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Interferon Regulatory Factor-Two Restricts Expression of Interferon-Stimulated Genes to the Endometrial Stroma and Glandular Epithelium of the Ovine Uterus[1]

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ABSTRACT

Interferon tau (IFNτ) is the signal for maternal recognition of pregnancy in ruminants. The positive effects of IFNτ on IFN-stimulated gene (ISG) expression are mediated by ISG factor 3 (ISGF3), which is composed of signal transducer and activator of transcription (Stat) 1, Stat 2, and IFN regulatory factor-9 (IRF-9), and by gamma-activated factor (GAF), which is a Stat 1 homodimer. Induction of ISGs, such as ISG17 and 2′,5′-oligoadenylate synthetase, by IFNτ during pregnancy is limited to the endometrial stroma (S) and glandular epithelium (GE) of the ovine uterus. The IRF-2, a potent transcriptional repressor of ISG expression, is expressed in the luminal epithelium (LE). This study determined effects of the estrous cycle, pregnancy, and IFNτ on expression of Stat 1, Stat 2, IRF-9, IRF-1, and IRF-2 genes in the ovine endometrium. In cyclic ewes, Stat 1, Stat 2, IRF-1, and IRF-9 mRNA and protein were detected at low levels in the S and GE. During pregnancy, expression of these genes increased only in the S and GE. Expression of IRF-2 was detected only in the LE and superficial GE (sGE) of both cyclic and pregnant ewes. In cyclic ewes, intrauterine administration of IFNτ stimulated Stat 1, Stat 2, IRF-9, and IRF-1 expression in the endometrium. Ovine IRF-2 repressed transcriptional activity driven by IFN-stimulated response elements that bind ISGF3, but not by gamma-activation sequences that bind GAF. These results suggest that IRF-2 in the LE and sGE restricts IFNτ induction of ISGs to the S and GE. In the S and GE, IFNτ hyperactivation of ISG expression likely involves formation and actions of the transcription factors ISGF3 and, perhaps, IRF-1.

conceptus, gene regulation, hormone action, interferon, IRF, signal transduction, Stat, uterus

INTRODUCTION

Tau interferons (IFNτ) are a unique subclass of the 172-amino acid type I omega (ω) IFNs produced exclusively by the trophectoderm of ruminant (i.e., sheep, cattle, and goat) conceptuses [1]. The mononuclear cells of the trophectoderm synthesize and secrete IFNτ between Day 11 and Days 20–24 of pregnancy in sheep [2, 3], which then acts in a paracrine manner on the endometrial luminal epithelium (LE) and superficial glandular epithelium (sGE) to suppress transcription of estrogen receptor alpha (ERα) and oxytocin receptor (OTR) genes [4–6]. These actions prevent development of the luteolytic mechanism by abrogating oxytocin-induced luteolytic pulses of prostaglandin $F_{2\alpha}$ [6–8]. In addition to suppressing or silencing transcription of ERα and OTR genes, IFNτ induces or increases expression of a number of IFN-stimulated genes (ISGs) in the ruminant endometrium as well as in endometrial and fibroblast cell lines. These ISGs include signal transducer and activator of transcription (Stat) 1 and 2 [9–11], β_2-microglobulin [12], IFN regulatory factor one (IRF-1) [6, 9, 10, 13], ISG17/ubiquitin cross-reactive protein [10, 14–16], Mx protein [17], granulocyte chemotactic protein-2 [18], and 2′,5′-oligoadenylate synthetase (OAS) [19–22]. In the endometrium of early pregnant ewes as well as cyclic ewes receiving intrauterine injections of recombinant ovine IFNτ, ISG17 and OAS genes are up-regulated only in the stroma (S) and middle- to deep uterine glands in the stratum spongiosum [15, 22, 23].

Induction and increases in ISG expression in response to IFNτ are mediated by an intracellular signal transduction system involving Stats and IRFs [6, 9, 10, 11, 13, 24]. The IFNτ activates a signal transduction pathway similar to that of IFNα/β in ovine endometrial cell lines [9, 10] and human fibroblast cell lines [11]. In ovine endometrial LE cells, IFNτ stimulates tyrosine phosphorylation and nuclear translocation of Stats 1, 2, 3, 5a/b, and 6 within 30 min [10]. However, exposure to IFNτ for more than 30 min extends Stat 1 and 2 tyrosine phosphorylation, whereas Stats 3, 5a/b, and 6 are rapidly dephosphorylated. The IFNτ induces homodimerization of Stat 1 to form the transcription factor gamma-activated factor (GAF) as well as heterodimerization of Stat 1 and Stat 2, which complex with IRF-9 (also known as ISGF3γ/p48) to form the transcription factor complex IFN-stimulated gene factor 3 (ISGF3). The GAF regulates transcription through γ-activated sequence (GAS) elements in the promoter region of selected ISGs such as IRF-1 [25, 26]. The ISGF3 binds to IFN-stimulated response elements (ISREs) to drive transcription of ISGs such as ISG17 [27], Mx [28], and OAS [29]. Similarly, expression of Stat1, Stat2, IRF-9, and IRF-1 genes is induced or increased by IFNτ and other type I IFNs via the ISGF3 and GAF transcription factor complexes [10, 11, 26, 30, 31]. The simultaneous induction of Stat 2 and IRF-9 gene expression by IFNτ appears to shift transcription factor formation from GAF toward predominantly ISGF3 [10, 11].

In the pregnant ovine uterus, ISGs including OAS and ISG17 are induced or up-regulated in the endometrial S and glandular epithelium (GE), but expression of these genes is

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not detectable in the endometrial LE or sGE [15, 22]. The lack of induction or increases in ISG expression in either LE or sGE during pregnancy and in response to IFNτ may be due either to the lack of Stat 1, Stat 2, IRF-9, or, perhaps, IRF-1 gene expression in the LE and sGE or the presence of IRF-2, a transcriptional repressor of ISGs as well as type I IFNs themselves [32–35]. In pregnant ewes, IRF-2 protein is present in the LE and sGE on Days 13, 15, 17, and 20 [6]. Therefore, the objectives of the present study were to determine effects of the estrous cycle, pregnancy, and intrauterine administration of recombinant ovine IFNτ on expression of the Stat 1, Stat 2, IRF-9, IRF-1, and IRF-2 genes in endometrium of the ovine uterus and to determine effects of ovine IRF-2 on transactivation of ISRE- and GAS-driven promoters.

MATERIALS AND METHODS

Animals and Experimental Design

Mature ewes of primarily Rambouillet breeding were observed daily for estrus using vasectomized rams. All ewes exhibited at least two estrous cycles of normal duration (~16–18 days). Experimental and surgical procedures involving animals were approved by the Agricultural Animal Care and Use Committee of Texas A&M University.

Study 1. At estrus, ewes were assigned randomly to cyclic or pregnant status. Ewes assigned to pregnant status were bred to intact rams during estrus. Ewes were then ovariohysterectomized (n = 4 ewes/day) on Days 11, 13, or 15 of the estrous cycle and Days 11, 13, 15, or 17 of pregnancy (Day 0 = estrus/mating). Pregnancy was confirmed by presence of an apparently normal conceptus in uterine flushings. At hysterectomy, several sections (~0.5 cm) 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% (v/v) ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). Several sections (1–1.5 cm) from the middle of each uterine horn were also embedded in Tissue-Tek Optimal Cutting Temperature (OCT) compound (Miles, Inc., Oneonta, NY), frozen in liquid nitrogen vapor, and stored at −80°C. The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at −80°C for RNA extraction. In monovulatory pregnant ewes, uterine tissue samples were marked as contralateral or ipsilateral to the ovary bearing the corpus luteum. No contralateral uterine samples were used for this study.

