cell cultures were then treated for 24 h with E₂: 10⁻¹⁰, 10⁻⁹ or 10⁻⁸ M E₂, or the E₂ vehicle (0.1% ethanol; '0 M E₂'). Samples of total cellular RNA were analyzed on Northern blots for ER mRNA concentration by hybridization to a radiolabeled ER cRNA probe. Hybridization signals of ER mRNA at a migration position of 6500 bases (Ing *et al.* 1996) increased significantly ($P < 0.05$) with 10⁻⁹ and 10⁻⁸ M E₂ treatments to levels about 50% higher than in vehicle-treated controls (Fig. 2). The 10⁻¹⁰ M E₂ treatment showed no effect on ER mRNA levels. In three additional experiments, 10⁻⁸ M E₂ significantly increased the concentration of ER mRNA even more: to 100% greater levels than in vehicle-treated control cells (data not shown).

To determine the earliest time of the increase in ER mRNA, RNA was prepared from cell cultures after the addition of E₂ at 0, 1, 4, 7 and 10 h. The Northern blot analyses indicated that ER mRNA concentrations significantly increased at 7 and 10 h post-E₂ ($P < 0.05$; Fig. 3). The levels of ER mRNA increased 150% over those in control (time=0 h) cells, a magnitude similar to that found in cells treated for 24 h (above).

E₂ stabilizes ER mRNA in Ishikawa cells: *in vivo* assays with inhibitors of transcription

Inhibition of mRNA transcription in cells with actinomycin D or DRB allows measurements of cellular RNAs over a subsequent time course to estimate their molecular stabilities. Two compounds with different modes of action are routinely used to block transcription: actinomycin D intercalates the DNA template, while DRB inhibits the transcription elongation factor TFIIF (Yankulov *et al.* 1995). In order to find the most efficacious agent in our cell system, we evaluated the inhibition of [³H]uridine incorporation into

Figure 2 E_2 regulation of ER mRNA levels in Ishikawa cells is dose-dependent. Ishikawa cells cultured as described in Fig. 1 were treated with E_2 at the indicated doses. The drug vehicle, 0.1% ethanol, was applied to the controls ('0 M treatment'). After 24 h of treatment, RNA was harvested from triplicate cultures, each with approximately 10^6 cells, and analyzed individually for ER mRNA on Northern blots. Levels of ER mRNA are reported as least squares means and standard errors relative to the '0 M' dose treatment set at 100 for three replicate experiments. E_2 treatment at 10^{-9} and 10^{-8} M doses significantly increased ER mRNA levels ($P < 0.05$) while 10^{-10} M E_2 had no effect.

poly(A)⁺ RNA in Ishikawa cells treated with actinomycin D (5 µg/ml) or DRB (75 µg/ml). Actinomycin D inhibited 93% while DRB blocked 50% of radiolabel incorporation compared with that in cultures treated with the respective drug's vehicle. Because of its higher efficacy, we used actinomycin D to inhibit transcription for our *in vivo* stability assays.

Ishikawa cells grown in steroid-depleted medium were treated with 10^{-9} M E_2 or vehicle for 24 h. At that time (time=0 h), 5 µg/ml actinomycin D was added to the medium of every cell culture. Total RNA was extracted from cells harvested at 3 h intervals and analyzed for ER mRNA concentrations on Northern blots. To contrast relative degradation rates, initial concentrations of

Figure 3 Time course of E_2 up-regulation of ER mRNA. Ishikawa cells were cultured as described in Fig. 1, then treated with 10^{-9} M E_2 as described in the Methods. Cells were harvested at time of E_2 treatment initiation ('0 h'), and at time points 1, 4, 7 and 10 h subsequent to it. Total cellular RNA was extracted from triplicate cultures of cells at the time of their harvest. Northern analyses indicated relative ER mRNA levels, reported as least squares means and standard errors, with the average hybridization signal of the RNA from the time '0 h' cultures set at 100. ER mRNA levels increased significantly at 7 and 10 h post-treatment ($P < 0.05$).

