The estrogen receptor α Σ3 mRNA splicing variant is differentially regulated by estrogen and progesterone in the rat uterus

J Varayoud*, J G Ramos*, L Monje, V Bosquiazzo, M Muñoz-de-Toro and **E H Luque**

Laboratorio de Endocrinología y Tumores Hormonodependientes, School of Biochemistry and Biological Sciences, Universidad Nacional del Litoral, C C 242, Santa Fe, Argentina

(Requests for offprints should be addressed to E H Luque; Email: eluque@fbcb.unl.edu.ar)

*(J Varayoud and J G Ramos contributed equally to this work)

Abstract

The gene for estrogen receptor α (ERα) has been shown to be under complex hormonal control and its activity can be regulated by mRNA alternative splicing. Here we examined the regulation of ERα transcription and translation in the rat uterus by ovarian steroid hormones. We examined whether expression of ERα mRNA splice isoforms is hormonally regulated in ovariectomized (OVX) and cycling rats. Adult OVX female rats were treated daily with 17-βestradiol (E_2) (0·05 μg/rat or 5 μg/rat), progesterone (P_4) (1 mg/rat) or a combination of both hormones for 4 days. Animals were killed 24 h after the last injection and uterine horns were removed. In order to determine whether ERα mRNA isoforms are differentially expressed under various physiological conditions, animals were evaluated at proestrus, estrus and diestrus. The ERα protein and mRNA were detected by immunohistochemistry and comparative RT-PCR analysis respectively. The presence of ERα mRNA isoforms was evaluated using a nested RT-PCR assay. In OVX control rats, ERα mRNA and protein levels were high, demonstrating a constitutive expression of the ERα gene in the uterus. When animals received P_4 or the high dose of E_2, a significant decrease in both ERα mRNA and protein was observed in the uterus. However, when rats were treated with the low dose of E_2, only the ERα protein was down-regulated; no changes were observed in ERα mRNA expression. In addition to the full-length ERα mRNA, OVX control rat uteri expressed three shorter transcripts: Σ3, Σ4 and Σ3,4 (lacking exon 3, exon 4, or both 3 and 4 respectively). Surprisingly, when OVX animals were treated with P_4, the low dose of E_2 or a combination of both steroids, expression of the Σ3 isoform was completely abolished. During the estrous cycle, all ERα mRNA splicing variants were detected at proestrus and estrus. However, in diestrus, significant low levels of the Σ3 isoform were observed. In summary, our results suggest a dose-dependent relationship between E_2 concentrations and the level of control in the ERα transcription–translation cascade. Moreover, the alternative splicing of the ERα primary transcript is influenced by the hormonal milieu, suggesting that these events could affect the estrogen responsiveness of the rat uterus during the estrous cycle.

Journal of Endocrinology (2005) **186,** 51–60

Introduction

Two different genes coding for the estrogen receptor (ER) have been identified: the classical ERα (Koike *et al.* 1987) and the more recently characterized ERβ (Kuiper *et al.* 1996). 17-β Estradiol (E_2) binds ERα with a higher affinity than ERβ, therefore promotes higher rates of ERα-mediated transcriptional activity at the estrogen response element (ERE) (Pettersson *et al.* 2000). Moreover, ERα and ERβ may have entirely opposite transcriptional effects at activating protein-1 (AP-1) sites, depending upon the structural properties of the ligand (Paech *et al.* 1997).

The rat uterus, a major target tissue for ovarian steroids, has served as an excellent model for studying hormonal regulation of ERα expression. Both, up-regulation and down-regulation of ERα by E_2 has been reported in the rat uterus, depending upon the physiological state of the animal and/or the experimental system employed (Zhou *et al.* 1993, Wang *et al.* 1999, Nephew *et al.* 2000). Since receptor levels influence target tissue responsiveness to the hormonal milieu, there has been a great interest in the understanding of how the ER is regulated.

It has been shown in the rat uterus that progesterone (P_4) selectively decreases ligand-bound ERα and inhibits recovery of ERα at transcriptional and translational levels (Clarke & Sutherland 1990). The repression of ERα-mediated transcriptional activity and the inhibition

DOI: 10.1677/joe.1.06099
0022–0795/05/0186–051
Online version via http://www.endocrinology-journals.org

of ERα retention by P_4 are mediated by the ligand-bound progesterone receptor (PR) (Kraus *et al.* 1995).

A great molecular diversity of ERs is achieved by the alternative splicing of mRNA isoforms that have been described in a variety of cells and tissues, particularly in cancer cell lines and tumors (Hopp & Fuqua 1998). Several of the variant transcripts generated by an exon skipping mechanism of the primary ERα pre-mRNA retain the same reading frame as the full-length transcript, and the corresponding variant proteins have been detected *in vivo* and *in vitro* (Pfeffer *et al.* 1995, Poola *et al.* 2000). It has been shown that the ERα splice variants lacking exon 3 and/or 4 (defined as Σ3, Σ4 and Σ3,4) are all translated into proteins *in vivo*; moreover, their expression levels varied during rat pituitary ontogeny and also according to the sex of the animal (Pasqualini *et al.* 1999). In addition, these splice variants were also observed in the rat uterus as exon-deleted transcripts (Friend *et al.* 1997). There is little information in the literature regarding steroid hormonal regulation of ERα mRNA isoform expression. Moreover, it has been suggested that ER mRNA splice variants might contribute to the deregulation of growth and differentiation in premalignant and malignant tissues in endocrine-related tumors (Hopp & Fuqua 1998).

In the present study, we examined the effects of E_2 and P_4 on the control of ERα transcription and translation in the uterus of adult ovariectomized (OVX) rats. The same animal model was used to determine whether these steroid hormones regulate the expression pattern of ERα mRNA splice variants. Furthermore, we studied if these isoforms are differentially expressed during the rat estrous cycle.

Materials and Methods

Experimental design

Sexually mature female rats of an inbred Wistar-derived strain bred at the Department of Human Physiology (Santa Fe, Argentina) were used. Animals were maintained under a controlled environment (22 ± 2 °C; lights on from 0600 to 2000 h) and had free access to pellet laboratory chow (Constantino, Córdoba, Argentina) and tap water. All rats were handled in accordance with the principles and procedures outlined in the FRAME's guidelines (http://www.frame.org.uk/reductioncommittee/journalguidelines.htm).

