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Expression of the Interferon Tau Inducible Ubiquitin Cross-Reactive Protein in the Ovine Uterus[1]

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ABSTRACT

Ubiquitin cross-reactive protein (UCRP) is a 17-kDa protein that shows cross-reactivity with ubiquitin antisera and retains the carboxyl-terminal Leu-Arg-Gly-Gly amino acid sequence of ubiquitin that ligates to, and directs degradation of, cytosolic proteins. It has been reported that bovine endometrial UCRP is synthesized and secreted in response to conceptus-derived interferon-tau (IFNτ). In the present studies, UCRP mRNA and protein were detected in ovine endometrium. Ovine UCRP mRNA was detectable on Day 13, peaked at Day 15, and remained high through Day 19 of pregnancy. The UCRP mRNA was localized to the luminal epithelium (LE), stromal cells (ST) immediately beneath the LE, and shallow glandular epithelium (GE) on Day 13, but it extended to the deep GE, deep ST, and myometrium of uterine tissues by Day 15 of pregnancy. Western blotting revealed induction of UCRP in the endometrial extracts from pregnant, but not cyclic, ewes. Ovine UCRP was also detected in uterine flushings from Days 15 and 17 of pregnancy and immunoprecipitated from Day 17 pregnant endometrial explant-conditioned medium. Treatment of immortalized ovine LE cells with recombinant ovine (ro) IFNτ induced cytosolic expression of UCRP, and intrauterine injection of roIFNτ into ovariectomized cyclic ewes induced endometrial expression of UCRP mRNA. These results are the first to describe temporal and spatial alterations in the cellular localization of UCRP in the ruminant uterus. Collectively, UCRP is synthesized and secreted by the ovine endometrium in response to IFNτ during early pregnancy. Because UCRP is present in the uterus and uterine flushings, it may regulate endometrial proteins associated with establishment and maintenance of early pregnancy in ruminants.

INTRODUCTION

Release of progesterone from the corpus luteum (CL) is essential for maintenance of early pregnancy in sheep [1]. In cyclic ewes, CL function is terminated and ovarian cyclicity is maintained by the action of the luteolytic hormone prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) [2]. Maternal recognition of pregnancy prevents luteolysis and extends CL life span. The ovine conceptus produces interferon tau (IFNτ), the antiluteolytic hormone in ruminants, between the 12th and 13th day of pregnancy [3–7]. IFNτ is thought to suppress transcription of the estrogen receptor gene. This prevents estrogen-induced increases in oxytocin receptor gene expression and formation in the luminal epithelium of the endometrium [8], which abrogates the production and release of luteolytic $PGF_{2\alpha}$.

IFNτ also regulates the de novo synthesis and/or secretion of several uterine proteins [9]. One of these is a 17-kDa endometrial protein that cross-reacts with ubiquitin antisera and, therefore, has been termed ubiquitin cross-reactive protein (UCRP) [10]. Human UCRP has been named IFN-stimulated gene product 15 (ISG15) and possesses homology to a tandem ubiquitin repeat including the carboxyl-terminal RGG amino acid sequence of ubiquitin that is essential for its conjugation to intracellular proteins [11]. ISG15 conjugates to intracellular proteins, and these conjugates are distinct from those of ubiquitin. Thus, interferons may induce an intracellular pathway for UCRP ligation that is parallel to, but distinct from, that of ubiquitin [12, 13]. Austin and coworkers linked UCRP expression to early pregnancy when they identified a 16-kDa protein that was secreted by the bovine endometrium in response to conceptus-derived IFNτ [10, 14]. Sequencing of a cDNA encoding bovine UCRP revealed that it shared 70% identity with ISG15 and 30% identity with a tandem ubiquitin repeat, and the Leu-Arg-Gly-Gly (LRGG) C-terminal sequence was conserved [15]. Bovine UCRP was later shown to conjugate to endometrial cytosolic proteins. These conjugates were distinct from those of ubiquitin and appeared only during early pregnancy or in response to IFNτ in vitro [16]. Up-regulation of UCRP in first-trimester decidua of both humans and baboons has also been reported [17]. Interestingly, monomeric UCRP was not found in these tissues but was always conjugated to other proteins. The objectives of the present experiments were to examine the temporal and spatial expression of UCRP in endometrium from cyclic and early pregnant ewes.

MATERIALS AND METHODS

Animals

Mature Western-range ewes of primarily Rambouillet breeding were observed daily for estrous behavior in the presence of vasectomized rams. After experiencing at least two estrous cycles of normal duration (16–18 days), ewes were assigned randomly on Day 0 (estrus/mating) to be either hysterectomized or fitted with uterine catheters. All experimental and surgical procedures involving animals were approved by the Agricultural Animal Care and Use Committee of Texas A&M University (Animal Use Protocols 7-286 and AG-239AG).

Experiment 1. At onset of estrus (Day 0), ewes were assigned randomly to cyclic or pregnant status. Ewes assigned to pregnant status were mated to intact rams three times at 12-h intervals beginning at estrus. Fifty-two ewes were ovariohysterectomized (n = 4 ewes/day) on Day 1, 3, 5, 7, 9, 11, 13, or 15 of the estrous cycle or Day 11, 13,

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15, 17, or 19 of gestation. At hysterectomy, uteri were flushed with 0.9% NaCl, and flushes were frozen at −80°C. Pregnancy was verified by recovery of an apparently normal conceptus in uterine flushes. Several sections (1–1.5 cm) of uterine wall from the middle of each uterine horn were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). The remaining endometrium was dissected from myometrium, frozen in liquid nitrogen, and stored at −80°C.

Experiment 2. Eight cyclic ewes were fitted with uterine catheters on Day 5 of the estrous cycle as previously described [18]. Ewes (n = 4 ewes per treatment) then received intrauterine injections (1 ml/horn) of either control proteins (ovine serum proteins; 6 mg/day) or recombinant ovine (ro) IFNτ (1×10^7 antiviral units/horn/day) from Days 11 to 15 postestrus. The uterine horns of each ewe received twice-daily (0700 h and 1900 h) injections of either roIFNτ (5×10^6 antiviral units/horn per injection) or control proteins (equal amount of total protein/horn per injection). All ewes were ovariohysterectomized on Day 16. Endometrium was dissected from myometrium and frozen in liquid nitrogen.