ER mRNA were set at 100% for each treatment group. Figure 4 shows that the degradation of ER mRNA over time after transcription blockade occurred much faster in control cells ('No E_2 ') compared with that in the E_2 -treated (' E_2 ') cells. E_2 treatment significantly increased ($P < 0.05$) the half-life (time to reduction to 50% of original levels) of the ER message from 6 h in control cells to 10 h in E_2 -treated cells.

E_2 treatment of Ishikawa cells stabilizes ER RNA: cell-free stability assays

In vitro transcription of the 3'UTR of the ER message provided a radiolabeled probe to assess

Figure 4 E_2 enhances ER mRNA stability in Ishikawa cells. Ishikawa cells were treated with 10^{-9} M E_2 or vehicle (0.1% ethanol) for 24 h. Then, at time '0 h', actinomycin D (5 μ g/ml) was added to the medium to inhibit transcription. Triplicate plates of 10^6 cells were harvested at 0, 3, 6, 9 and 12 h time points. RNA was prepared from each plate and analyzed for ER mRNA on Northern blots. Relative levels of ER mRNA at each time point are presented as a percentage of the average time 0 value for the treatment group: vehicle-treated cells ('No E_2 ', open circles) and E_2 -treated cells (' E_2 ', filled squares). Least squares means and standard errors are from three replicate experiments. E_2 treatment of Ishikawa cells significantly stabilized ER mRNA from a half-life (time to 50% of initial concentration) of 6 h to one of 10 h ($P < 0.05$).

whether the RNA was more stable in cytosolic extracts from E_2 -treated Ishikawa cells (24 h with 10^{-9} M E_2) compared with extracts from untreated cells. After a 30-min incubation of probe and extract at 37 °C, degradation of the radiolabeled probe was analyzed on a denaturing acrylamide gel (Fig. 5). The two lanes at left show the migration of duplicate samples of the intact 4000 base ER RNA probe ('Input') near the starting point of electrophoresis (upper thin arrow). The two middle lanes

Figure 5 ER RNA stability is enhanced in extracts from E_2 -treated Ishikawa cells. High-speed cytosolic extracts prepared from Ishikawa cells were incubated with radiolabeled ER RNA encompassing the entire 3'UTR at 37 °C for 30 min in buffer conditions described in the text. This Figure shows direct scanning results of RNA analysis on a denaturing 5% acrylamide gel. Lanes (left to right) contained duplicate samples of unincubated ER RNA ('Input') and ER RNA incubated with untreated ('Ishikawa- E_2 ') and E_2 -treated ('Ishikawa+ E_2 ') cell extracts. The full-length ER RNA in the left lanes (4000 bases long, migration position indicated by thick arrow) was rapidly degraded to short fragments in extracts from untreated cells (near the bottom of the gel in the middle lanes, indicated by the lower thin arrow at left). E_2 treatment of cells, however, greatly reduced degradation of the ER RNA (right lanes) so more RNA migrated less far from the wells (upper thin arrow). These are representative results from three replicate experiments.

show severe degradation of the RNA probe to small fragments that migrate near the dye front of electrophoresis (lower thin arrow), a result of incubation with extract from untreated Ishikawa cells ('Ishikawa- E_2 '). In contrast, the duplicate RNA samples in the right lanes show much less degradation of the probe after incubation with the extract from E_2 -treated Ishikawa cells ('Ishikawa+ E_2 '). Multiple experiments were analyzed for the percentage of intact RNA probe

Figure 6 E_2 increases ER RNA half-life in Ishikawa cell extracts. ER RNA half-lives were determined with data from three replicate experiments, including the one shown in Fig. 5. Least squares means and standard errors for percentages of ER 3'UTR RNA probe remaining after 0- and 30-min incubations with cell extracts from untreated ('No E_2 ') and E_2 -treated (' E_2 ') Ishikawa cells were plotted as in Fig. 4. E_2 treatment increased ER RNA half-life (time to 50% of initial concentration) significantly ($P < 0.05$), from 7.5 to 30 min.