For steroid treatments, female rats were OVX and then rested for 14 days. Rats that exhibited at least 7 days of atrophic vaginal smears (Montes & Luque 1988) were injected s.c. daily for 4 days with vehicle (sesame oil), E_2 (0·05 or 5 μg/rat), P_4 (1 mg/rat) or a combination of both steroids. Rats were included in one of six experimental groups (n=7–8 rats/group): sesame oil (control), low dose of E_2 (0·05 E), high dose of E_2 (5 E), P_4 (P) or combinations 0·05 E+P and 5 E+P. The low dose of E_2 and the dose of P_4 were chosen because they reproduced the physiological plasma levels described in pregnant rats (Downing & Sherwood 1986, Luque *et al.* 1996). Moreover, a 100 times higher dose of E_2 was also tested. Animals were killed 24 h after the last injection and uteri were isolated. One uterine horn from each rat was placed immediately in liquid nitrogen and stored at −80 °C for RNA extraction. The other uterine horn was fixed by immersion in 10% buffer formalin for 6 h at 4 °C, embedded in paraffin, and used for immunohistochemical staining.

Intact 12-week-old female rats were used to determine expression of uterine ERα splice isoforms during the normal estrous cycle. Daily vaginal cytology was examined to establish different stages of the estrous cycle (Montes & Luque 1988). Only rats exhibiting three consecutive regular estrous cycles were employed. Animals were killed and uteri collected between 1000 and 1100 h at proestrus, estrus or diestrus (n=5 rats per stage of estrous cycle). The uterine horns from each rat were placed immediately in liquid nitrogen and stored at −80 °C for RNA extraction.

ERα expression detected by immunohistochemistry

Serial sections (5 μm in thickness) of paraffin-embedded uterine horns were mounted on 3-aminopropyl triethoxysilane (Sigma)-coated slides and dried for 24 h at 37 °C.

A standard immunohistochemical technique (avidin–biotin-peroxidase) was used to visualize ERα immunostaining intensity and distribution. In brief, for detection of ERα, a microwave (MW) pretreatment for antigen retrieval was performed following the protocol previously described (Muñoz-de-Toro *et al.* 1998). After washing in buffer (0·05 M PBS, pH 7·5), endogenous peroxidase activity and non-specific binding sites were blocked. Sections were incubated overnight at 4 °C in a humidified chamber with a diluted mouse monoclonal antibody raised to the full-length recombinant human ERα (working dilution 1:60; clone 6F11, Novocastra, Newcastle upon Tyne, UK), followed by incubation with a biotinylated secondary antibody (Sigma). Negative controls were obtained by replacing the primary antibody with non-immune mouse serum (Sigma). The reaction was developed using the avidin–biotin-peroxidase method and diaminobenzidine (DAB) (Sigma) as a chromogen substrate. Samples were dehydrated and mounted with permanent mounting medium (PMyR, Buenos Aires, Argentina).

Quantitation of ERα expression by image analysis

Image analysis was performed using the Image Pro-Plus 4·1·0·1 system (Media Cybernetics, Silver Spring, MA, USA) as previously described (Ramos *et al.* 2002). In brief, images were recorded by a Sony ExwaveHAD color video camera attached to an Olympus BH2 microscope (illumination: 12-V halogen lamp, 100 W, equipped with a stabilized light source), using a Dplan ×100 objective

Table 1 Positions of primers and PCR product sizes for RT-PCR experiments

	Position of primers on cDNA	PCR product size (bp)
cDNA (GenBank reference		
ERα (NM_012689)	E1: 681–701 E2: 1003–1025	345
GADPH (AF106860)	GADPH1: 94–111 GADPH2: 439–462	466
ERα First round	E3: 819–841 E4: 1422–1442	Full length: 624, Σ3: 507, Σ4: 288, and Σ3,4: 171
ERα Second round	E5: 836–856 E6: 1372–1389	Full length: 552, Σ3: 435, Σ4: 216, and Σ3,4: 99

(numerical aperture, 1·25). The microscope was set up properly for Koehler illumination. Correction of unequal illumination (shading correction) and calibration of the measurement system were done with a reference slide. Using Auto-Pro macro language, an automated standard sequence operation was created to measure optical density (OD). In this automated analysis process, the images from immunostained slides were converted to a gray scale and the OD was measured as an average gray, being equal to the sum of the gray intensity of each pixel divided by the number of pixels measured. The resolution of the images was set to 640 × 480 pixels and the final screen resolution was 0·103 μm/pixel.

For each uterine horn specimen, 3 sections separated at 20 μm intervals were evaluated and 20 microscopic fields were analyzed in each tissue compartment (luminal epithelium, glandular epithelium, subepithelial stroma and muscular stroma).

ERα mRNA analysis by comparative RT-PCR

Reverse transcription and PCR amplification were performed following the technique previously described by Ramos *et al.* (2003) with minor modifications. Total RNA was isolated from frozen uterine tissue using Trizol reagent (Life Technologies). The total RNA concentration was assessed by A_{260} and isolated RNA was stored at − 80 °C until needed. Equal quantities (4 μg) of total RNA from each animal were reverse-transcribed into cDNA with avian myoblastosis virus (AMV) reverse transcriptase (12·5 U; Promega) using 200 pmol of random hexamers (Promega) to prime the reaction; 20 U ribonuclease inhibitor (RNse OUT; Invitrogen) and 200 nmol of a deoxy NTP mixture were added to each reaction tube in a final volume of 30 μl of 1 × AMV-RT buffer. Reverse transcription was performed at 42 °C for 90 min. Reactions were terminated by heating at 97 °C for 5 min. Reactions were next cooled on ice, followed by dilution of the reverse-transcribed cDNA with RNAse-free water to a final volume of 60 μl. RNA incubated under identical conditions, but without reverse transcriptase served as a negative control.