Experiment 3. Endometrium from three Day 17 pregnant ewes was collected by dissection and placed into warm Dulbecco's Modified Eagle's medium (DMEM)/F12 culture medium (Sigma, St. Louis, MO) containing penicillin G (100 IU/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 μg/ml; Gibco-BRL, Grand Island, NY). The endometrium was then minced with scalpel blades into small pieces (2–3 cubic mm). Aliquots of 500 mg minced endometrium were placed into culture dishes (100 × 15 mm) with 5 ml of cysteine-methionine-deficient DMEM culture medium (Sigma) containing 50 μCi/ml of [^{35}S]methionine-cysteine (Promix; Amersham Life Sciences, Arlington Heights, IL) for incorporation into newly synthesized proteins. Recombinant oIFNτ was added to cultures at 10^4 antiviral units/ml, and cultures were incubated for 24 h with rocking under an atmosphere of 45% nitrogen, 5% carbon dioxide, and 50% oxygen. The culture medium was then harvested, centrifuged (3000 × *g* for 10 min at 4°C), and stored at −80°C.

Experiment 4. At onset of estrus (Day 0), a ewe was assigned to be hysterectomized on Day 5 of the estrous cycle. Uterine luminal epithelium (LE) cells were isolated as previously described [19]. Primary ovine LE cells were immortalized by transduction with a retroviral vector (LXSN-16E6E7) packaged by the amphoteric fibroblast line PA317 as previously described [20]. Culture supernatants from the PA317 producer cells (American Type Culture Collection) were used with polybrene (4 μg/ml; Sigma) to transfect primary cell lines in their third passage. Cells having integrated the vector were selected by resistance to the neomycin analog G418 (800 μg/ml; Gibco-BRL). The G418-resistant LE populations were subcultured and maintained in G418-supplemented (100 μg/ml) medium for > 30 passages.

Northern Blot Analysis

Complementary cDNA probes were generated through random primer reactions (Life Technologies Inc., Grand Island, NJ). Bovine UCRP cDNA [15] was labeled using 50 μCi [^{32}P-α]dCTP (New England Nuclear, Boston, MA) and Klenow enzyme. One hundred milligrams of homogenized endometrial tissue was extracted for total cellular RNA using Tri Reagent (Molecular Research Inc., Cincinnati, OH) as previously described [21]. For Northern blot analysis, total cellular RNA was loaded (10 μg/lane) onto 1.5% agarose gels, electrophoresed, transferred to 0.2-μm nylon membranes, prehybridized, hybridized, and placed on Kodak XAR film (Eastman Kodak, Rochester, NY) for 48 h as previously described [21].

Slot Blot Hybridization Analysis

Total cellular RNA was isolated from endometrium using Trizol reagent (Gibco-BRL). The quantity of RNA was assessed spectrophotometrically, and integrity of RNA was examined by electrophoresis in a denaturing 1% agarose gel [22]. Steady-state levels of UCRP mRNA were measured in endometrial samples using slot blot hybridization analysis. For each ewe, denatured total cellular RNA (20 μg) was hybridized with radiolabeled antisense cRNA probes generated by in vitro transcription with [α-^{32}P]UTP (Amersham) as previously described [18]. Plasmid templates for bovine UCRP (pKA16) [15] and 18 S rRNA (pT718S; Ambion, Austin, TX) were used. The radioactivity in each slot was quantitated using an Instant Imager (Packard, Meriden, CT) and expressed as total counts.

In Situ Hybridization Analysis

The UCRP mRNA was localized in uterine tissue sections by in situ hybridization analysis. Uterine tissue sections were deparaffinized in xylene and then rehydrated to water through a graded series of alcohol. Tissue sections were postfixed in 4% paraformaldehyde in PBS and then digested with proteinase K (20 μg/ml) in PK digestion buffer (50 mM Tris, 5 mM EDTA, pH 8) for 8 min at 37°C. Sections were then refixed for 5 min in 4% paraformaldehyde, rinsed twice for 5 min each in PBS, dehydrated through a graded series of alcohol, and then dried at room temperature for 30 min. Sections were hybridized with radiolabeled antisense or sense cRNA probes generated from a linearized bovine UCRP (pKA16) plasmid template using in vitro transcription with [α-^{35}S]UTP (specific activity: 3000 Ci/mmol; Amersham). Radiolabeled cRNA probes (5×10^6 cpm/slide) were denatured in 75 μl hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris-HCl [pH 8], 5 mM EDTA [pH 8], 10 mM sodium phosphate [pH 8], single-strength Denhardt's solution, 10% dextran sulfate, 0.5 mg/ml yeast RNA, 100 mM dithiothreitol [DTT]) at 70°C for 10 min. Hybridization solution was applied to the middle of each slide, and a coverslip was placed gently on top. Slides were then incubated in a humidified chamber containing 50% formamide/5-strength SSC (single-strength SSC is 0.15 M sodium chloride, 0.015 M sodium citrate) and hybridized overnight at 55°C. Coverslips were floated off the slides by placement in 5-strength SSC/10 mM β-mercaptoethanol (βME) for 30 min at 55°C, and remaining coverslips were gently removed with a pair of forceps. Sections were then washed as follows: 50% formamide/double-strength SSC/50 mM βME for 20 min at 65°C; single-strength TEN (0.05 M NaCl/10 mM Tris [pH 8]/5 M EDTA) for 10 min at room temperature; and then three times in single-strength TEN for 10 min at 37°C. Sections were then digested with deoxyribonuclease (DNase)-free ribonuclease (RNase; 10 μg/ml) in single-strength TEN for 30 min at 37°C to remove nonspecifically bound probe and washed as follows: single-strength TEN for 30 min at 37°C; 50% formamide/double-strength SSC/50 mM βME for 20 min at 65°C; double-strength SSC for 15 min at room tem-