(migrating at the position of the full length RNA probe) remaining after the incubations. E_2 treatment significantly increased ($P < 0.001$) the percentage of intact RNA remaining from $9 \pm 4.7\%$ in controls to $45 \pm 4.4\%$. The half-lives of ER RNA in E_2 -treated and untreated cell extracts were estimated from a semilogarithmic plot of the data (Fig. 6). E_2 significantly increased ER RNA half-life, from 7.5 to 30 min. This E_2 effect was specific for ER RNA because a 28S rRNA probe was equally stable in extracts from control and E_2 -treated Ishikawa cells, with 72% of the latter probe remaining intact after incubation in either extract (data not shown).

Discussion

This is the first report of E_2 up-regulating ER gene expression in an endometrial cell line. Only one *ex vivo* system of endometrial cell culture has previously recapitulated the *in vivo* up-regulation of ER gene expression by E_2 (Xiao & Goff 1999). Those were primary cultures of epithelial cells from bovine endometrium, which demonstrated a 120% increase in ER protein levels after treatment with 10^{-9} M E_2 for 4 days. We set up similar experiments with primary cultures of sheep endometrial cells but had variable results (data not shown). As reported here, the Ishikawa cell line reliably reproduces the E_2 up-regulation of ER gene expression that occurs in endometrium.

A conflict in the literature exists between those reporting that Ishikawa cells have little or no expression of ER gene (Mueller *et al.* 2000) and those who observe robust E_2 responses and ER gene expression in their Ishikawa cells (Barsalou *et al.* 1998, Wormke *et al.* 2000). Therefore, an initial concern of ours was to demonstrate expression of the ultimate ER gene product, ER protein, in our cultures of Ishikawa cells. When we tested our Ishikawa cells for activation of the transfected ERE_2 tkCAT reporter gene, our data nearly matched the 700% increase in CAT gene expression in response to 2.5×10^{-9} M E_2 for 24 h (Wormke *et al.* 2000). These studies also prove that Ishikawa cells are suitable for genetic manipulations, such as transfection of foreign DNA.

Our focus then turned to E_2 -regulated expression of the endogenous ER gene in Ishikawa cells. The same low dose of E_2 (10^{-9} M) that increased expression of p ERE_2 tkCAT in transfected Ishikawa cells also up-regulated ER mRNA in untransfected cells. The magnitude of the E_2 -induced increase in ER mRNA levels in Ishikawa cells is consistent with that in the endometrium of ovariectomized ewes in response to a physiological dose of E_2 (Robertson *et al.* 1998, Zou & Ing 1998, Robertson *et al.* 2001). E_2 levels of 10^{-9} and 10^{-8} M have been used to up-regulate expression of the PR gene and other genes in Ishikawa cells (Holinka *et al.* 1986, Jamil *et al.* 1991, Lessey *et al.* 1996, Pinelli *et al.* 1998, Lovely *et al.* 2000). Both are within the physiological range of circulating estrogen concentrations in female mammals during estrous and menstrual cycles (Guillemot *et al.* 1993).

Our time course study also illustrates the exquisite sensitivity of Ishikawa cells to E_2 , with the response reaching the peak induction within 7 h of treatment. We are aware of no other studies showing such a rapid effect of E_2 on Ishikawa cells: others have used 24 h as an initial time point (Lessey *et al.* 1996, Wormke *et al.* 2000) and/or treated for 3 or more days (Holinka *et al.* 1986, Jamil *et al.* 1991, Barsalou *et al.* 1998, Lovely *et al.* 2000). The E_2 response measured here was faster than the up-regulation of ER mRNA in endometrium (present at 24 but not 12 h post-injection; Ing *et al.* 1996, Ing & Tornesi 1997). One possible reason for the faster response in cell culture is the lack of metabolism and clearance of the hormone during the treatment period.