Oligonucleotides primers for PCR were synthesized by Invitrogen. The locations of primers on the cDNAs, the GenBank accession numbers and the size of amplified fragments are listed in Table 1. A pair of primers was used to quantify the ERα gene transcription level: sense primer, E1, 5′-AATTCTGACAATCGACGCCAG-3′; antisense primer, E2, 5′-GTGCTTCAACATTCTCCC TCCTC-3′. In addition, as an internal control of reverse transcription and reaction efficiency, amplification of GAPDH mRNA was carried out in parallel in each sample using the following primers: sense primer, GAPDH1, 5′-CAGCCGCATCTTCTTGTG-3′; antisense primer, GAPDH2, 5′-AGTTGTCATATTTCTC GTGGTTCA-3′. All amplifications were performed in duplicate. To perform comparative PCR, aliquots of cDNA samples equivalent to 800 ng total RNA input were used in each PCR amplification. Each reaction mixture contained: 2·5 U Taq-DNA polymerase (Promega), 1·5 mM $MgCl_2$ (Promega), 0·2 mM of the dNTPs mix (Promega) and 20 pmol of each primer in a final volume of 25 μl of 1 × PCR Taq buffer. After initial denaturation at 97 °C for 2 min, the reaction mixture was subjected to successive cycles of denaturation at 96 °C for 1 min, annealing at 58 °C for 45 s, and extension at 72 °C for 1 min. A final extension cycle at 72 °C for 10 min was included. The optimal number of cycles for each reaction was determined experimentally to yield linear relationships between signal intensity and cycle number (see Results).

ERα mRNA splicing variants analysis by RT-nested PCR

In order to amplify full-length ERα mRNA and the exon-deleted splicing variants Σ3, Σ4 and Σ3,4 (lacking exon 3, exon 4, or both 3 and 4 respectively), we performed a nested-PCR with ERα-specific primers located upstream of exon 3 and downstream of exon 4 (Koike *et al.* 1987). We used nested-PCR to improve the specificity and accuracy of the PCR amplification process by making a second round of amplification using primers placed internally of the first pairs of primers (see Table 1). Reverse transcription of total RNA and PCR reaction

Table 2 OD of ERα-immunostained cells in luminal epithelium (LE), glandular epithelium (GE), subepithelial stroma (SS) and muscular stroma (MS)

	ERα (OD)			
	LE	GE	SS	MS
Groups				
C	49·7 ± 8·06	73·4 ± 7·78	42·2 ± 3·67	43·6 ± 7·31
5 E	18·5 ± 4·40**	12·5 ± 1·34**	25·4 ± 4·42**	37·7 ± 7·23
0·05 E	37·0 ± 3·21*	45·3 ± 10·3*	33·6 ± 2·13*	36·9 ± 6·96
5 E+P	56·9 ± 7·24	79·1 ± 11·9	41·0 ± 3·61	36·1 ± 7·41
0·05 E+P	45·7 ± 12·7	64·6 ± 12·6	46·5 ± 11·2	29·5 ± 6·94
P	10·3 ± 5·89**	72·5 ± 8·45	40·7 ± 8·13	21·6 ± 2·67*

Values are means ± S.E.M. Asterisks indicate statistically significant differences between control and hormone-treated grups (*P<0·05; **P<0·01). C; control group.

mixtures was performed as described above. To perform the first round of amplification, the following pair of primers was used: sense primer, E3, 5′-GTCTGGTCCT GTGAAGGCTGCAA- 3′; antisense primer, E4, 5′-AGG AGCAAACAGGAGCTTCCC- 3′. The PCR conditions included an initial denaturation step at 95 °C for 30 s, followed by 38 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. At the end of the cycling program, a final extension cycle at 72 °C for 5 min was included. Products from the first reaction were diluted 1/2000 and used in the second round of amplification with the following primers: sense primer, E5, 5′-CTGCAAGGCTTTCTTTAA GAG-3′; antisense primer, E6, 5′-TCATCAGGATCTC CAACC- 3′. The second round of PCR was performed using the same conditions as described for the first round.

Detection and identification of PCR products

The generated cDNA fragments were resolved in 1·5% agarose gels containing ethidium bromide (Sigma) and their molecular sizes determined by comparison with DNA standards (Cien Marker; Biodynamics, Buenos Aires, Argentina). Agarose gel images were digitized using a Sony ExwaveHAM color video camera (Sony Electronics Inc, Sony Drive, Park Ridge, NJ, USA) and the Image Pro-Plus 4·1·0·1 image system analyzer (Media Cybernetics, Silver Spring, MD, USA). GAPDH mRNA was selected as an internal control since expression of GAPDH mRNA remained constant in all experimental conditions. In comparative PCR analysis, the absolute OD values for each PCR product were obtained by densitometry and were normalized with GAPDH levels. Relative levels of the specific mRNAs were expressed in arbitrary units. In all assays, negative controls using RNA without reverse transcription and Taq-DNA polymerase-negative tubes were performed in order to minimize the introduction of potential artifacts. All PCR products were cloned using the TA cloning kit (Invitrogen) and specificity was confirmed by DNA sequencing (data not shown).

Data analysis

The statistical analysis was performed by the Kruskal–Wallis one-way ANOVA and significance between groups was determined by Dunn's post-test (Siegel 1956).

Results

Cell type-expression of ERα protein in the uterus of OVX rats

The relative ERα expression level in each uterine cellular compartment was determined by measuring the OD of nuclei in ERα-immunostained tissue sections (Table 2). OD levels for ERα in the uterus of control adult OVX rats were high, demonstrating a constitutive expression of ERα in epithelial, stromal and myometrial compartments (Fig. 1A). In controls, glandular epithelium showed the strongest signal, with all cell nuclei highly immunostained (Figs 1A and 2).

Treatment with E_2 resulted in significant changes in ERα expression patterns (Figs 1B and 2). OVX rats treated with E_2 exhibited a dramatic decrease in the intensity of ERα staining in the luminal epithelium, glandular epithelium and subepithelial stroma; in contrast, the high ERα level was maintained in the muscular compartment (Fig. 2). The E_2-induced down-regulation of ERα was detected with both low and high doses of E_2; however, a greater reduction in ERα was observed in the high dose-group (Fig. 2). Addition of P_4 to the E_2 treatment inhibited the down-regulation of ERα observed in E_2-treated animals (Fig. 1C). When OVX animals received P_4 alone, the immunohistochemical results revealed a clear down-regulation of ERα only in luminal epithelium and