Study 2. Eight cyclic ewes were fitted with uterine catheters on Day 5 of the estrous cycle (Day 0 = estrus) as described previously [23]. Ewes (n = 4 ewes/treatment) were then allotted randomly to receive intrauterine injections of either control serum proteins (6 mg/day) or recombinant ovine IFNτ (roIFNτ; 2×10^7 antiviral units/day) [36] on Days 11–15 after estrus. For intrauterine administration, the uterine horns of each ewe received injections of either roIFNτ (5×10^6 antiviral units/horn per injection) or control ovine serum proteins (CX; equal amount of total protein/horn per injection) twice daily. This regimen of roIFNτ treatment has been shown to mimic the antiluteolytic effects of the conceptus on the endometrium during the pregnancy-recognition period [23, 37]. All ewes were ovariohysterectomized on Day 16. The endometrium was dissected from the myometrium, frozen in liquid nitrogen, and stored at −80°C for RNA extraction.

Cloning of a Partial cDNA for Ovine IRF-9 and Full-Length cDNA for Ovine IRF-1 and IRF-2

Partial cDNAs for ovine IRF-1 and IRF-9 were amplified by reverse transcription-polymerase chain reaction (PCR) of total RNA from Day 15 pregnant ovine endometrium using primers based on the human IRF-1 sequence (GenBank accession no. NM_002198; forward: 5′-TCC ACC CAC CAA GAA CC-3′, reverse: 5′-TTC TGG CTC CTC CTT ACA GC-3′) or human IRF-9 sequence (NM_006084; forward: 5′-TGA ATT TAA GGA GGT TCC-3′, reverse, 5′-GCA GCA GTG AGT AGT CTG-3′). Reverse transcription of endometrial total RNA was performed as described previously [37]. The PCR was conducted as follows for IRF-1 using PCR Optimized Buffer A (Invitrogen, Carlsbad, CA): 1) 95°C for 2 min; 2) 30 sec at 95°C, 30 sec at 57°C, and 30 sec at 72°C for 30 cycles; and 3) 72°C for 10 min. For IRF-9, PCR was performed using PCR Optimized Buffer C (Invitrogen) as follows: 1) 95°C for 2 min; 2) 30 sec at 95°C, 1 min at 42°C, 1 min at 72°C for four cycles; 3) 30 sec at 95°C, 1 min at 53°C, and 1 min at 72°C for 35 cycles; and 3) 72°C for 10 min. The amplified IRF-1 (502 base pairs [bp]) and IRF-9 (392 bp) PCR products were subcloned into pCRII cloning vector using a T/A Cloning Kit (Invitrogen) and sequenced in both directions using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA) to confirm identity.

Full-length cDNAs for ovine IRF-1 and ovine IRF-2 were obtained by conventional screening of a Lambda ZAP II cDNA library of Day 15 pregnant ovine endometrium (Stratagene, La Jolla, CA) at low stringency using radiolabeled cDNA probes of an ovine IRF-1 partial cDNA and murine IRF-2 cDNA. Positive clones containing entire ovine IRF-1 and IRF-2 coding sequences were sequenced in both directions and deposited in GenBank (GenBank accession no. AF331970 and AF228445, respectively). Mammalian expression vectors for ovine IRF-1 and IRF-2 were constructed by subcloning the *Eco*RV/*Not*I and *Bam*HI/*Not*I fragments containing full-length ovine IRF-1 and IRF-2 cDNA sequences into *Bam*HI (blunt end)/*Not*I-digested and *Bam*HI/*Not*I-digested pEF1-Myc/His-LacZ mammalian expression vector (Invitrogen), respectively.

RNA Isolation and Analyses

RNA isolation. Total cellular RNA was isolated from endometrium using Trizol reagent (Gibco-BRL, Bethesda, MD). The quantity of RNA was assessed spectrophotometrically and the integrity of RNA examined by gel electrophoresis in a denaturing 1% (w/v) agarose gel.

Slot blot hybridization analysis. Steady-state levels of Stat and IRF mRNAs were assessed by slot blot hybridization using methods described previously [37, 38]. Denatured total endometrial RNA (20 μg) from each ewe was analyzed using a radiolabeled antisense human Stat 1 (M97935) [39], human Stat 2 (NM005419) [40], ovine IRF-9, ovine IRF-1, or ovine IRF-2 cRNA. To correct for variation in total RNA loading, a duplicate RNA slot membrane was hybridized with radiolabeled antisense 18S rRNA cRNA (pT718S; Ambion, Austin, TX). Following washing, nonspecific hybridization was removed by RNase A digestion [41]. The radioactivity associated with each slot was quantitated by electronic autoradiography using an Instant Imager (Packard Instrument Company, Meridian, CT) and is expressed as total counts (TC).

In Situ Hybridization Analysis

The mRNAs for Stat 1, Stat 2, IRF-9, IRF-1, and IRF-2 were localized in uterine tissue sections (5 μm) by in situ hybridization analysis as described previously [38]. Deparaffinized, rehydrated, and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized human Stat 1, human Stat 2, ovine IRF-9, ovine IRF-1, or ovine IRF-2 cRNA cDNAs using in vitro transcription with α-^{35}S-uridine triphosphate. After hybridization, washing, and ribonuclease A digestion, slides were dipped in NTB-2 liquid photographic emulsion (Eastman Kodak, Rochester, NY), stored at 4°C for 1 wk (Stat 1, Stat 2, IRF-9, and IRF-1) or 3 wk (IRF-2), and developed in Kodak D-19 developer. Slides were then counterstained with Harris modified hematoxylin (Fisher Scientific, Fairlawn, NJ), dehydrated through a graded series of alcohol to xylene, and protected with a coverslip.

Generation of Polyclonal Antibodies to Ovine IRF-2 in Chickens

Single Comb White Leghorn hens (13 wk old) were used for production of polyclonal antibodies against ovine IRF-2 protein. An IRF-2-specific peptide segment was selected based on the predicted amino acid sequence of ovine IRF-2 (AAF34781.1) by use of the EMBL Predict-Protein server. The selected antigenic sequence was NH2-CITQARVKSY-COOH (IRF-2 CT) corresponding to the C-terminus of the native protein. An N-terminal cysteine residue was added for ease of coupling to a carrier protein. The peptide was synthesized by Sigma Genosys (Woodlands, TX) and coupled to thyroglobulin (Sigma, St. Louis, MO) with *m*-maleimidobezoyl-*N*-hydroysuccinimide ester (Pierce, Rockford, IL) using an approximately 40-fold molar excess of peptide over protein (2 mg peptide and 15 mg of thyroglobulin). The conjugates (400 mg) were solubilized in 2 ml of PBS buffer (0.15 M), emulsified with 2 ml of Freunds Complete Adjuvant (Sigma), and used to immunize four birds in the breast muscle, with each bird receiving 100 mg of the conjugate. The chickens were boosted at 2-wk intervals using the same dose and volume of the respective conjugates emulsified in Freunds Incomplete Adjuvant (Sigma). Eggs were collected for purification of IgY from the yolks. Briefly, yolk lipids were removed from the emulsified yolks by euglobulin precipitation, and

TABLE 1. Effects of day and pregnancy status on steady-state levels of Stat 1, Stat 2, IRF-9, IRF-1, and IRF-2 mRNAs in ovine endometrium.[a]

Ewe		Endometrial mRNA expression (total counts)				
Day	Status[b]	Stat 1	Stat 2	IRF-9	IRF-1	IRF-2
11	C	18 317	2 205	8 535	9 346	18 668
13	C	15 874	1 944	8 091	10 525	15 549
15	C	11 123	1 955	4 552	12 565	12 461
11	P	12 574	1 689	7 737	8 388	12 963
13	P	18 612	2 883	10 872	13 304	13 501
15	P	43 591	4 654	17 387	34 388	15 699
17	P	28 951	3 983	15 632	18 347	12 540
SEM[c]		1 450	157	555	850	512

[a] See text for description of statistical analyses.
[b] C, Cyclic; P, pregnant.
[c] Overall SEM.

chicken IgY was then further purified by ammonium sulfate precipitation [42]. Peptide-specific antibodies were purified by immunoaffinity chromatography over a 1-ml SulfoLink Coupling gel (Pierce) derivatized with 950 mg of the respective peptides. Peptide-specific antibodies were eluted with 50 mM glycine (pH 2.5). The purified fractions were collected in Tris buffer (2.0 M, pH 8.0) for immediate neutralization and stored at 4°C.