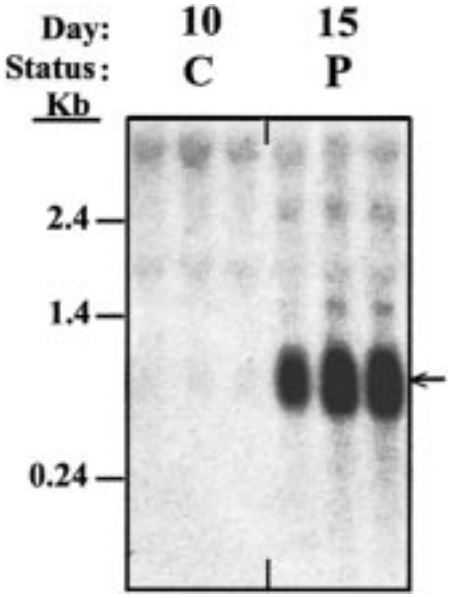

FIG. 1. Northern blot analysis of UCRP mRNA in ovine endometrium from cyclic (C) and pregnant (P) ewes. Each lane (10 μg/lane) represents total endometrial mRNA from a different ewe. Positions of RNA standards (kb) are indicated. The UCRP mRNA (arrow) was detected only in endometrium from pregnant ewes.

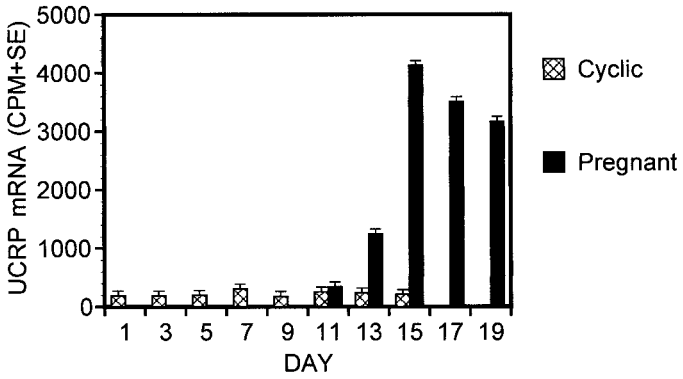

FIG. 2. Quantitation of UCRP mRNA (20 μg/slot) in ovine endometrium during the estrous cycle and pregnancy using slot blot hybridization and an Instant Imager (cpm). All cpm values were normalized against quantitation of duplicate blots hybridized with an 18 S rRNA probe (n = 3 to 4 ewes/day). There was a day-by-pregnancy status interaction ($p < 0.05$) and, within the pregnant ewes, an effect of day ($p < 0.05$) was detected.

perature; 0.1-strength SSC for 12 min at room temperature; 70% ethyl alcohol, ethyl hydroxide containing 0.3 M ammonium acetate for 5 min at room temperature; 95% ethanol containing 0.03 M ammonium acetate for 1 min at room temperature; twice in 100% ethanol; and three times in single-strength TEN for 10 min at 37°C. Liquid film emulsion autoradiography was performed using Kodak NTB-2 liquid photographic emulsion. Slides were stored at 4°C for 5 days, developed in Kodak D-19 developer, counterstained with Harris' modified hematoxylin in acetic acid (Fisher, Fairlawn, NJ), dehydrated through a graded series of alcohol to xylene, coverslipped, and evaluated by both brightfield and darkfield microscopy with a Zeiss Photomicroscope III (Carl Zeiss Inc., Thornwood, NY).

Western Blot Analysis

Endometrium was thawed and immediately homogenized in extraction buffer (10 mM Tris [pH 7], 1 mM EDTA, 1 mM DTT, 100 μg/ml PMSF) at a ratio of 1 g tissue per 5 ml buffer. Homogenates were sonicated for 30 sec with a Mini Ultrasonic Cell Disrupter (Sonics & Materials, Inc., Danbury, CT), and cellular debris was cleared by centrifugation (10 000 × *g* for 15 min at 4°C). Uterine flushings were thawed and concentrated by ultrafiltration (1 h; 2000 × *g*) over M_r 3000 cut-off membranes (Amicon, Danvers, MA).

Concentrations of protein in endometrial extracts and uterine flushings were determined using a Bradford protein assay (Bio-Rad Laboratories, Richmond, CA) with BSA as the standard. Proteins in endometrial extracts (150 μg) or uterine flushings (60 μg) were denatured in Laemmli buffer, separated on 15% (total monomer) SDS-PAGE gels, and transferred to nitrocellulose as previously described [23]. Blots were blocked overnight in TBST (20 mM Tris [pH 7.5], 137 mM NaCl, 0.05% Tween 20) containing 5% dried milk. Blots were washed three times for 5 min each in TBST and then incubated with polyclonal rabbit anti-human (h) UCRP serum (5 μg/ml), kindly donated by Dr. Ernest Knight, or normal rabbit serum (5 μg/ml) in TBST containing 2% dried milk while rocking overnight at 4°C. Blots were washed three times for 10 min each in TBST and placed in goat anti-rabbit IgG-horseradish peroxidase conjugate (1:15 000 dilution of 1 mg/ml stock; KPL, Bethesda, MD) for 1 h at room temperature while rocking. Blots were washed three times for 10 min each in TBST, and immunoreactive proteins were detected using enhanced chemiluminescence (Amersham) and X-OMAT AR film (Kodak).

Immunoprecipitation Assay

Proteins in endometrial explant culture medium (200 μl) were immunoprecipitated as previously described [23] with modifications. Explant culture medium was placed in microfuge tubes and brought to 1 ml with IPH lysis buffer (50 mM Tris-HCL [pH 8], 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 0.1 mM PMSF). Polyclonal rabbit anti-h-UCRP serum (5 μg) or normal rabbit serum (5 μg) was added to relevant tubes and incubated with rocking at 4°C for 1 h. Protein A/G-sepharose beads (20 μl; Santa Cruz Biotechnology, Santa Cruz, CA) were added, and the mixture was incubated with rocking at 4°C overnight. Beads were then pelleted by centrifugation (1000 × *g* for 3 min) and washed four times with 1 ml of IPH lysis buffer. All centrifuging and washing steps were performed at 4°C. Remaining wash buffer was removed from pellets, beads were solubilized in 40 μl SDS-PAGE loading buffer and then denatured at 70°C for 5 min, cooled on ice for 2 min, and centrifuged at 12 000 × *g* for 10 min. Supernatants were loaded onto a 15% SDS-PAGE gel along with prestained molecular weight standards. Gels were impregnated with a fluorographic enhancer (Amplify; Amersham), dried, and placed on Kodak XRP film for 7 days at −80°C.