Next, we determined whether Ishikawa cells shared a post-transcriptional mechanism of E_2 response with endometrium for up-regulation of ER mRNA. The stabilities of ER mRNAs were analyzed in both transcription inhibitor and cell-free assays. The results demonstrated that E_2 -treatment of Ishikawa cells significantly increased ER mRNA half-life by 70% and approximately 300% in the respective assays, similar to the 170% increase in ER RNA half-life in endometrium of E_2 -treated ovariectomized ewes (Ing & Ott 1999). The ER mRNA half-life in Ishikawa cells was similar to that measured in MCF7 breast cancer cells (Saceda *et al.* 1998). The half-lives in the cell-free assay were much shorter than those in living cells, probably due to lack of subcellular structures restricting RNase access to RNA and the lack of polyadenylation and capping on the synthetic RNA in the cell-free assay. We have seen similar differences between absolute values of half-lives of ER RNA in endometrial tissue and their cell-free extracts (Ing & Ott 1999, NH Ing & D Mitchell submitted). As for the Ishikawa assays reported here, the increased ER RNA stability with E_2 treatment was consistent in both endometrial RNA stability assays.

Our conclusions are that the Ishikawa cell line maintains ER gene expression in culture and that the resultant ER protein powerfully activates transfected estrogen-responsive genes in a ligand-dependent manner. In addition, Ishikawa cells up-regulate the expression of their ER genes in response to E_2 by a post-transcriptional mechanism, as endometrium does *in vivo*. By transfecting reporter genes linked to ER mRNA sequences, the

elements that confer its E_2 -modulated stability can now be defined in this physiologically relevant model system. Functional comparisons to the E_2 modulated stability of vitellogenin mRNA, sequences and the binding protein 'vigilin' that post-transcriptionally regulate gene expression in frog and chicken liver (Dodson & Shapiro 1997) may link this ancient mechanism of E_2 action to ER mRNA regulation in a mammalian cell.

Acknowledgements

The authors gratefully acknowledge kind gifts of the Ishikawa cells from Dr BA Lessey (Department of Obstetrics and Gynecology, University of North Carolina at Chapel Hill) and pERE₂tkCAT from Drs Ming-Jer Tsai and Sophia Tsai (Department of Cell and Molecular Biology, Baylor College of Medicine), as well as the outstanding technical assistance provided by Cindy Balog. Research was conducted through the Texas Agricultural Experiment Station with funding provided by grants to N.H.I. from the USDA/CSREES (NRI-CGP 98-35203-6272) and to JR from the Lalor Foundation.

References

- Anderson E, Clarke RB & Howell A 1998 Estrogen responsiveness and control of normal human breast proliferation. *Journal of Mammary Gland Biology and Neoplasia* **3** 23–35.
- Anzai Y, Holinka CF, Kuramoto H & Gurside E 1989 Stimulatory effects of 4-hydroxytamoxifen on proliferation of human endometrium adenocarcinoma cells (Ishikawa Line). *Cancer Research* **49** 2362–2365.
- Anzai Y, Gong Y, Holinka CF, Murphy LJ, Murphy LC, Kuramoto H & Gurside E 1992 Effects of transforming growth factors and regulation of their mRNA levels in two human endometrial adenocarcinoma cell lines. *Journal of Steroid Biochemistry and Molecular Biology* **42** 449–455.
- Barsalou A, Gao W, Anghel SI, Carriere J & Mader S 1998 Estrogen response elements can mediate agonist activity of anti-estrogens in human endometrial Ishikawa cells. *Journal of Biological Chemistry* **273** 17138–17146.
- Bergman MD, Schachter BS, Karelus K, Combatsiaris EP, Garcia T & Nelson JF 1992 Up-regulation of the uterine estrogen receptor and its messenger ribonucleic acid during the mouse estrous cycle: the role of estradiol. *Endocrinology* **130** 1923–1930.
- Berkenstam A, Glaumann H, Martin M, Gustafsson J-A & Norstedt G 1989 Hormonal regulation of estrogen receptor messenger ribonucleic acid in T47D_{co} and MCF-7 breast cancer cells. *Molecular Endocrinology* **3** 22–28.
- Bernstein PL, Herrick DJ, Prokipcak RD & Ross J 1992 Control of *c-myc* mRNA half-life in vitro by a protein capable of binding to a coding region stability determinant. *Genes and Development* **6** 642–654.