Immunocytochemical Analyses

Frozen sections (4–8 μm) of uterine tissues embedded in OCT compound in study 1 were cut with a cryostat and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Sections were fixed in −20°C methanol for 10 min, permeabilized with 0.3% Tween-20 in 0.02 M PBS, and then blocked in antibody dilution buffer (two parts 0.02 M PBS, 1.0% BSA, and 0.3% Tween-20 [pH 8.0] to one part glycerol) containing 5% normal goat serum for 1 h at room temperature. Sections were rinsed in PBS and incubated overnight at 4°C with antibodies. Antibodies were mouse anti-Stat 1 (#S21120) at 30 μg/ml and mouse anti-IRF-9/ISGF3γ (#I29320) at 20 μg/ml from Transduction Laboratories (Lexington, KY); rabbit anti-Stat 2 (#sc-476) at 20 μg/ml and rabbit anti-IRF-1 (#sc-497) at 20 μg/ml from Santa Cruz Biotechnology (Santa Cruz, CA); chicken anti-IRF-2 at 20 μg/ml; and normal rabbit IgG (#I5006), normal mouse IgG (#I5381), or normal chicken IgY (Sigma) at the same concentrations as primary antibodies. Following three rinses in PBS for 10 min each, sections were incubated with fluorescein-conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA) for 1 h at room temperature and again washed in PBS three times for 10 min each. Sections were then overlaid with a coverslip and Prolong Antifade mounting reagent (Molecular Probes, Eugene, OR).

Photomicroscopy and Digital Imaging

Images of representative fields of in situ hybridization and immunofluorescence slides were recorded using a Zeiss Axioplan2 microscope (Carl Zeiss, Thornwood, NY) fitted with a Hamamatsu C-5810 chilled three-color CCD camera (Hamamatsu Corporation, Bridgewater, NJ). Digital images were captured and/or assembled using Adobe Photoshop 4.0 (Adobe Systems, Seattle, WA) and a MacIntosh PowerMac G3 computer (Apple Computer, Cupertino, CA). Black-and-white prints were electronically printed using a Kodak DS8650 color printer.

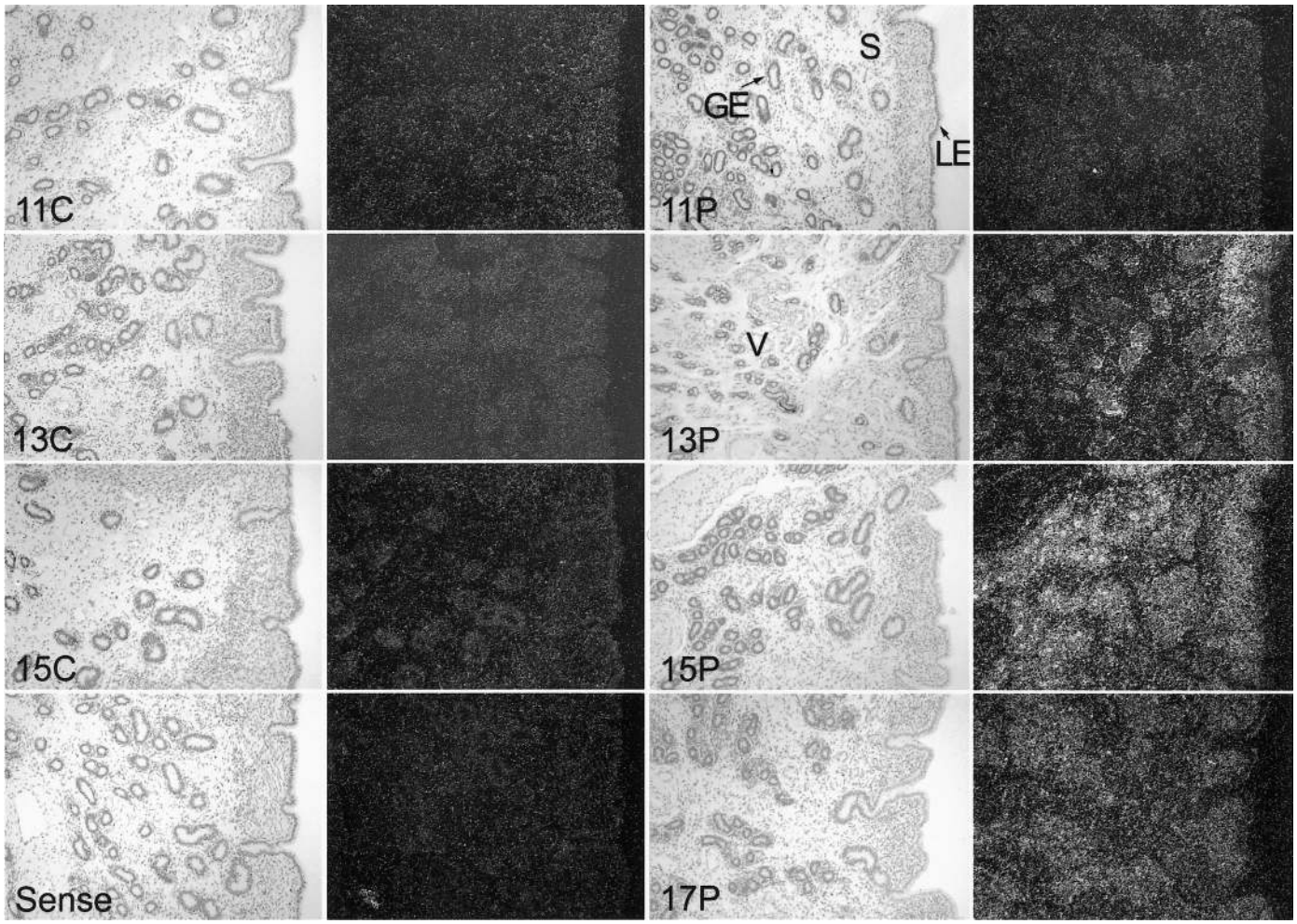

FIG. 1. In situ localization of Stat 1 mRNA in the endometrium of cyclic and early pregnant ewes from study 1. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with α-^{35}S-labeled antisense or sense human Stat 1 cRNA probes. Protected transcripts were visualized by liquid emulsion autoradiography and imaged under bright- or dark-field illumination. GE, Glandular epithelium; LE, luminal epithelium; S, stroma; V, blood vessel. ×260.