Statistical Analysis

Data were subjected to least-squares ANOVA using the general Linear Models (GLM) procedures of the Statistical Analysis System [24]. Slot blot hybridization data (total counts) were normalized for differences in sample loading using the 18 S rRNA data as a covariate. All tests of statistical significance were performed using the appropriate error terms according to the expectation of mean squares. Data are presented as least-squares means (LSM) with standard errors (SE).

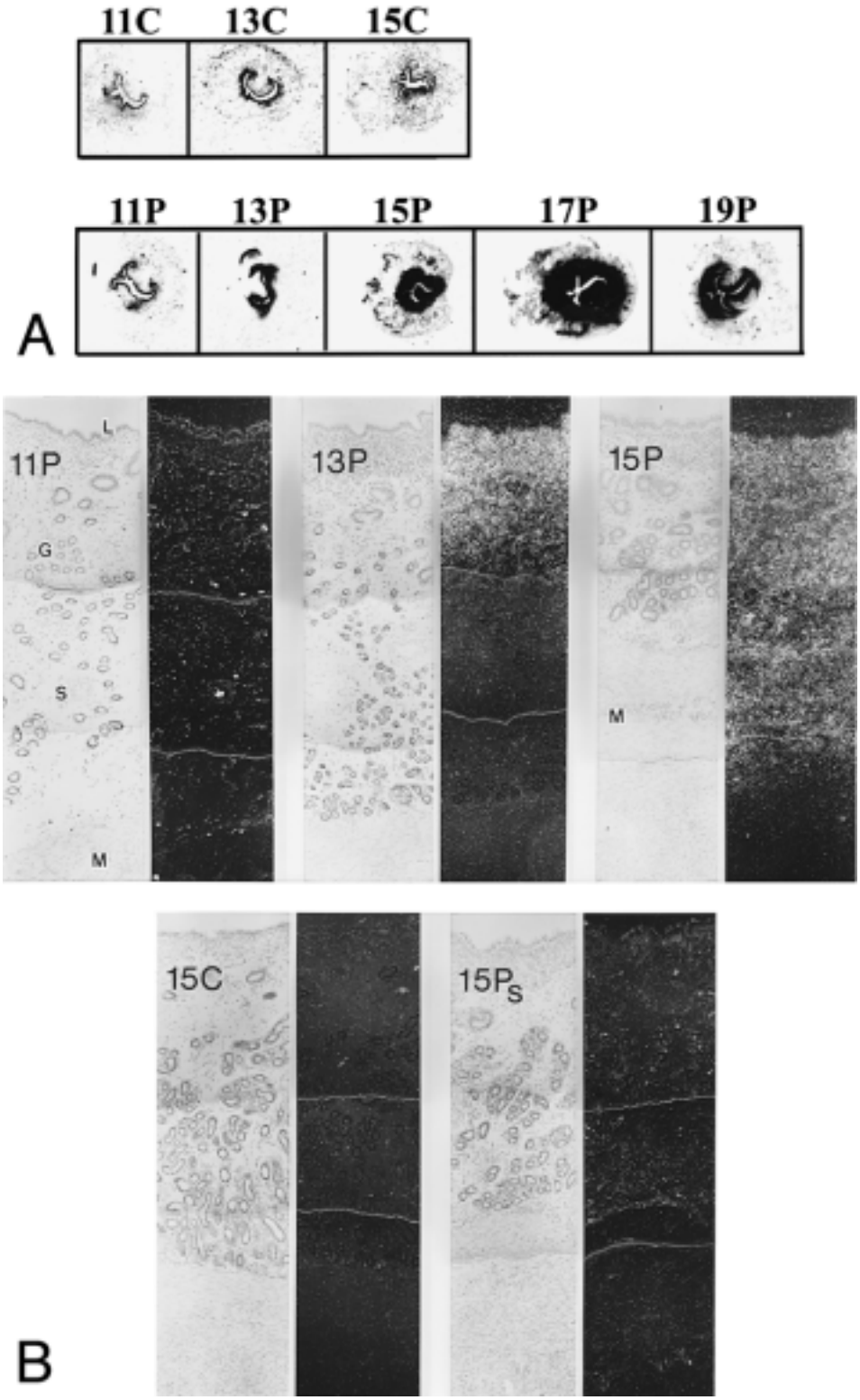

FIG. 3. Expression of UCRP mRNA in ovine endometrium during the estrous cycle and pregnancy using in situ hybridization. **A**) Representative autoradiographic images (Biomax-MR; Kodak) showing entire cross sections of the uterine wall from Days 11, 13, and 15 in cyclic (C) ewes and from Days 11, 13, 15, 17, and 19 in pregnant (P) ewes. Magnification = ×1 (published at 70%). **B**) Representative photomicrographs of sectioned uteri in both brightfield and darkfield illumination from Days 11, 13, and 15 of pregnancy. Representative sections from a Day 15 cyclic ewe and a Day 15 pregnant ewe (negative control) hybridized with radiolabeled sense cRNA probe (15 P_s) are shown. Sections were counterstained with hematoxylin. L, Luminal epithelium; G, glandular epithelium; S, stroma; M, myometrium. ×60 (published at 70%).

RESULTS

Northern Blot, Slot Blot, and In Situ Hybridization Analysis of UCRP mRNA in Ovine Endometrial Total RNA Extracts and Paraffin-Embedded Sections

Detection of UCRP mRNA in ovine endometrium during the estrous cycle and pregnancy by Northern blotting (Fig. 1), and quantitation of temporal changes in expression by slot blot hybridization (Fig. 2) indicated that UCRP mRNA was not present in endometrium from cyclic ewes. However, UCRP mRNA expression increased ($p < 0.05$) between Days 13 and 15 of pregnancy and remained high through Day 19 of pregnancy. The mRNA transcript of 0.65 kilobases (kb) was similar in size to that described in bovine endometrium [21].