- Borras M, Hardy L, Lempereur F, El Khissi AH, Legros N, Gol-Winkler R & Leclercq G 1994 Estradiol-induced down-regulation of estrogen receptor: effect of various modulators of protein synthesis and expression. *Journal of Steroid Biochemistry and Molecular Biology* **48** 325–336.
- Brenner RM, McClellan MC, West NB, Novy MJ, Haluska GJ & Sternfeld MD 1991 Estrogen and progesterone receptors in the macaque endometrium. *Annals of the New York Academy of Sciences* **622** 149–166.
- Dodson RE & Shapiro DJ 1997 Vigilin, a ubiquitous protein, with 14K homology domains, is the estrogen-inducible vitellogenin mRNA 3'-untranslated region-binding protein. *Journal of Biological Chemistry* **272** 12249–12252.
- Flouriort G, Pakdel F & Valotaire Y 1996 Transcriptional and post-transcriptional regulation of rainbow trout estrogen receptor and vitellogenin gene expression. *Molecular and Cellular Endocrinology* **124** 173–183.
- Fujimoto J, Hori M, Ichigo S, Hirose R & Tamaya T 1997 Antiestrogenic compounds inhibit estrogen-induced expression of basic fibroblast growth factor and its mRNA in well-differentiated endometrial cancer cells. *General Pharmacology* **28** 215–219.
- Geisert RD, Brenner RM, Moffatt RJ, Harney JP, Yellin T & Bazer FW 1993 Changes in oestrogen receptor protein, mRNA expression and localization in the endometrium of cyclic and pregnant gilts. *Reproduction, Fertility and Development* **5** 247–260.
- Gonsalez IL, Gorski JL, Campen TS, Dorney DJ, Erickson JM, Sylvester JE & Schmickel RD 1985 Variation among human 28S ribosomal RNA genes. *PNAS* **82** 7666–7670.
- Guillomot M, Flechon J-E & Leroy F 1993 Blastocyst development and implantation. In *Reproduction in Mammals and Man*, pp 387–410. Eds C Thibault, M-C Levasseur & RHF Hunter. Paris: Ellipses.
- Hargrove JL, Hulsey MG & Beale EG 1991 The kinetics of mammalian gene expression. *Bioassays* **13** 667–674.
- Hata H & Kuramoto H 1992 Immunocytochemical determination of estrogen and progesterone receptors in human endometrial adenocarcinoma cell (Ishikawa cells). *Journal of Steroid Biochemistry and Molecular Biology* **42** 201–210.
- Helftenbein G, Misseyanni A, Hagen G, Peter W, Slater EP, Wiehle RD, Suske G & Beato M 1991 Expression of the uteroglobin promoter in epithelial cell lines from endometrium. *Annals of the New York Academy of Sciences* **622** 69–79.
- Holinka CF, Hata H, Kuramoto H & Gurpide E 1986 Responses to estradiol in a human endometrial adenocarcinoma cell line (Ishikawa). *Journal of Steroid Biochemistry* **24** 85–89.
- Ing NH & Tornesi MB 1997 Estradiol up-regulates estrogen receptor and progesterone receptor gene expression in specific ovine uterine cells. *Biology of Reproduction* **56** 1205–1215.
- Ing NH & Ott TL 1999 Estradiol up-regulates estrogen receptor alpha messenger ribonucleic acid in sheep endometrium by increasing its stability. *Biology of Reproduction* **60** 134–139.
- Ing NH, Spencer TE & Bazer FW 1996 Estrogen enhances endometrial estrogen receptor gene expression by a posttranscriptional mechanism in the ovariectomized ewe. *Biology of Reproduction* **54** 591–599.
- Jamil A, Croxtall JD & White JO 1991 The effect of anti-oestrogens on cell growth and progesterone receptor concentration in human endometrial cancer cells. *Journal of Molecular Endocrinology* **6** 215–221.
- Keaveney M, Parker MG & Gannon F 1993 Identification of a functional role for the 3' region of the human estrogen receptor gene. *Journal of Molecular Endocrinology* **10** 143–152.
- Kenealy M-R, Flouriort G, Sonntag-Buck V, Dandekar T, Brand H & Gannon F 2000 The 3'-untranslated region of the human estrogen receptor α gene mediates rapid messenger ribonucleic acid turnover. *Endocrinology* **141** 2805–2813.