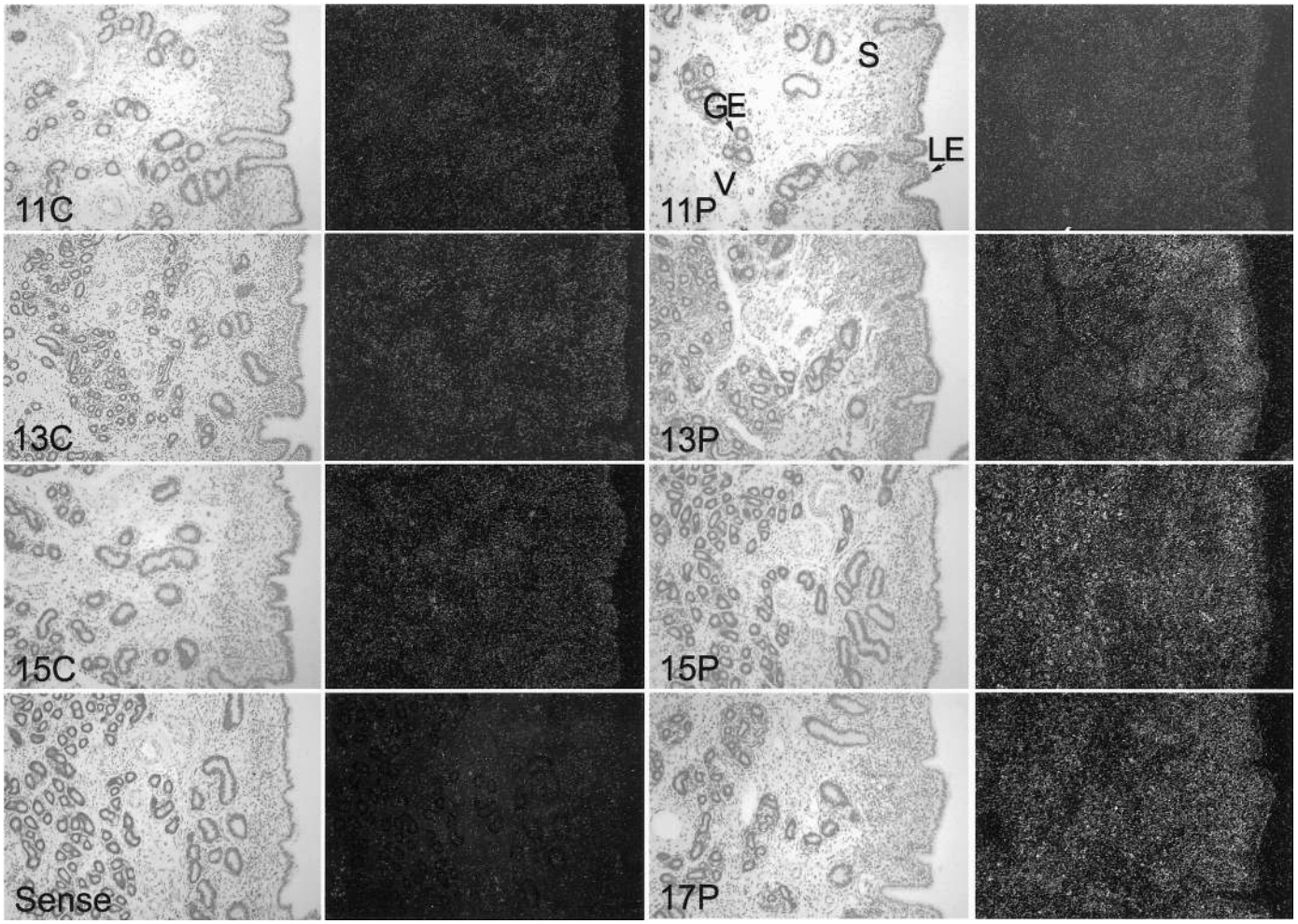

FIG. 2. In situ localization of Stat 2 mRNA in the endometrium of cyclic and early pregnant ewes from study 1. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with α-^{35}S-labeled antisense or sense human Stat 2 cRNA probes. Protected transcripts were visualized by liquid emulsion autoradiography and imaged under bright- or dark-field illumination. See Figure 1 for abbreviations. ×260.

Transient Transfection Assays

Constructs. The 5xISRE-TK-LUC (pISRE-LUC) and TK-LUC (pTAL-LUC) constructs were purchased from Clontech (Palo Alto, CA). The 3xGAS-TK-LUC constructs contain three copies of the rat IRF-1 GAS element as described previously [10].

Transfection procedure. Immortalized ovine endometrial stromal cells [9] were subcultured into 12-well plates (70–80% confluent) and transiently cotransfected (n = 4 wells per construct and treatment) with the indicated LUC reporter construct (0.5 μg/well) and pEF1-Myc/His-LacZ (0.05 μg/well; Invitrogen) as a marker for transfection efficiency using the GenePORTER transfection reagent (Gene Therapy Systems, Inc., San Diego, CA) according to the manufacturer's recommendations. After transfection, the cells were left untreated as a control or were treated with roIFNτ (10 000 antiviral units/ml) for 24 h. Cells were then harvested using Cell Culture Lysis Buffer (Promega, Madison, WI).

Luciferase and β-galactosidase assays. Luciferase expression assays were conducted using the Promega Luciferase Assay System according to the manufacturer's recommendations and quantitated using a Packard Luminometer. β-Galactosidase assays were performed using a Galacto-Light Plus Kit (Tropix, Bedford, MA) according to the manufacturer's recommendations. For each experiment, luciferase expression was analyzed by least-squares ANOVA procedures using the β-galactosidase values as a covariate to correct for differences in transfection efficiency between wells and plates within time points. Data are reported as fold-induction or fold-suppression of IFNτ based on least-squares mean relative light units. Each transient transfection experiment was repeated a minimum of four times.

Statistical Analyses

Data from slot blot hybridization analyses were subjected to least-squares ANOVA using the General Linear Models procedures of the Statistical Analysis System [43]. Slot blot hybridization data for Stat 1, Stat 2, IRF-9, IRF-1, and IRF-2 mRNAs (TC) were normalized for differences in sample loading using the 18S rRNA data as a covariate in least-squares ANOVA. Data from study 1 were analyzed for effects of day, pregnancy status (cyclic or pregnant), and their interaction. Within pregnancy status, least-squares regression analyses were used to determine effects of day on endometrial mRNA levels. Data from study 2 were subjected to one-way least-squares ANOVA. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error [44]. Fold-induction or fold-suppression data derived from each transfection experiment were analyzed by one-way ANOVA to determine effects of ovine IRF-1 and IRF-2 expression on activity of the promoter-reporter constructs. A P value of 0.05 or less was considered to be statistically significant. Data are presented as least-square means (TC) with SEM.

RESULTS

Effects of the Estrous Cycle and Pregnancy on Stat and IRF Expression in the Ovine Endometrium (Study 1)

Slot blot hybridization analyses. Effects of day and pregnancy status on steady-state levels of Stat and IRF mRNAs in the ovine endometrium are summarized in Table 1. In cyclic ewes, Stat 1, IRF-9, and IRF-2 mRNA levels decreased ($P < 0.05$, linear) between Days 11 and 15 of the cycle. Endometrial levels of Stat 2 and IRF-1 mRNA did not decline in cyclic ewes ($P > 0.10$). Pregnancy affected expression of Stat 1 ($P = 0.003$), Stat 2 ($P = 0.03$), IRF-