In situ hybridization analysis of uterine cross sections revealed increased abundance of UCRP mRNA between Days 11 and 13 of pregnancy (Fig. 3A). The transcript was local-

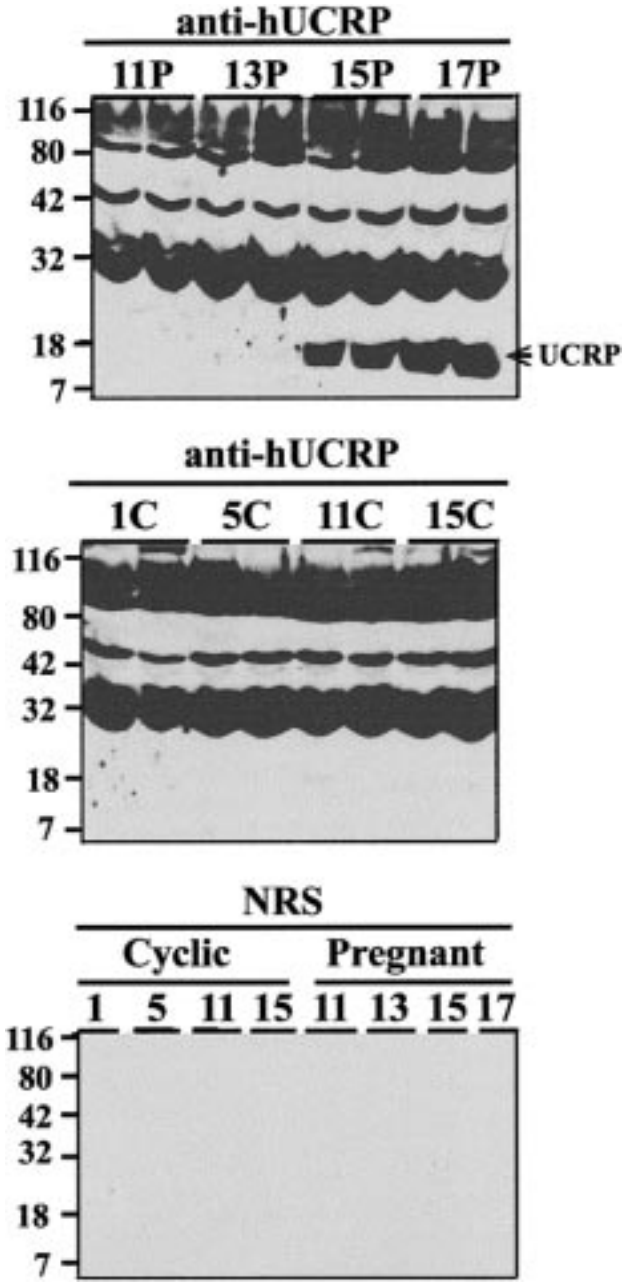

FIG. 4. Detection of UCRP (15% PAGE) in ovine endometrial extracts from cyclic (C) and pregnant (P) ewes using Western blotting. Endometrium is from Days 1, 5, 11, or 15 of the estrous cycle or Days 11, 13, 15, or 17 of pregnancy. Each lane (150 μg/lane) represents endometrial cytosolic extracts from a different ewe. Immunoreactive proteins were detected using either polyclonal rabbit anti-hUCRP or NRS as a control. Positions of prestained molecular weight standards ($\times 10^{-3}$) are indicated. The UCRP was detected only in endometrial extracts from ewes on Days 15 and 17 of pregnancy. Because the polyclonal rabbit anti-hUCRP serum cross-reacts with ubiquitin as well as proteins conjugated to both UCRP and ubiquitin, the immunoreactive bands of higher molecular weight in these blots are presumed to be proteins conjugated to UCRP and/or ubiquitin [12, 16].

ized in low amounts to the LE on Day 11 and was observed in the stratum compactum layer of the stromal cells (ST) and the shallow glandular epithelium (GE) on Day 13; then expression extended into the deep GE, stratum spongiosum layer of the ST, and myometrium on Days 15 through 19 in pregnant ewes (Fig. 3B). The UCRP mRNA decreased to undetectable levels in the LE coincident with expression of transcript in deeper endometrial cells and myometrium.

Western Blot Analysis of Immunoreactive UCRP in Endometrial Extracts and Uterine Flushings

Evidence for immunoreactive UCRP in endometrium from cyclic and pregnant ewes is shown in Figure 4. The UCRP (17 kDa) was detected in endometrial extracts from pregnant ewes, but not in extracts from cyclic ewes. UCRP in endometrium was not detectable on Days 11 and 13 of pregnancy but was detectable on Days 15 and 17 of pregnancy. Controls in which normal rabbit serum (NRS) replaced polyclonal anti-hUCRP primary antibody showed no cross reacting 17-kDa bands. The UCRP was also detected in uterine flushings by Western blotting on Days 15 and 17 of pregnancy (Fig. 5).

Immunoprecipitation Analysis of UCRP in Endometrial Explant Culture Medium

Figure 6 illustrates results of an immunoprecipitation assay using proteins in Day 17 pregnant ovine endometrial explant-conditioned culture medium 24 h after culture in the presence of roIFNτ. Rabbit anti-hUCRP serum, but not normal rabbit serum, specifically immunoprecipitated a radiolabeled protein of 17 kDa, which was identical in size to immunoreactive UCRP in ovine endometrial extracts and uterine flushings.

In Vivo Up-Regulation of Uterine UCRP mRNA by roIFNτ

Injection of roIFNτ into uteri of cyclic ewes increased ($p < 0.05$) steady-state levels of endometrial UCRP mRNA (1065 ± 50 cpm) compared to levels in ewes that had received injections of control proteins (275 ± 50 cpm).

In Vitro Up-Regulation of UCRP by roIFNτ

Incubation of immortalized ovine LE cells with roIFNτ induced cytosolic expression of UCRP after 6 h, and expression was maintained through 48 h (Fig. 7). Expression of ubiquitin in LE cells was not altered by treatment with roIFNτ.