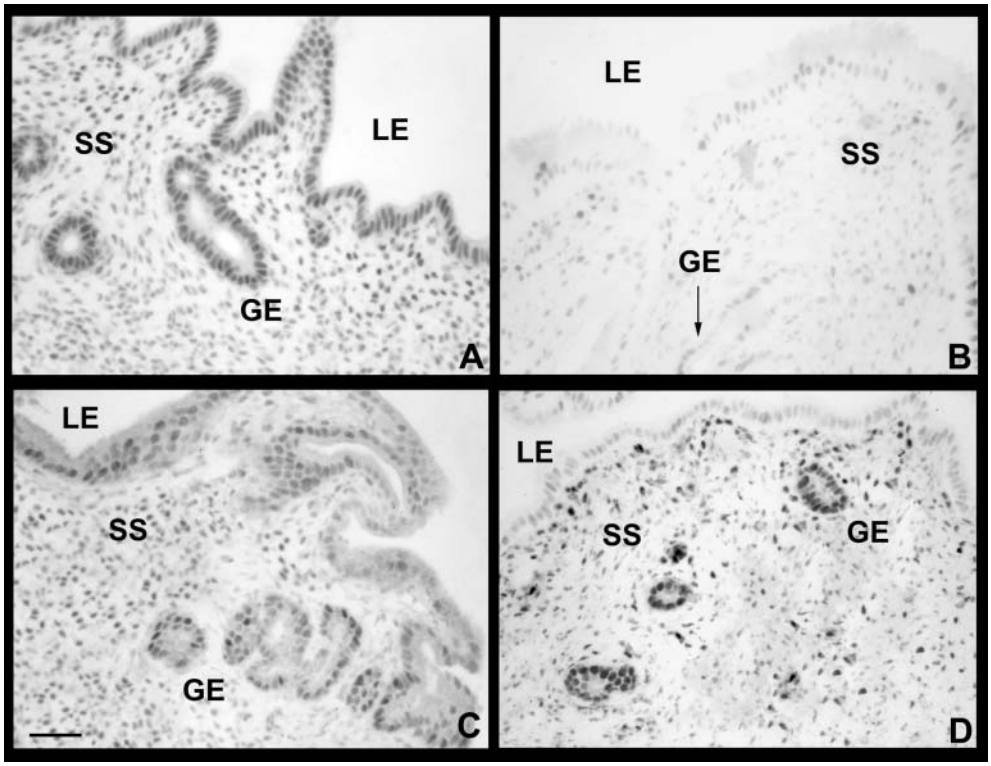

Figure 1 Representative photomicrographs of immunohistochemical detection of uterine ERα from adult OVX rats injected with: (A) sesame oil (control); (B) E_2 (5 μg/rat); (C) E_2 (5 μg/rat)+P_4 (1 mg/rat); (D) P_4 (1 mg/rat). (A) The high constitutive expression of ERα in luminal and glandular epithelium, and subepithelial stroma from control rats is shown. (B) A significant reduction in ERα staining in luminal and glandular epithelium and subepithelial stroma in rats treated with the high dose of E_2 was observed. (C) Down-regulation of ERα observed in E_2-treated rats was lost when P_4 was added. (D) When animals received P_4 alone, immunostaining revealed a clear down-regulation of ERα in luminal epithelium and muscular stroma. LE, luminal epithelium; GE, glandular epithelium; SS, subepithelial stroma. Scale bar represents 50 μm.

muscular stroma. No significant changes in glandular epithelium and subepithelial stroma were observed following P_4 administration (Figs 1D and 2).

Effects of E_2 and P_4 on total ERα mRNA in OVX rat uterus

Expression levels of ERα and GAPDH mRNAs in the uterus of experimental groups were evaluated by RT-PCR. Optimization of assays for semiquantitative analysis was carried out for each target by correlating the number of PCR cycles with the OD of the PCR products, using total RNA from a pool of control and treated rat uteri. This method revealed a linear relationship for amplification between cycles 28–36 for ERα and cycles 22–32 for GADPH. All linear correlation coefficients were greater than 0·97. Based on these results, and in accordance with our previously published protocols (Ramos *et al.* 2003, Kass *et al.* 2004), we have chosen 30 and 28 cycles for ERα and GAPDH respectively, using separate reactions for each target gene.

Representative ethidium bromide-stained images of ERα and GADPH mRNA by RT-PCR analysis are shown in Fig. 3. The relative expression of ERα mRNA was determined in each uterus (n=7–8 animals/group), with the results showing a differential modulation dependent upon the hormonal treatment. An abundant ERα PCR product of the expected size (345 bp) was generated after 30 cycles from reverse-transcribed uterine mRNA from adult OVX rats (Fig. 3). The identity of this product was confirmed by cloning and sequencing the PCR products (data not shown). The high dose of E_2 produced a significant reduction in the level of ERα mRNA. In contrast, the expression of ERα mRNA was unaffected when animals received the low dose of E_2. In addition, there were no significant changes in OD of ERα mRNA when combining low or high doses of E_2 with P_4 compared with controls. However, when animals were