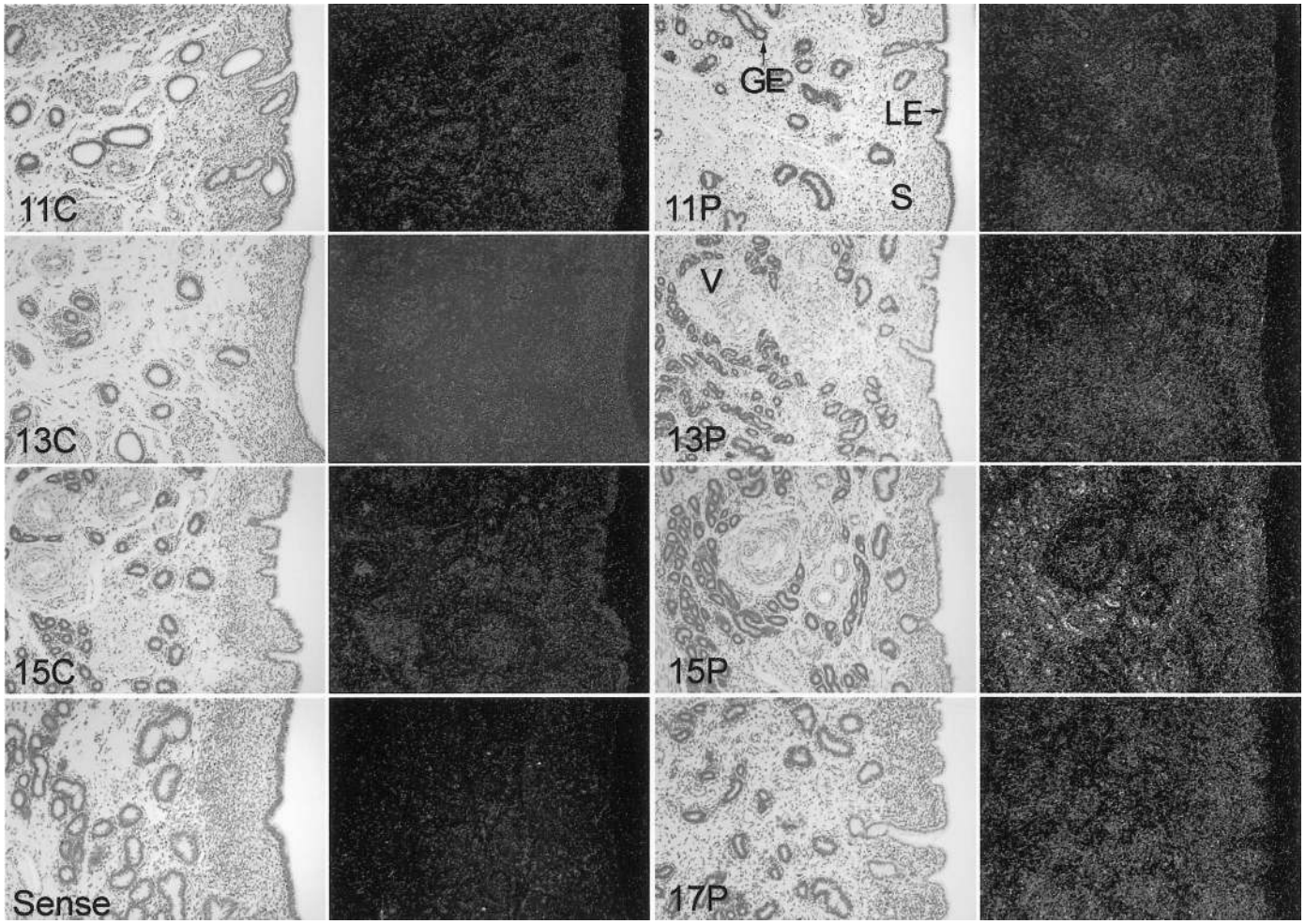

FIG. 3. In situ localization of IRF-9 mRNA in the endometrium of cyclic and early pregnant ewes from study 1. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with α-^{35}S-labeled antisense or sense human IRF-9 cRNA probes. Protected transcripts were visualized by liquid emulsion autoradiography and imaged under bright- or dark-field illumination. See Figure 1 for abbreviations. ×260.

9 ($P = 0.05$), IRF-1 ($P = 0.0001$), and IRF-2 ($P = 0.05$) mRNAs in the ovine endometrium (day × status interaction). In pregnant ewes, steady-state levels of Stat 1, Stat 2, IRF-9, and IRF-1 mRNAs increased in the endometrium ($P < 0.05$; quadratic for Stat 1, Stat 2, and IRF-9; cubic for IRF-1).

In situ hybridization analyses. The overall pattern of expression of Stat and IRF mRNAs during the cycle and early pregnancy paralleled that found by slot blot hybridization analysis of endometrial total RNA. During the estrous cycle, Stat 1, Stat 2, IRF-9, and IRF-1 mRNA expression was detected at relatively low levels in all cell types (Figs. 1–4). In the intercaruncular endometrium, expression of these mRNAs was higher in the stratum compactum S than in the stratum spongiosum S, LE, or GE. In cyclic ewes, IRF-1 mRNA increased in the LE between Days 11 and 15 (Fig. 4). In contrast, IRF-2 mRNA was expressed in the LE and sGE to middle GE during the estrous cycle but was not detected in S as compared to the background present in the sense control (Fig. 5).

Expression of Stat 1, Stat 2, IRF-9, and IRF-1 mRNAs increased in the endometrium during early pregnancy (Figs. 1–4). Relative levels of expression of these mRNAs were not different in the endometrium of cyclic and pregnant ewes on Day 11, except for IRF-1 mRNA, which was more abundant in focal areas of S in the caruncular areas of the uterus. On Day 13 of pregnancy, an increase in Stat 1, Stat 2, and IRF-9 mRNA expression was detected in the stratum compactum S and middle GE. The IRF-1 mRNA increased in stratum compactum S but not in GE on Day 13. By Day 15 of pregnancy, strong expression of Stat 1, Stat 2, IRF-9, and IRF-1 mRNAs was detected throughout the S and GE of the intercaruncular endometrium. No increase in expression of these Stat and IRF mRNAs was detected in the LE or sGE. By Day 17 of pregnancy, relative levels of Stat 1, Stat 2, IRF-9, and IRF-1 mRNA expression declined in the GE but remained higher in the S than observed in endometrium from Day 11 of pregnancy.

During early pregnancy, IRF-2 mRNA was expressed in LE and sGE (Fig. 5). No difference in endometrial expression of IRF-2 mRNA was detected in these epithelia between cyclic and pregnant ewes on Days 11 and 13. However, IRF-2 mRNA levels were greater in the LE and sGE on Day 15 of pregnancy, and they remained elevated on Day 17 of pregnancy.

Immunofluorescence Localization of Stat 1, Stat 2, IRF-9, IRF-1, and IRF-2 Protein in Ovine Endometrium (Study 1)

Immunoflourescence localization of Stat and IRF proteins was conducted to confirm Stat and IRF mRNA expression results obtained by in situ hybridization. As com-