DISCUSSION

This is the first report of UCRP expression in the ovine uterus. More importantly, the expression of UCRP is coordinate with ovine IFNτ production [25]. Indeed, intrauterine infusion of roIFNτ into cyclic ewes induced expression of endometrial UCRP mRNA. Both mRNA and protein were expressed maximally in endometrium by Day 15 of pregnancy, and UCRP was detectable in uterine flushings from pregnant ewes by Day 15. Of particular interest is the temporal and spatial expression of UCRP mRNA in the uterine wall. In pregnant ewes, UCRP mRNA increased first in the LE, then in the shallow GE and stratum compactum layer of the ST. This was followed by a rapid increase in expression throughout the endometrium and extending through the myometrium, which was coincident with a loss of UCRP mRNA in the LE. This pattern of expression is similar to that for the IFNτ-induced Mx protein [26]. IFNτ binds type I IFN receptors that are present on cells of the uterine endometrium [27] but accessible only when expressed by endometrial LE and shallow GE [28]. Because IFNτ cannot be detected in venous or lymphatic drainage [29], this trophectoderm-specific cytokine may induce secretion of an ''interferonmedin'' from the basolateral surface of the epithelium that acts as a paracrine stimulator of IFNτ responses in stroma and myometrium [30].

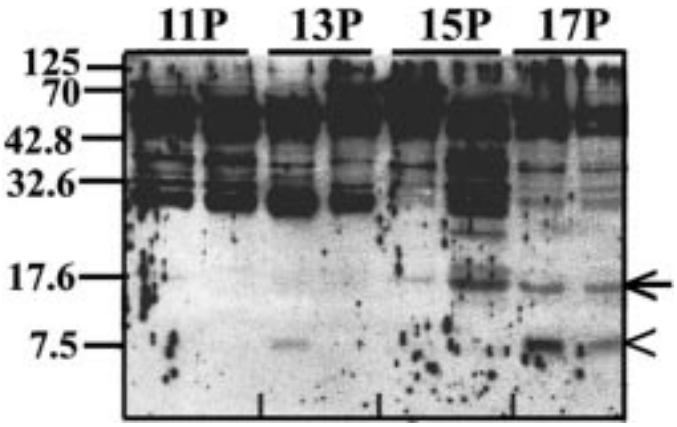

FIG. 5. Detection of UCRP (15% PAGE) in ovine uterine flushings using Western blotting. Each lane (60 μg/lane) represents proteins in uterine flushings (concentrated over 3000 *M*, cut-off membrane) from different ewes. Immunoreactive proteins were detected using polyclonal rabbit anti-hUCRP serum. Positions of prestained molecular weight standards ($\times 10^{-3}$), UCRP (closed arrow), and ubiquitin (open arrow) are indicated. The UCRP was detected in uterine flushings from ewes on Days 15 and 17 of pregnancy. The immunoreactive bands of higher molecular weight are presumed to be proteins conjugated to UCRP and/or ubiquitin.

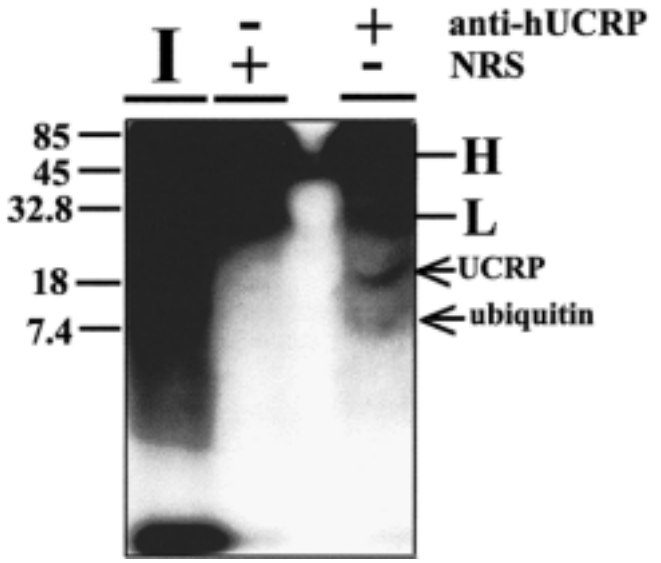

FIG. 6. Fluorograph and one-dimensional PAGE (15%) of immunoprecipitation of radiolabeled UCRP in Day 17 pregnant endometrial explants after culture for 24 h with roIFNτ. The input (I; endometrial explant culture medium before immunoprecipitation) lane is on the left. The middle lane contains proteins immunoprecipitated with NRS. Proteins in the right lane were immunoprecipitated with polyclonal rabbit anti-hUCRP serum. Positions of the heavy (H) and light (L) IgG chains, UCRP, and ubiquitin are indicated. Positions of prestained molecular weight standards ($\times 10^{-3}$) are indicated on the left.

However, we note that IFNτ may act directly on each cell type within the uterus. Isolated ovine LE, GE, and stromal cell lines all express UCRP in response to roIFNτ treatment (unpublished observation).

Ubiquitin cross-reactive protein is a functional ubiquitin homolog [12, 16, 17] that retains the C-terminal RGG amino acid sequence [10, 11] required for the first step in covalent conjugation to cytosolic proteins [31]. Activated ubiquitin ligates through the carboxyl terminal glycine residue to a lysine side-chain of the substrate protein. Additional ubiquitination can be accepted by the Lys-48 of ubiquitin [32]. This ligation process is termed ubiquitination or conjugation, and the target protein/ubiquitin complexes are referred to as conjugates. The primary site for targeted protein degradation is a 26 S cytosolic ubiquitin receptor and protease complex called the proteasome [33]. Substrate proteins are cleaved from ubiquitin moieties by isopeptidases, and ubiquitin is recycled [34]. However, conjugated proteins are not always targeted for proteasomal degradation. Monoubiquitinated proteins can be stabilized, modified, or activated by the conjugation pathway [35–39].