- Kingston RE 1997 Preparation of a poly(A)⁺ RNA. In *Current Protocols in Molecular Biology*, pp 4.5.1–4.5.3. Eds FM Ausubel, R Brent, RE Kingston, DD Moore, JG Seidman, JA Smith & K Struhl. New York: John Wiley and Sons.
- Klein-Hitpass L, Tsai SY, Greene GL, Clark JH, Tsai M-J & O'Malley BW 1989 Specific binding of estrogen receptor to the estrogen response element. *Molecular and Cellular Biology* **9** 43–49.
- Klein-Hitpass L, Tsai SY, Weigel NL, Allan GF, Riley D, Rodriguez R, Schrader WT, Tsai M-J & O'Malley BW 1990 The progesterone receptor stimulates cell-free transcription by enhancing the formation of a stable preinitiation complex. *Cell* **60** 247–257.
- Kuiper GJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S & Gustafsson J-A 1997 Comparison of the ligand binding specificity and transcript distribution of estrogen receptors α and β . *Endocrinology* **138** 863–870.
- Lee H, Jiang F, Wang Q, Nicosia SV, Yang J, Su B & Bai W 2000 MEKK1 activation of human estrogen receptor α and stimulation of the agonistic activity of 4-hydroxyl tamoxifen in endometrial and ovarian cancer cells. *Molecular Endocrinology* **14** 1882–1896.
- Lessey BA, Killam AP, Metzger DA, Haney AF, Greene GL & McCarty KS 1988 Immunohistochemical analysis of human uterine estrogen and progesterone receptor throughout the menstrual cycle. *Journal of Clinical Endocrinology and Metabolism* **67** 334–340.
- Lessey BA, Ilesanmi AO, Castelbaum AJ, Yuan L, Somkuti SG, Satyaswaroop PG & Chwalisz K 1996 Characterization of the functional progesterone receptor in an endometrial adenocarcinoma cell line (Ishikawa): progesterone-induced expression of the α integrin. *Journal of Steroid Biochemistry and Molecular Biology* **59** 31–39.
- Lovely LP, Rao KBCA, Gui Y & Lessey BA 2000 Characterization of androgen receptors in a well-differentiated endometrial adenocarcinoma cell line (Ishikawa). *Journal of Steroid Biochemistry and Molecular Biology* **74** 235–241.
- Margot JB & Williams DL 1996 Estrogen induces the assembly of a multiprotein messenger ribonucleoprotein complex on the 3' untranslated region of chicken apolipoprotein II mRNA. *Journal of Biological Chemistry* **271** 4452–4460.
- Mueller MD, Vigne J-L, Minchenko A, Lebovic DI, Leitman DC & Taylor RN 2000 Regulation of vascular endothelial growth factor (VEGF) gene transcription by estrogen receptors α and β . *PNAS* **97** 10972–10977.
- Nishida M, Kasahara K, Kaneko M, Iwasaki H & Hayashi K 1985 Establishment of a new human endometrial adenocarcinoma cell line, Ishikawa cells, containing estrogen and progesterone receptors. *Acta Obstetrics Gynaecology Japan* **37** 1103–1111.
- Nuttall ME, Pendrak I, Emery JG, Nadeau DP, Fisher PW, Nicholson TA, Zhu Y, Suva LJ, Kingsbury WD & Gowen M 2001 Antagonism of oestrogen action in human breast and endometrial cells in vitro: potential novel antitumour agents. *Cancer Chemotherapy and Pharmacology* **47** 437–443.
- Okulicz WC, Savasta AM, Hoberg LM & Longcope C 1990 Biochemical and immunohistochemical analyses of estrogen and progesterone receptors in rhesus monkey uterus during the proliferative and secretory phases of artificial menstrual cycles. *Fertility and Sterility* **53** 913–920.
- Ott TL, Zhou Y, Miranda MA, Stevens C, Harney JP, Ogle TF & Bazer FW 1993 Changes in progesterone and oestrogen receptor mRNA and protein during maternal recognition of pregnancy and luteolysis in ewe. *Journal of Molecular Endocrinology* **10** 171–183.
- Paech K, Webb P, Kuiper GJM, Nilsson S, Gustafsson J-A, Kushner PJ & Scanlan TS 1997 Differential ligand activation of estrogen receptors ER α and ER β at AP1 sites. *Science* **277** 1508–1510.
- Pinelli DM, Drake J, Williams MC, Cavanagh D & Becker JL 1998 Hormonal modulation of Ishikawa cells during three-dimensional growth in vitro. *Journal of the Society for Gynecologic Investigations* **5** 217–223.