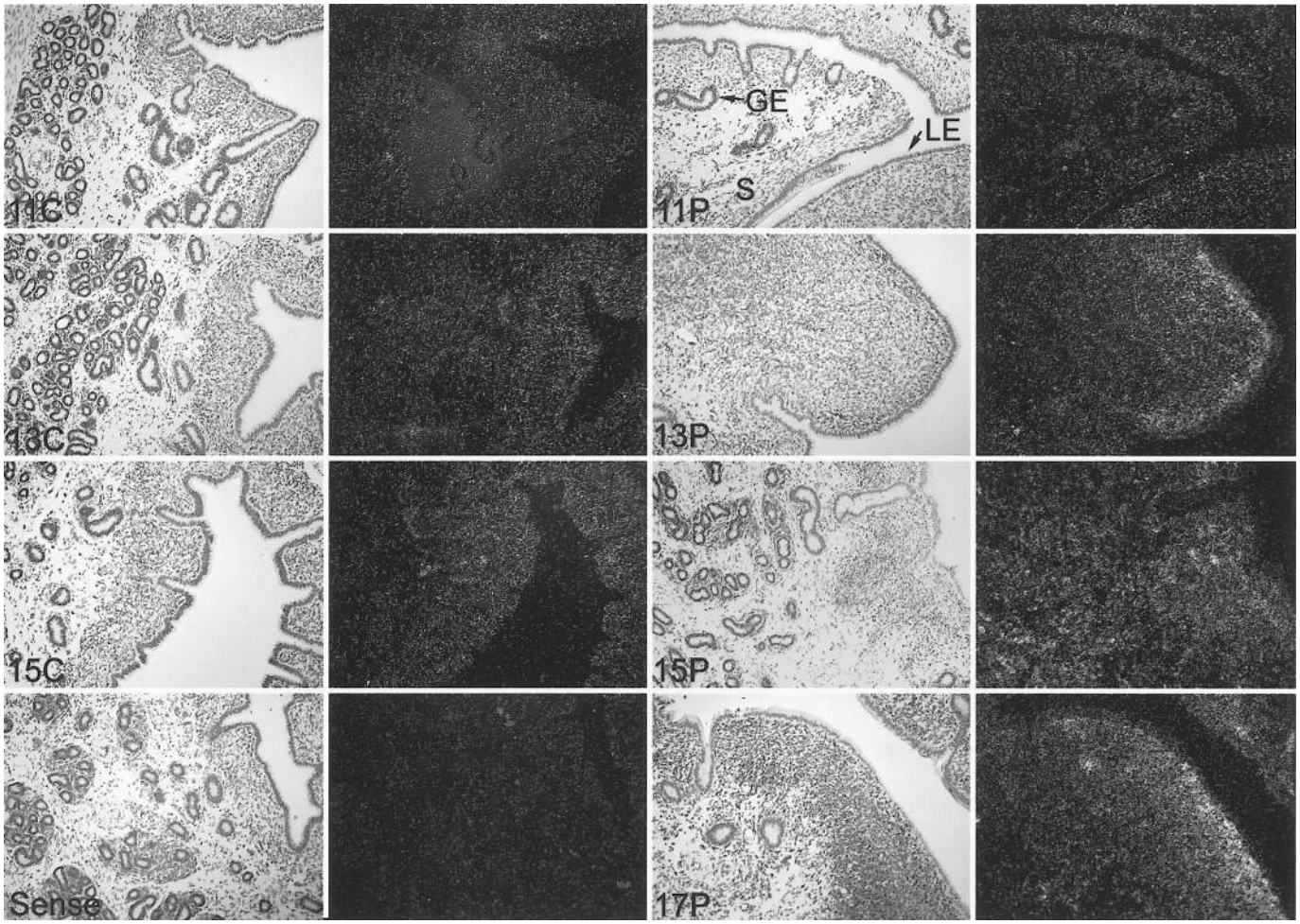

FIG. 4. In situ localization of IRF-1 mRNA in the endometrium of cyclic and early pregnant ewes from study 1. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with α-^{35}S-labeled antisense or sense ovine IRF-2 cRNA probes. Protected transcripts were visualized by liquid emulsion autoradiography and imaged under bright- or dark-field illumination. See Figure 1 for abbreviations. ×260.

pared to the IgG controls, immunoreactive Stat 1, Stat 2, and IRF-9 proteins were not detected in either LE or GE in endometrium from cyclic or early pregnant ewes (Fig. 6). However, a notable increase in Stat 1, Stat 2, and IRF-9 protein expression in S and GE was detected between Days 11 and 15 of early pregnancy (Fig. 6). These proteins were detected in both the nucleus and cytoplasm. The rabbit polyclonal IRF-9 antibody nonspecifically cross-reacted with an unidentified antigen in the basal lamina of the endometrial LE in all uterine samples regardless of day. All antibodies used in this study have been used previously for Western blot analyses of ovine endometrial cell lines as well as human fibroblasts [10, 11].

The IRF-1 protein was present only in the S of Day 11 cyclic and pregnant ewes (Fig. 7). On Day 15 of the estrous cycle, IRF-1 protein was detected in LE and sGE as well as in S. Between Days 11 and 15 of pregnancy, IRF-1 protein increased in the S and the middle to deep GE. However, no increase in IRF-1 protein expression was detected in the LE and sGE of pregnant ewes. In the middle to deep uterine glands, a punctate pattern of immunoreactive IRF-1 protein was observed in the uterine glands. Although IRF-1 protein was present in the GE cells, expression was predominantly located in the nuclei of intraepithelial cells, which are presumed to be immune cells.

Chicken anti-ovine IRF-2 IgY detected only recombinant ovine IRF-2, not recombinant ovine IRF-1 or IRF-6 proteins, in Western blot assays (data not shown). In addition, the purified chicken anti-ovine IRF-2 IgY detected the appropriately sized protein in extracts of immortalized ovine LE cells as well as Cos-7 cells overexpressing ovine IRF-2, but in not ovine IRF-1 or ovine IRF-6 (data not shown). Immunoreactive IRF-2 protein was detected in the endometrial LE and sGE of cyclic and pregnant ewes (Fig. 7). As for immunoreactive IRF-1, abundant IRF-2 protein was detected in intraepithelial immune cells in the middle to deep GE regardless of day. On Day 15 of pregnancy, levels of IRF-2 protein were higher in the nuclei of LE and sGE cells on Day 15 of pregnancy compared to Day 11 of pregnancy or Day 15 of the cycle. Preadsorption of the antipeptide IRF-2 IgY with recombinant ovine IRF-2 eliminated L, GE, and immune cell staining, but it also revealed low levels of nonspecific binding of the chicken anti-ovine IRF-2 IgY to the basal lamina of the endometrial epithelia as well as blood vessels in a manner similar to IgY alone (data not shown).

Effects of Intrauterine Administration of Recombinant Ovine IFNτ on Stat and IRF mRNA Expression in Ovine Endometrium (Study 2)

To determine if the effects of pregnancy on endometrial Stat and IRF gene expression in vivo were due to IFNτ from the conceptus, cyclic ewes were fitted with uterine

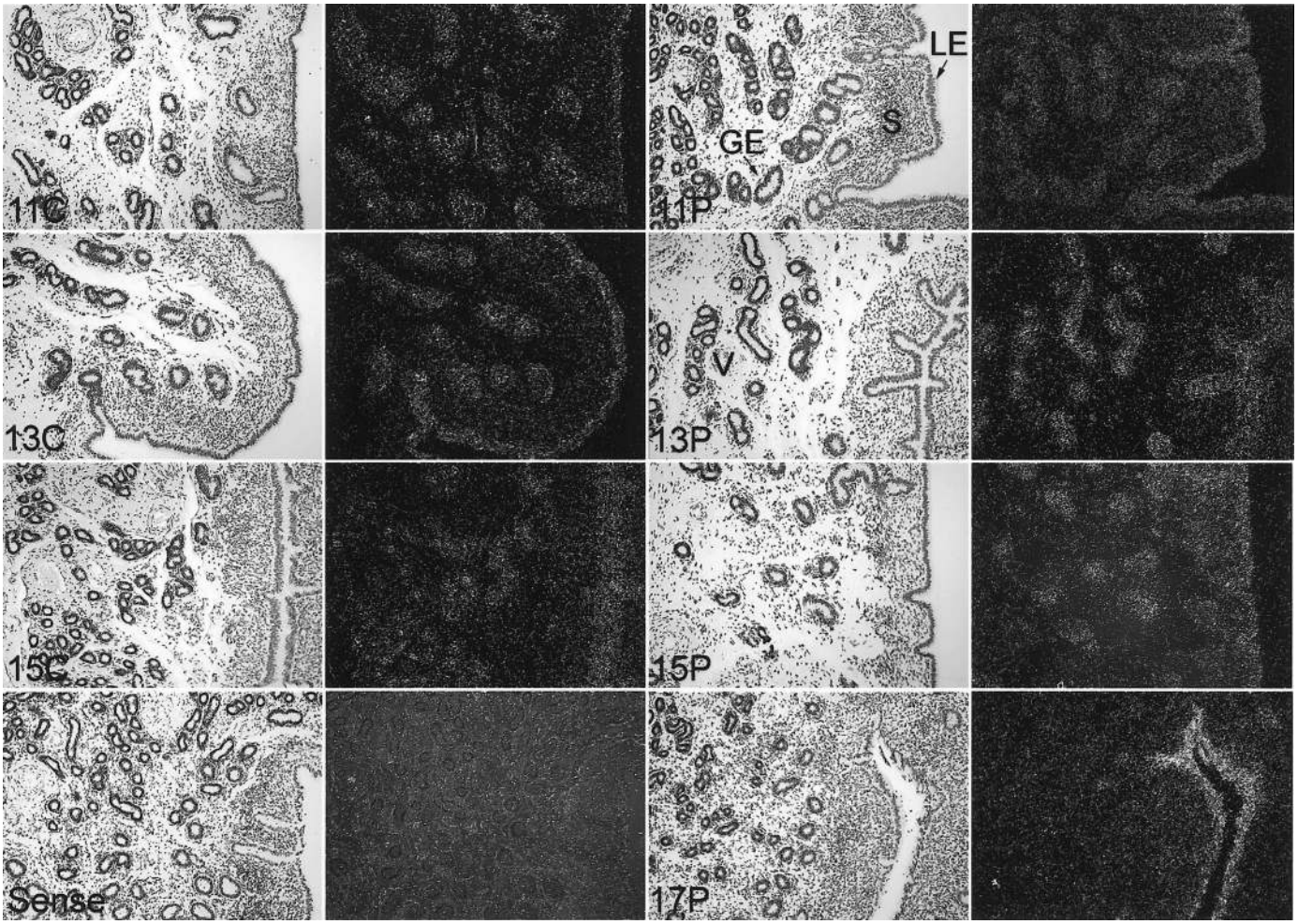

FIG. 5. In situ localization of IRF-2 mRNA in the endometrium of cyclic and early pregnant ewes from study 1. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with α-^{35}S-labeled antisense or sense ovine IRF-2 cRNA probes. Protected transcripts were visualized by liquid emulsion autoradiography and imaged under bright- or dark-field illumination. See Figure 1 for abbreviations. ×260.