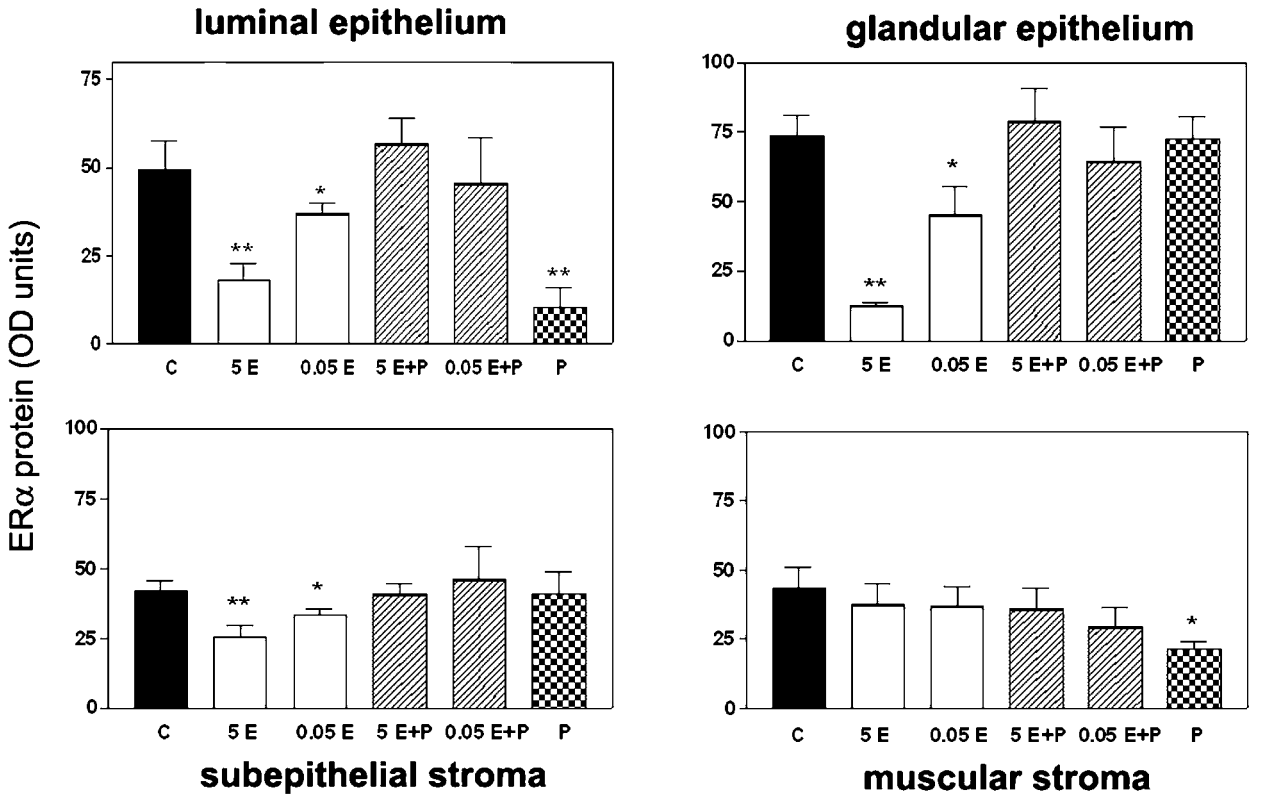

Figure 2 Quantitative measurement of ERα protein expression in different uterine cellular compartments following ovarian steroid treatment. Adult OVX animals were injected with sesame oil (C group), high dose of E_2 (5 E group), low dose of E_2 (0·05 E group), a combination of E_2+P_4 (5 E+P group and 0·05 E+P group), or P_4 alone (P group). Bars represent means+S.E.M. (n=7–8 animals/group). Asterisks indicate significant differences between control and hormone-treated rats (*P<0·05; **P<0·01).

injected with P_4 alone, a significant down-regulation of ERα mRNA expression was observed in comparison to controls.

The expression of alternatively spliced receptor variants in OVX rats following steroid treatment suggests a differential hormonal control

To investigate the presence of alternatively spliced receptor variants, we applied an RT-nested PCR procedure employing two pairs of specific oligonucleotides. The second-round PCR products revealed the presence of the full-length ERα mRNA (552 bp) in all samples. In addition, three exon-skipping isoforms could be detected with this protocol, the Σ3 (lacking exon 3), Σ4 (lacking exon 4) and the Σ3,4 variant (lacking exons 3 and 4). The amplification products were subsequently cloned and sequenced and were shown to represent splicing variants of the entire ERα mRNA which could maintain the in-phase reading frame of the ERα.

In the uterus of control OVX animals, we detected the four transcripts with a predominant signal of the full-length amplification product (Fig. 4). When animals received the high dose of E_2, a down-regulation of both the full-length ERα mRNA and also of the three exon-skipping isoforms was observed. However, when rats were treated with the high dose of E_2 plus P_4, no differences were observed in comparison to controls (Fig. 4). Surprisingly, in animals treated with either the low dose of E_2, P_4 alone or the combination of both hormones, the expression of the Σ3 isoform was completed abolished (Fig. 4). Interestingly, nucleotide sequence analysis revealed that this isoform is an exon-skipping variant that lacks the coding region for the second Zn-finger of the DNA-binding domain.

Expression of ERα mRNA splicing variants depends upon the estrous cycle

As in the uterus of OVX control animals, the four ERα mRNA transcripts were detectable at all stages of the estrous cycle (Fig. 5). The pattern of expression was similar between proestrus and estrus, with a predominant expression of the full-length amplification product. Levels of the Σ3 isoform were highest at proestrus and estrus, while in diestrus, a very low expression of this isoform was detected. The Σ4 and Σ3,4 isoforms did not exhibit

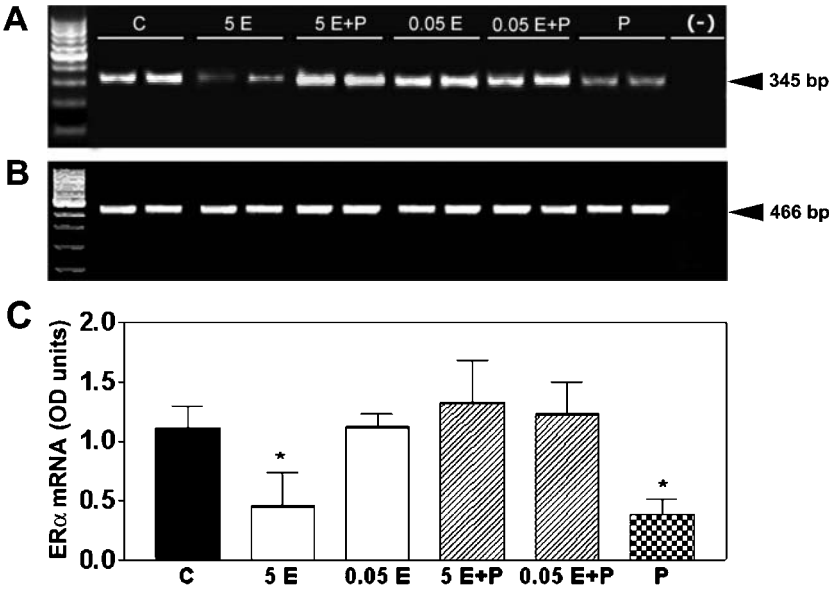

Figure 3 Expression of total ERα mRNA in the uterus of OVX control rats and following ovarian steroid treatment. Representative ethidium bromide-stained gel electrophoresis of uterine ERα mRNA from two independent samples per experimental group are presented. (A) Very low expression of ERα mRNA was exhibited by animals treated with the high dose of E_2 (5 E group) and with P_4 alone (P group), when compared with controls. (B) Parallel amplification of GADPH mRNA served as an internal control. In each gel, representative non-RT negative controls are presented (−). Abbreviations of experimental groups are detailed in legend of Fig. 2. (C) Densitometric values of ERα mRNA, expressed in arbitrary OD units relative to GADPH mRNA levels are shown. Bars represent mean values (+S.E.M.) of at least 7 animals/group. Asterisks indicate significant ($P<0{\cdot}05$) differences compared with controls.

changes in the uterus with changes in the rat estrous cycle, suggesting that expression of these isoforms is not regulated by sex hormones.