Whether UCRP helps to prepare the ovine uterus for the implanting conceptus is highly speculative yet intriguing in the context of its potential functionality. UCRP might ligate to and alter or initiate proteasomal degradation of cytosolic uterine proteins that are involved with uterine $PGF_{2\alpha}$ release. Candidates include estrogen and/or oxytocin receptors [8], enzymes including prostaglandin synthase [40], or transcription factors regulating genes encoding proteins such as the signal transducers and activators of transcription (STATs) or interferon regulatory factors (IRFs) [25, 28]. Likewise, enzymes involved in synthesis of PGE_2 could be activated or stabilized by conjugation to UCRP [41]. UCRP may selectively modulate or degrade elements of the immune response within the uterus [42]. Ligation of UCRP to intracellular proteins might influence molecular interactions involved in apposition and attachment of trophectoderm to the uterine LE surface to coordinate the implantation adhesion cascade [43]. Also, UCRP might conjugate to proteins in uterine tissues to inhibit the induction of apoptosis by type I IFNs, and thus circumvent the toxic effects of high doses of IFNτ. Indeed, sentrin, a ubiquitin-related protein, conjugates to Fas and tumor necrosis factor to inhibit induction of cell death by these proteins [44, 45].

Although UCRP probably functions to modulate and eliminate proteins within the cell, the physiological relevance of UCRP secretion or release by cells is not clear. Secretion of ubiquitin has only recently been reported, and a specific role for extracellular ubiquitin has not been advanced [10]. It is possible that UCRP release is simply a by-product of increased protein synthesis within the cell and is not a direct response to early pregnancy signals. However, since UCRP is found in ovine uterine flushings and is released from endometrial explants in response to IFNτ, it may have extracellular endocrine, exocrine, or paracrine roles that are conducive to the maintenance of early pregnancy.

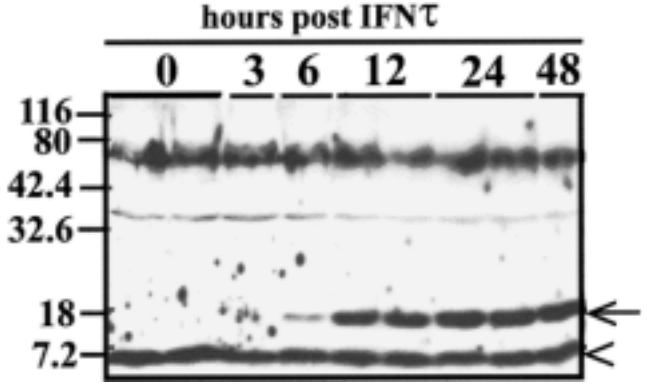

FIG. 7. Detection of UCRP (12% PAGE) in immortalized LE cells that were incubated in the presence of roIFNτ for 0–48 h. Immunoreactive UCRP (closed arrow) was detected using rabbit anti-hUCRP serum between 6 and 48 h, while expression of ubiquitin (open arrow) was constitutive. Positions of prestained molecular weight standards ($\times 10^{-3}$) are indicated. The immunoreactive bands of higher molecular weight are presumed to be proteins conjugated to UCRP and/or ubiquitin.