- Prokipcak RD, Herrick DJ & Ross J 1994 Purification and properties of a protein that binds to the C-terminal coding region of human c-myc mRNA. *Journal of Biological Chemistry* **269** 9261–9269.
- Ratnasabapathy R, Hwang SPL & Williams DL 1990 The 3' untranslated region of apolipoprotein II mRNA contains two independent domains that bind distinct cytosolic factors. *Journal of Biological Chemistry* **265** 14050–14055.
- Ree AH, Landmark BF, Eskild W, Levy FO, Lahooti H, Jahnsen T, Aakvaag A & Hansson V 1989 Autologous down-regulation of messenger ribonucleic acid and protein levels for estrogen receptor in MCF-7 cells: an inverse correlation to progesterone receptor levels. *Endocrinology* **124** 2577–2583.
- Robertson JA, Bhattacharyya S & Ing NH 1998 Tamoxifen up-regulates oestrogen receptor- α , c-fos and glyceraldehyde 3-phosphate dehydrogenase mRNAs in ovine endometrium. *Journal of Steroid Biochemistry and Molecular Biology* **67** 285–292.
- Robertson JA, Zhang Y & Ing NH 2001 ICI 182,780 acts as a partial agonist and antagonist of estradiol effects in specific cells of the sheep uterus. *Journal of Steroid Biochemistry and Molecular Biology* **77** 281–287.
- Saceda M, Lippman ME, Chambon P, Lindsey RL, Ponglikitmongkol M, Puente M & Martin MB 1988 Regulation of the estrogen receptor in MCF-7 cells by estradiol. *Molecular Endocrinology* **2** 1157–1162.
- Saceda M, Lindsey RK, Solomon H, Angeloni SV & Martin MB 1998 Estradiol regulates estrogen receptor mRNA stability. *Journal of Steroid Biochemistry and Molecular Biology* **66** 113–120.
- Wormke M, Castro-Rivera E, Chen I & Safe S 2000 Estrogen and aryl hydrocarbon receptor expression and crosstalk in human Ishikawa endometrial cancer cells. *Journal of Steroid Biochemistry and Molecular Biology* **72** 197–207.
- Xiao CW & Goff AK 1999 Hormonal regulation of oestrogen and progesterone receptors in cultured bovine endometrial cells. *Journal of Reproduction and Fertility* **115** 101–109.
- Yankulov K, Yamashita K, Roy R, Elgy JM & Bentley DL 1995 Transcriptional elongation inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole inhibits transcription factor IIH-associated kinase. *Journal of Biological Chemistry* **270** 23922–23925.
- Zheng J, Johnson ML, Redmer DA & Reynolds LP 1996 Estrogen and progesterone receptors, cell proliferation, and c-fos expression in the ovine uterus during early pregnancy. *Endocrinology* **137** 340–348.
- Zou K & Ing NH 1998 Oestradiol up-regulates oestrogen receptor, cyclophilin, and glyceraldehyde phosphate dehydrogenase mRNA concentrations in endometrium, but down-regulates them in liver. *Journal of Steroid Biochemistry and Molecular Biology* **64** 231–237.

Received in final form 25 February 2002

Accepted 28 February 2002