Discussion

The mechanisms that control ERα expression remain to some extent poorly understood. Although the expression of ERα is differentially regulated within individual cell types, the role of individual promoters, *cis*-acting elements, transcription factors, ERα itself and the cellular context remain to be elucidated (Reid *et al.* 2002).

In OVX control rats, we observed a constitutive expression of ERα protein in the nucleus of epithelial (glandular and luminal), stromal and myometrial uterine compartments. In accordance with previous studies, E_2 treatment down-regulated the expression of ERα protein in specific cellular types in the rat uterus (Manni *et al.* 1981, Nephew *et al.* 2000). The expression of ERα in epithelium and subepithelial stroma was shown to be very sensitive to estrogen treatment, since the low dose of E_2 used in this study significantly diminished receptor expression. In contrast, the myometrial compartment maintained high levels of nuclear ERα immunostaining even if the animals were treated with the high dose of E_2. Interestingly, the levels of ERα mRNA decreased only when animals received the high dose of E_2, indicating that this steroid can control the ERα transcription–translation cascade at different levels.

An interesting finding from this study is that luminal and glandular epithelium display differential regulation of ERα expression when OVX animals received P_4 alone. While E_2 alone down-regulated ERα protein immunostaining in both epithelial cell types, P_4 induced a selective inhibition of ERα in the luminal epithelial compartment. It should be noted that this P_4-dependent down-regulation of ERα expression was induced without any E_2 priming, demonstrating that the OVX rat uterus is constitutively responsive to P_4. Moreover, we have shown that P_4 action maintained a high expression of ERα in the glandular epithelium. In accordance with our results, Katsuda *et al.* (1999) have found a positive correlation between serum P_4 levels during metestrus and ERα expression in glandular epithelial uterine cells. Moreover, they have reported that a significant increase of bromodeoxyuridine-labeled cells occurred in the uterine glands at metestrus. Therefore, it is necessary to further investigate the role of P_4 in the regulation of ERα in glandular epithelial cells as an indirect mechanism of controlling cell proliferation. Regarding the differential

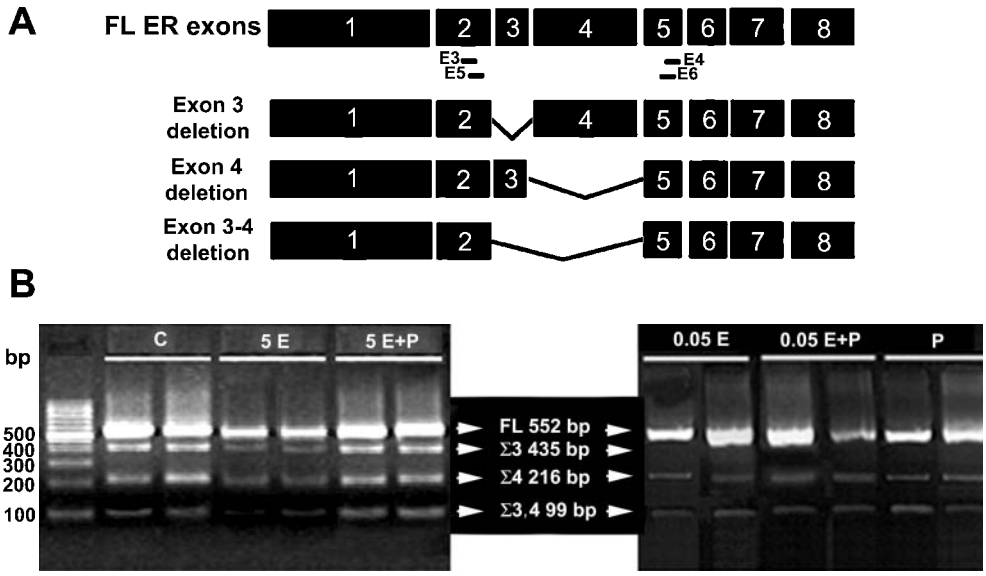

Figure 4 Expression of ERα mRNA alternative spliced variants in rat uterus detected by RT-nested PCR in adult OVX controls and steroid-treated animals. Abbreviations of experimental groups are detailed in the legend of Fig. 2. (A) Schematic representation of the structure of full-length (FL) ERα mRNA and splicing variants. Σ3, Σ4 and Σ3,4 isoforms were created by exon-skipping mechanisms from the ERα primary transcript. The locations of primers on the cDNA for performing the first (E3, E4) and second (E5, E6) round of nested PCR are shown according to the Materials and Methods and Table 1. (B) The existence of four ERα transcripts in the uterus of controls rats with a differential pattern of expression according to each steroid treatment was observed. The lengths of the amplification products are indicated in the center of the figure. Surprisingly, in rats treated with either the low dose of E_2, P_4 alone or the combination of both steroids, expression of the Σ3 isoform was completed abolished. Assays from two independent RNA samples per experimental group are presented (n=7–8 animals/group).

expression of ERα between luminal and glandular cells during preparation of the uterus for implantation, it has been suggested that these two epithelial cell types respond differentially to P_4 and E_2 action (Tan *et al.* 1999).

The ERα gene constitutes a complex unit exhibiting alternative splicing and promoter usage in a tissue-specific manner (Flouriot *et al.* 1998). A relatively high mRNA turnover rate could be one mechanism, in conjunction with transcription controls, in order to maintain the ERα protein at suitable levels. Our results suggest ERα expression in the uterus of adult rats is dependent upon the dose of E_2 and/or co-treatment with P_4. It is interesting to note that only when animals received the high dose of E_2 or P_4 alone did the uterus exhibit a down-regulation of both ERα protein and ERα mRNA.