catheters on Day 5 and given daily intrauterine injections of either control proteins or roIFNτ from Days 11 to 15. As illustrated in Figure 8, intrauterine administration of roIFNτ into cyclic ewes induced an approximately 3-fold increase ($P < 0.01$, CX vs. IFN) in steady-state levels of endometrial Stat 1 mRNA. Similarly, roIFNτ increased steady-state levels of endometrial Stat 2 and IRF-9 mRNA by approximately 2-fold ($P < 0.01$) and of IRF-1 mRNA by approximately 1.4-fold ($P < 0.10$, CX vs. IFN). However, IRF-2 mRNA levels were not affected by roIFNτ treatment ($P > 0.10$).

Effects of Ovine IRF-1 and Ovine IRF-2 on Activity of Promoters Containing ISRE or GAS Sequences in Transient Transfection Assays

An immortalized ovine endometrial S cell line was transiently transfected with mammalian overexpression vectors for ovine IRF-1, ovine IRF-2, or the combination of ovine IRF-1 and IRF-2 to determine their effects on transactivation of a promoter-reporter construct containing five consensus ISRE-binding sites (Fig. 9). Ovine IRF-1 alone transactivated (2 ± 0.2-fold) the 5xISRE-TK-LUC reporter. In contrast, ovine IRF-2 alone strongly repressed (24 ± 3-fold) transcriptional activity of the 5xISRE-TK-LUC reporter. Cotransfection of ovine IRF-1 with ovine IRF-2 also resulted in repression (17 ± 3-fold) of the 5xISRE-TK-LUC reporter. Transcriptional activity of a reporter containing three IRF-1 GAS elements (3xGAS-TK-LUC) was not affected by overexpression of either ovine IRF-1 or ovine IRF-2 in the ovine LE cells (data not shown). Activity of the minimal TK-LUC reporter was also not affected by overexpression of either ovine IRF-1 or ovine IRF-2 (data not shown).

DISCUSSION

To our knowledge, this is the first report of temporal and spatial alterations in Stat 1, Stat 2, and IRF-9 gene expression in the ovine uterus. Results presented here confirm and extend those of previous reports that these classical ISGs are directly regulated by IFNτ in the ovine endometrium [9–11, 15, 22, 23]. Similar to expression of OAS, Mx, and ISG17 [15, 17, 22], expression of Stat 1, Stat 2, IRF-9, and IRF-1 in the ovine endometrial S and GE during early pregnancy was coordinate with temporal production of IFNτ by the conceptus [2, 3]. The increases in expression of these ISGs during pregnancy can be attributed to IFNτ from the conceptus, because intrauterine infusion of cyclic ewes with roIFNτ alone increased Stat 1, Stat 2, IRF-9, and IRF-1 expression in the endometrium. Originally, Stats 1 and 2 and IRF-9 were discovered as ISGs and termed ISG factors 1 (p91), 2 (p113), and 3γ (p48), respectively [45, 46]. Expression of these genes is increased by type I IFNs [31] via

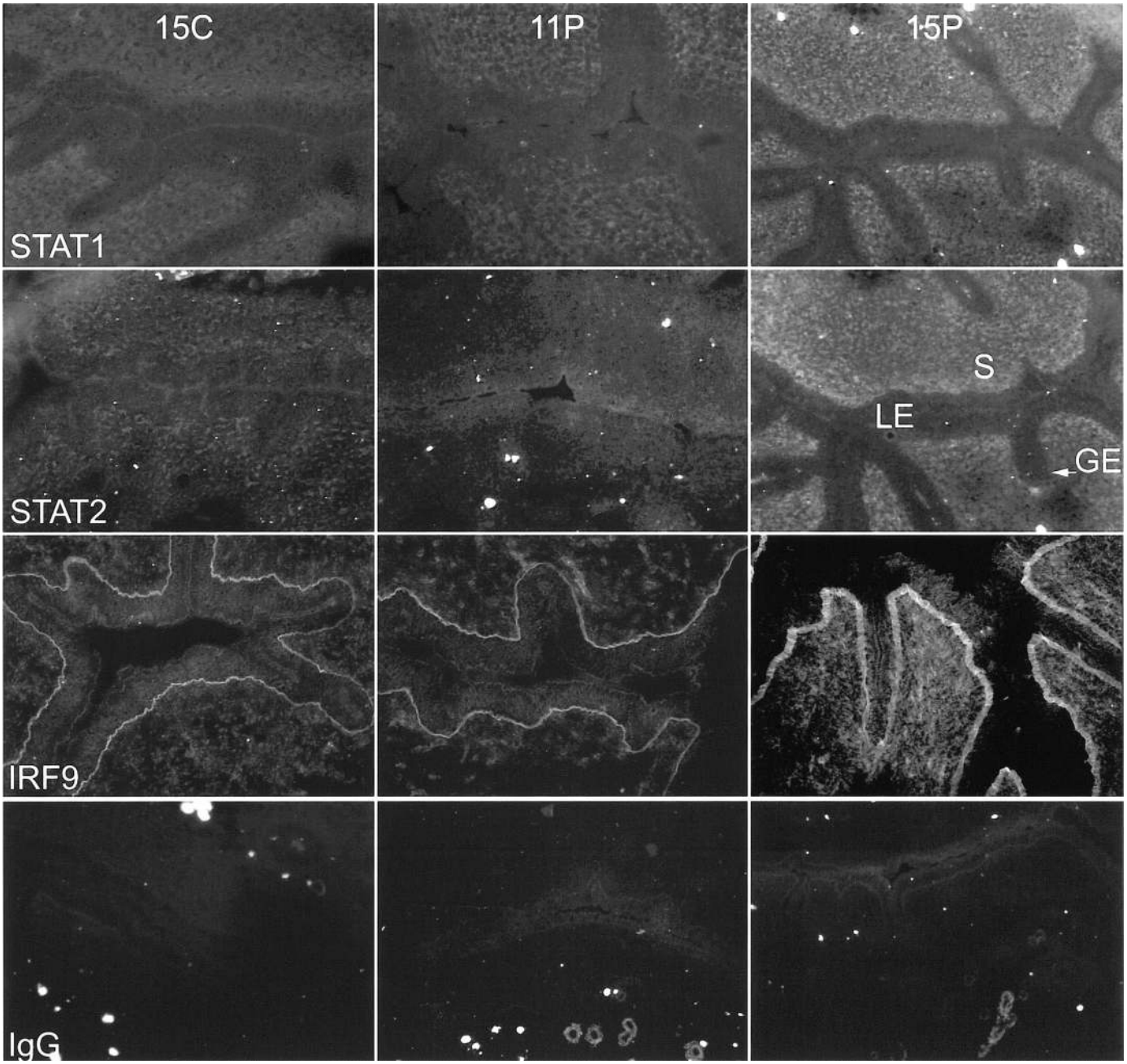

FIG. 6. Detection of Stat 1, Stat 2, and IRF-9 protein in uterine endometrium from cyclic and pregnant ewes from study 1. Immunofluorescence staining of frozen uterine sections from cyclic (C) and pregnant (P) ewes was conducted using specific antibodies or irrelevant IgG as a control. See Figure 1 for abbreviations. ×230.

multiple ISREs in their promoter regions [47, 48]. Likewise, the 5′ promoter/enhancer region of other ISGs in the ovine uterus, including ISG17, Mx, and OAS, contain multiple ISREs that bind ISGF3 and induce or increase their expression in response to type I IFNs [13, 27–30].