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REFERENCES

1. Short RV. Implantation and the maternal recognition of pregnancy, In: Foetal Autonomy. Churchill, London: Ciba Foundation Symposium; 1969: 2.
2. Horton EW, Poyser NL. Uterine luteolytic hormones: a physiological role for prostaglandin F2α. Physiol Rev 1976; 56:595–651.
3. Thatcher WW, Bartol FF, Knickerbocker JJ, Curl JS, Wolfenson D, Bazer FW, Roberts RM. Maternal recognition of pregnancy in cattle. J Dairy Sci 1985; 67:2791–2811.
4. Moor RM, Rowsen LEA. The corpus luteum of sheep: effect of the removal of embryos on luteal function. J Endocrinol 1966; 34:497–502.
5. Godkin JD, Bazer FW, Thatcher WW, Roberts RM. Proteins released by cultured day 15–16 conceptuses prolong luteal maintenance when introduced into the uterine lumen of cyclic ewes. J Reprod Fertil 1984; 71:57–64.
6. Vallet JL, Bazer FW, Fliss MFV, Thatcher WW. Effect of ovine conceptus secretory proteins and purified ovine trophoblast protein-1 on interoestrous interval and plasma concentrations of prostaglandins F2α and E2 and of 13,14 dihydro-15-keto prostaglandin F2α in cyclic ewes. J Reprod Fertil 1988; 84:493–504.
7. Ott TL, Van Heek G, Johnson HM, Bazer FW. Cloning and expression in *S. cerevisiae* of a synthetic gene for the type-I trophoblast interferon ovine trophoblast protein-1: purification and antiviral activity. J Interferon Res 1991; 11:357–364.
8. Spencer TE, Becker WC, George P, Mirando MA, Ogle TF, Bazer FW. Ovine interferon-τ regulates expression of endometrial receptors for estrogen and oxytocin but not progesterone. Biol Reprod 1995; 53:732–745.
9. Vallet JL, Bazer FW, Roberts RM. The effect of ovine trophoblast protein-one on endometrial protein secretion and cyclic nucleotides. Biol Reprod 1987; 37:1307–1316.
10. Austin KJ, Ward SK, Teixeira MG, Dean VC, Moore DW, Hansen TR. Ubiquitin cross-reactive protein is released by the bovine uterus in response to interferon during early pregnancy. Biol Reprod 1996; 54:600–606.
11. Haas AL, Ahrens D, Bright PM, Amhel H. Interferon induces a 15-kilodalton protein exhibiting marked homology to ubiquitin. J Biol Chem 1987; 262:11315–11323.
12. Loeb KR, Haas AL. The interferon-inducible 15-kDa ubiquitin homolog conjugates to intracellular proteins. J Biol Chem 1992; 267: 7806–7813.
13. Narasimhan J, Potter JL, Haas AL. Conjugation of the 15-kDa interferon-induced ubiquitin homolog is distinct from that of ubiquitin. J Biol Chem 1996; 271:324–330.
14. Naivar KA, Ward SK, Austin KJ, Moore DW, Hansen TR. Secretion of bovine uterine proteins in response to type-1 interferons. Biol Reprod 1995; 52:848–854.
15. Austin KJ, Pru JK, Hansen TR. Complementary deoxyribonucleic acid sequence encoding bovine ubiquitin cross-reactive protein: a comparison with ubiquitin and a 15-kDa ubiquitin homolog. Endocrine 1996; 5:191–197.
16. Johnson GA, Austin KJ, Van Kirk EA, Hansen TR. Pregnancy and interferon-tau induce conjugation of bovine ubiquitin cross-reactive protein to cytosolic uterine proteins. Biol Reprod 1998; 58:898–905.
17. Bebington C, Fleming S, Doherty F, Tippler C, Fazleabas A. Detection and localization of ubiquitin and ubiquitin cross-reactive protein in the primate endometrium and decidua. In: Serono Symposia USA. International Symposium on Embryo Implantation: Molecular, Cellular and Clinical Aspects; 1997: 29 (abstract).
18. Spencer TE, Ing NA, Ott TL, Mayes JS, Becker WC, Watson GH, Mirando MA, Bazer FW. Intrauterine injection of ovine interferon-tau (oIFN-τ) alters oestrogen receptor and oxytocin receptor expression in the endometrium of cyclic ewes. J Mol Endocrinol 1995; 15:203–220.
19. Newton GR, Weise DW, Bowen JA, Woldensenbet S, Burghardt RC. Regulation of protein and prostaglandin secretion in polarized primary cultures of caprine uterine epithelial cells. In Vitro Cell Dev Biol 1998; 34:578–584.
20. Finchorova RW, Rheinwald JG, Anderson DJ. Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins. Biol Reprod 1997; 57:847–855.
21. Hansen TR, Austin KJ, Johnson GA. Transient ubiquitin cross-reactive protein gene expression in the bovine endometrium. Endocrinology 1997; 138:5079–5082.
22. Ausebel FM, Brent R, Kinston RE, Moore DD, Seidmen JG, Smith HA, Struhl KA. Current Protocols in Molecular Biology. New York: John Wiley and Sons; 1992.
23. Spencer TE, Jenster G, Burcin M, McKenna N, Allis CD, Zhou J, Onate S, Tsai SY, Tsai M-J, O'Malley BW. Steroid receptor coactivator one (SRC-1) is a histone acetyltransferase. Nature 1997; 389: 194–196.
24. SAS. SAS User's Guide: Statistics, Version 6. Cary, NC: Statistical Analysis System Institute, Inc.; 1990.
25. Bazer FW, Spencer TE, Ott TL. Interferon tau: a novel pregnancy recognition signal. Am J Reprod Immunol 1996; 37:412–420.
26. Ott TL, Spencer TE, Lin JY, Kim H-T, Gerami B, Bartol FF, Wiley AA, Bazer FW. Effects of the estrous cycle and early pregnancy on uterine expression of Mx protein in sheep (*Ovis aries*). Biol Reprod 1998; 59:784–789.
27. Hansen TR, Kazemi M, Keisler DH, Malathy PV, Imakawa K, Roberts RM. Complex binding of the embryonic interferon, ovine trophoblast protein-1, to endometrial receptors. J Interferon Res 1989; 9:215–225.
28. Spencer TE, Ott TL, Bazer FW. Expression of interferon regulatory factors one and two in the ovine endometrium: effects of pregnancy and ovine interferon tau. Biol Reprod 1998; 58:1154–1162.
29. Lamming GE, Wathes DC, Flint APF, Payne JH, Stevenson KR, Vallet JL. Local actions of trophoblast interferons in suppression of the development of oxytocin and oestradiol receptors in ovine endometrium. J Reprod Fertil 1995; 105:165–175.
30. Spencer TE, Ott TL, Bazer FW. τ-Interferon: pregnancy recognition signal in ruminants. Proc Soc Exp Biol Med 1996; 213:215–229.
31. Wilkinson KD, Audhya TK. Stimulation of ATP-dependent proteolysis requires ubiquitin with the COOH-terminal sequence Arg-Gly-Gly*. J Biol Chem 1981; 256:9235–9241.
32. Finley D, Chau V. Ubiquitination. Annu Rev Cell Biol 1991; 7:25–69.
33. Hochstrasser M. Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. Curr Opin Cell Biol 1995; 7:215–223.
34. Wilkinson KD, Tashayev VL, O'Connor LB, Larsen CN, Kasperek E, Pickart CM. Metabolism of the polyubiquitin degradation signal; structure mechanism, and role of isopeptidase T. Biochemistry 1995; 34:14535–14546.
35. Murti KG, Smith HT, Fried VA. Ubiquitin is a component of the microtubule network. Proc Natl Acad Sci USA 1988; 85:3019–3023.
36. Ball E, Karlik CC, Beall CJ, Seville DL, Sparrow JC. Athrin, a myofibrillar protein of insect flight muscle is an actin-ubiquitin conjugate. Cell 1987; 51:221–228.
37. Cenciarelli C, Damen H, Kou-Ching H, Rellahan BL, Wiest DL, Smith HT, Fried VA, Weissman AM. Activation-induced ubiquitination of the T cell antigen receptor. Science 1992; 257:795–797.
38. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T. The ubiquitin-proteasome pathway is required for processing of the NF-κB1 precursor protein and the activation of NF-κB. Cell 1994; 78:773–785.
39. Hicke L, Riezman H. Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. Cell 1996; 84:277–287.
40. Thatcher WW, Meyer MD, Danet-Desmoyers G. Maternal recognition of pregnancy. J Reprod Fertil Suppl 1995; 49:15–28.
41. Asselin E, Lacroix D, Fortier MA. IFN-τ increases PGE_2 production and Cox-2 gene expression in the bovine endometrium in vitro. Mol Cell Endocrinol 1997; 132:117–126.
42. Beer AE. The paradox of feto-placental units as allografts. In: Talevar GP (ed.), Recent Advances in Reproduction and Regulation of Fertility. New York: Elsever's/North-Holland Biomedical Press; 1979.
43. Burghardt RC, Bowen JA, Newton GR, Bazer FW. Extracellular matrix and the implantation cascade in pigs. J Reprod Fertil Suppl 1997; 52:151–164.
44. Okura T, Gong L, Kamitani T, Wada T, Okura I, Wei C, Chang H, Yeh ETH. Protection against Fas/APO-1 and tumor necrosis factor-mediated cell death by a novel protein, sentrin. J Immunol 1996; 157: 4277–4281.
45. Gong L, Kamitani T, Fujise K, Caskey LS, Yeh ETH. Preferential Interaction of sentrin with a ubiquitin-conjugating enzyme, Ubc9. J Biol Chem 1997; 272:28198–28201.