Alternative splicing of pre-mRNAs is thought to be one of the cellular mechanisms for generating a functionally diverse pool of gene products derived from a single gene and is also recognized as an important mechanism for regulating wild-type proteins (Poola *et al.* 2002). In this work, we observed that three splice variants of ERα mRNA – exhibiting in-frame deletions of exon 3, exon 4, or both – are present in the uterus of both OVX adult rats and normal cycling females. Moreover, for the first time,

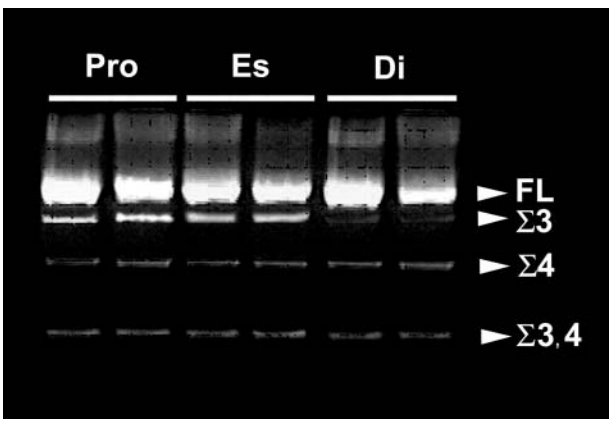

Figure 5 Representative RT-nested PCR assays showing ERα mRNA full length (FL) and the three spliced isoforms (Σ3, Σ4 and Σ3,4) at different stages of the estrous cycle. Animals were killed and uteri collected between 1000 and 1100 h at proestrus (Pro), estrus (Es) or diestrus (Di). Note the very low expression of the Σ3 isoform at diestrus. Assays from two independent RNA samples per experimental group are presented (n=5 animals per stage of estrus cycle).

we observed that alternative splicing of ERα transcripts is influenced by the ovarian steroid hormonal milieu.

The biological relevance of the presence of ERα isoforms in normal tissues is not well established. Their functions may be partially predicted by the structures of those isoforms compared with that of the wild-type ERα (Pasqualini *et al.* 1999). The isoform originating from the deletion of exon 3 was shown to be unable to bind to a canonical ERE (Wang & Miksicek 1991). In the MCF-7 breast cancer cell line, Σ3 inhibits the activation of estrogen-dependent transcription in a dominant negative fashion when cotransfected with the full-length ERα. It has been suggested that Σ3 expression in normal tissue may provide a means of decreasing or blocking estrogen responsiveness (Pasqualini *et al.* 2001).

The presence of the Σ3 isoform may be necessary for regulating the cellular response to estrogens. In our OVX model, those animals that received the high dose of E_2 expressed this splicing isoform, suggesting that Σ3 could exhibit a 'buffer action' in regulating estrogen responsiveness. This 'buffer action' was observed during pituitary gland ontogeny, allowing regulation of ERα activities by differential splicing of the primary transcript (Pasqualini *et al.* 1999). The physiological relevance of the Σ3 isoform is suggested by the fact that it is present during the normal estrous cycle. Moreover, Σ3 isoform mRNA levels are higher during proestrus and estrus when estrogenic effects are maximal. These results support the hypothesis that the Σ3 isoform may play a significant physiological role that is under steroidal control. A recent report indicates that endogenous estrogen differentially regulates pituitary expression of mRNAs encoding several ER isoforms with distinct functional properties (Tena-Sempere *et al.* 2004). This, together with our current results, allows us to postulate that interaction of ERα protein with its ligand not only activates signaling but also turns on different mechanisms for the fine tuning of uterine responsiveness to estrogen. These would include repression of inhibitory isoforms, which may be essential for the full expression of estrogen effects at the ERα, as well as induction of dominant negative isoforms, which may participate in the auto-limitation of estrogen effects. In addition, since the combination of the high dose of E_2 plus P_4 maintained a high expression of the Σ3 isoform, this result might suggest that, at least in part, the antagonistic action of P_4 on E_2-mediated effects may be regulated by the presence of the Σ3 exon-skipping variant. The observation that expression of this variant was not observed with the low dose of E_2 suggests that the ligand-mediated regulation of ERα exon-skipping variant expression may be dose dependent. It has been demonstrated that steroid receptor isoforms are differentially expressed depending on the type of tissue examined and the anatomical localization (Guerra-Araiza *et al.* 2003, Patchev *et al.* 2004). Moreover, the splicing isoforms of ERα are distributed in different subcellular compartments (Pasqualini *et al.* 1999). These results, taken together, strongly support the hypothesis that differential splicing of the ERα transcripts may be an extensive mechanism for controlling ERα-mediated cellular responses.

In summary, this is the first time that the presence of the ERα Σ3 exon-skipping variant has been shown to be regulated by E_2 and P_4 in the rat uterus. The data presented here suggest that this regulation is dose dependent and could be a regulatory mechanism of E_2 action. Actually, we are evaluating ERα mRNA isoforms in each individual cellular compartment of the rat uterus, using a combination of laser capture microdissection and nested real time-PCR. This technology could allow us to better understand the complex regulation of ERα mRNA differential splicing in each individual cell type. Further characterization of this receptor isoform may increase the understanding of the mechanisms of action of therapeutic and environmentally relevant estrogen-like compounds.

Acknowledgements

We are very grateful to Dr Charles E. Powell (Nova Southeastern University, Ft. Lauderdale, FL, USA) for critical reading of the manuscript and helpful discussions, and to Mr Juan Grant and Mr Juan C. Villarreal for technical assistance and animal care.

Funding

This study was supported by grants from the Argentine National Council for Science and Technology (CONICET) (PIP 528/98), the Argentine National Agency for the Promotion of Science and Technology (ANPCyT) (PICT-99 no. 5–7001) and the Universidad Nacional del Litoral (Santa Fe, Argentina). J V, L M and V B are Fellows and J G R and E H L are Career Investigators of CONICET. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

Clarke CL & Sutherland RL 1990 Progestin regulation of cellular proliferation. *Endocrine Reviews* **11** 266–301.

Downing SJ & Sherwood OD 1986 The physiological role of relaxin in the pregnant rat. IV. The influence of relaxin on cervical collagen and glycosaminoglycans. *Endocrinology* **118** 471–479.

Flouriot G, Griffin C, Kenealy M, Sonntag-Buck V & Gannon F 1998 Differentially expressed messenger RNA isoforms of the human estrogen receptor-α gene are generated by alternative splicing and promoter usage. *Molecular Endocrinology* **12** 1939–1954.

Friend KE, Resnick EM, Ang LW & Shupnik MA 1997 Specific modulation of estrogen receptor mRNA isoforms in rat pituitary throughout the estrous cycle and in response to steroid hormones. *Molecular and Cellular Endocrinology* **131** 147–155.

Guerra-Araiza C, Villamar-Cruz O, Gonzales-Arenas A, Chavira R & Camacho-Arroyo I 2003 Changes in progesterone receptor isoforms content in the rat brain during the oestrous cycle and after oestradiol and progesterone treatments. *Journal of Neuroendocrinology* **15** 984–990.

Hopp TA & Fuqua SA 1998 Estrogen receptor variants. *Journal of Mammary Gland Biology and Neoplasia* **3** 73–83.

Kass L, Durando M, Ramos JG, Varayoud J, Powell CE, Luque EH & Muñoz-de-Toro, M 2004 Association of increased estrogen receptor β2 expression with parity-induced alterations in the rat mammary gland. *Journal of Steroid Biochemistry and Molecular Biology* **91** 29–39.

Katsuda SI, Yoshida M, Watanabe T, Kuroda H, Ando-Lu J, Takahashi M, Hayashi H & Maekawa A 1999 Estrogen receptor mRNA in uteri of normal estrous cycling and ovariectomized rats by *in situ* hybridization. *Proceedings of the Society for Experimental Biology and Medicine* **221** 207–214.

Koike S, Sakai M & Muramatsu M 1987 Molecular cloning and characterization of rat estrogen receptor cDNA. *Nucleic Acids Research* **15** 2499–2513.

Kraus WL, Weis KE & Katzenellenbogen BS 1995 Inhibitory cross-talk between steroid hormone receptors: differential targeting of estrogen receptor in the repression of its transcriptional activity by agonist- and antagonist-occupied progestin receptors. *Molecular and Cellular Biology* **15** 1847–1857.

Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S & Gustafsson J-A 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *PNAS* **93** 5925–5930.

Luque EH, Ramos JG, Rodríguez HA & Muñoz-de-Toro MM 1996 Dissociation in the control of cervical eosinophilic infiltration and collagenolysis at the end of pregnancy or after pseudopregnancy in ovariectomized steroid-treated rats. *Biology of Reproduction* **55** 1206–1212.

Manni A, Baker R, Arafah BM & Pearson OH 1981 Uterine oestrogen and progesterone receptors in the ovariectomized rat. *Journal of Endocrinology* **91** 281–287.

Montes GS & Luque EH 1988 Effects of ovarian steroids on vaginal smears in the rat. *Acta Anatomica* **133** 192–199.

Muñoz-de-Toro MM, Maffini MV, Giardina RH & Luque EH 1998 Processing fine needle aspirates of prostate carcinomas for standard immunocytochemical studies and *in situ* apoptosis detection. *Pathology Research and Practice* **194** 631–636.

Nephew KP, Long X, Osborne E, Burke KA, Ahluwalia A & Bigsby RM 2000 Effect of estradiol on estrogen receptor expression in the rat uterine cell types. *Biology of Reproduction* **62** 168–177.

Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ & Scanlan TS 1997 Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. *Science* **277** 1508–1510.

Pasqualini C, Guivarc'h D, Boxberg Y, Nothias F, Vincent JD & Vernier P 1999 Stage- and region-specific expression of estrogen receptor α isoforms during ontogeny of the pituitary gland. *Endocrinology* **140** 2781–2789.

Pasqualini C, Guivarc'h D, Barnier JV, Guibert B, Vincent JD & Vernier P 2001 Differential subcellular distribution and transcriptional activity of ΣE3, ΣE4, and ΣE3–4 isoforms of the rat estrogen receptor-α. *Molecular Endocrinology* **15** 894–908.

Patchev AV, Gotz F & Rohde W 2004 Differential role of estrogen receptor isoforms in sex-specific brain organization. *FASEB Journal* **18** 1568–1570.

Pettersson K, Delaunay F & Gustafsson JA 2000 Estrogen receptor β acts as a dominant regulator of estrogen signaling. *Oncogene* **19** 4970–4978.

Pfeffer U, Fecarotta E & Vidali G 1995 Coexpression of multiple estrogen receptor variant messenger RNAs in normal and neoplastic breast tissues and in MCF-7 cells. *Cancer Research* **55** 2158–2165.

Poola I, Koduri S, Chatra S & Clarke R 2000 Identification of twenty alternatively spliced estrogen receptor α mRNAs in breast cancer cell lines and tumors using splice targeted primer approach. *Journal of Steroid Biochemistry and Molecular Biology* **72** 249–258.

Poola I, Abraham J & Baldwin K 2002 Identification of ten exon deleted ERβ mRNAs in human ovary, breast, uterus and bone tissues: alternate splicing pattern of estrogen receptor β mRNA is distinct from that of estrogen receptor α. *FEBS Letters* **516** 133–138.

Ramos JG, Varayoud J, Bosquiazzo V, Luque EH & Muñoz-de-Toro M 2002 Cellular turnover in the rat uterine cervix and its relationship to estrogen and progesterone receptor dynamics. *Biology of Reproduction* **67** 735–742.

Ramos JG, Varayoud J, Kass L, Rodríguez H, Costabel L, Muñoz-de-Toro M & Luque EH 2003 Bisphenol A induces both transient and permanent histofunctional alterations of the hypothalamic-pituitary-gonadal axis in prenatally exposed male rats. *Endocrinology* **144** 3206–3215.

Reid G, Denger M, Kos M & Gannon F 2002 Review. Human estrogen receptor α: regulation by synthesis, modification and degradation. *Cellular and Molecular Life Sciences* **59** 821–831.

Siegel S 1956 *Nonparametric Statistics for the Behavioral Sciences*. New York: McGraw-Hill.

Tan J, Paria BC, Dey SK & Das SK 1999 Differential uterine expression of estrogen and progesterone receptors correlates with uterine preparation for implantation and decidualization in the mouse. *Endocrinology* **140** 5310–5321.

Tena-Sempere M, Navarro VM, Mayen A, Bellido C & Sánchez-Criado JE 2004 Regulation of estrogen receptor (ER) isoform messenger RNA expression by different ER ligands in female rat pituitary. *Biology of Reproduction* **70** 671–678.

Wang H, Masironi B, Eriksson H & Sahlin L 1999 A comparative study of estrogen receptors α and β in the rat uterus. *Biology of Reproduction* **61** 955–964.

Wang Y & Miksicek RJ 1991 Identification of a dominant negative form of the human estrogen receptor. *Molecular Endocrinology* **5** 1707–1715.

Zhou Y, Chorich LP, Mahesh VB & Ogle TF 1993 Regulation of estrogen receptor protein and messenger ribonucleic acid by estradiol and progesterone in rat uterus. *Journal of Steroid Biochemistry and Molecular Biology* **46** 687–698.

Received 28 March 2005
Accepted 15 April 2005
Made available online as an Accepted Preprint
29 April 2005