The relative abundance of Stat 1, Stat 2, IRF-9, and IRF-1 is hypothesized to dictate the temporal and spatial influences of conceptus IFNτ on ISG expression in the endometrial S and GE as determined from studies in ovine endometrial cell lines [9, 10] as well as human fibroblast cell lines lacking specific IFN signaling components [11]. Collectively, available results support the concept that the continual, elevated expression of ISGs in the endometrial S and GE during pregnancy can be attributed to IFNτ stimulation of janus kinase (JAK), JAK1 and Tyk2, activation of Stats 1 and 2 by phosphorylation, formation of the transcription factor complexes GAF and ISGF3, and increased expression of IRF-1 and then Stat 1, Stat 2, and IRF-9 (Fig. 10). Similar to in vitro observations on IFNτ signal transduction pathways [9–11], IFNτ activation of Stats 1 and 2 is proposed to result in ISGF3 formation and hyperactivated expression of ISRE-containing target genes, such as Stat 1, Stat 2, IRF-9, ISG17, and OAS, in the S and GE. Similarly, activation of Stat 1 elicits formation of Stat 1 homodimers or GAF that activates GAS-containing target genes, such as IRF-1 [10, 25, 49]. The IRF-1 can bind to ISREs [50] and may assist in maintaining activation of ISRE-containing target genes by type I IFNs [13, 27], because it contains a transactivation domain [51]. The simultaneous induction of Stat 2 and IRF-9 gene expression by IFNτ appears to

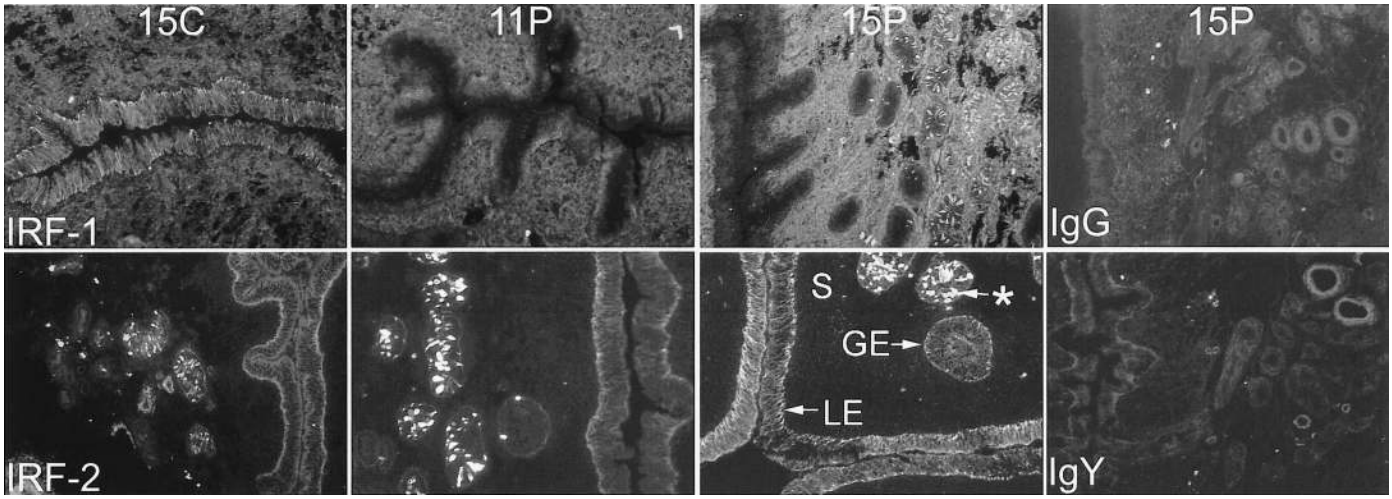

FIG. 7. Detection of IRF-1 and IRF-2 protein in uterine endometrium from cyclic and pregnant ewes from study 1. Immunofluorescence staining of frozen uterine sections from cyclic (C) and pregnant (P) ewes was conducted using specific antibodies or irrelevant IgG as a control (see *Materials and Methods*). See Figure 1 for abbreviations. ×230.

shift transcription factor formation from GAF toward predominantly ISGF3 [10, 11]. The persistent formation of ISGF3 would favor the expression of ISGs in the S and GE.

In this study, low levels of IRF-2 gene expression were detected in the LE and sGE from ewes on Days 11, 13, and 15 of the estrous cycle. These findings conflict with those of a previous report [6], perhaps due to differences in sensitivities of antibodies or methods for immunohistochemistry. In the present study, a peptide-specific antibody generated to ovine IRF-2 was used in immunofluorescence analyses of frozen ovine uterine tissue. Results indicated that IRF-2 mRNA and protein expression increased in the endometrial LE and sGE between Days 11 and 15 of pregnancy, as reported previously [6]. The increase in IRF-2 gene expression in the LE and sGE likely involves other factors besides IFNτ, because IFNτ does not induce or increase IRF-2 expression in the endometrium of cyclic ewes (Fig. 8) or in bovine and ovine endometrial epithelial cell lines [13, 52]. The decline in IRF-2 mRNA expression in the whole endometrium between Days 15 and 17 of pregnancy is likely due to loss of some LE during initial synepithelial placentation [53].

Expression of Stat 1, Stat 2, and IRF-9 mRNA and protein was not detected in the LE and sGE on Days 11–19 of early pregnancy in this study. These results agree with those reported for ISG17 and OAS expression in the ovine uterus [15, 16, 22, 23]. The absence of these ISGs in the LE and sGE during early pregnancy is hypothesized to result from expression of IRF-2, a potent repressor and attenuator of IFNα/β-induced gene expression [33, 34, 54–57]. In the present and a previous study [6], IRF-2 protein was detected only in the LE and sGE on Days 13, 15, 17, and 20 of pregnancy. Results from the transient transfection studies presented here further support the hypothesis that IRF-2 expression in the LE and sGE restricts and specifies increases in ISG expression to the S and GE in response to

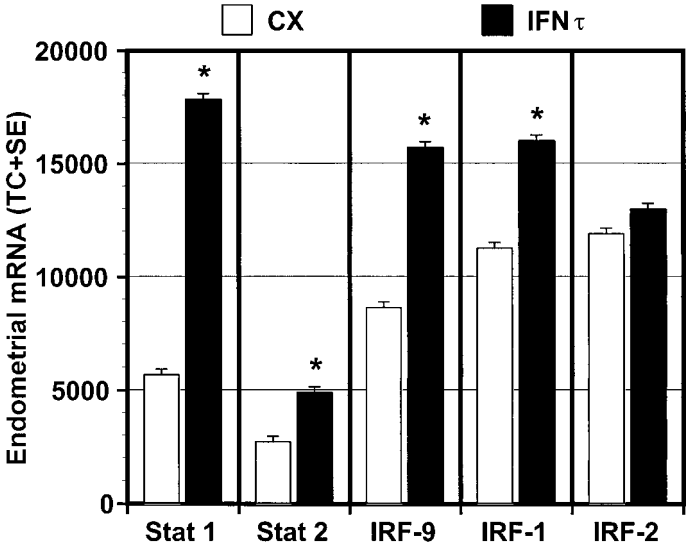

FIG. 8. Effects of IFNτ on steady-state levels of Stat and IRF mRNAs in ovine endometrium from study 2. Slot blot analyses were conducted on total endometrial RNA obtained from uteri of Day 16 cyclic ewes receiving intrauterine injections of roIFNτ or control proteins from Days 11 to 15 (n =5 ewes/treatment). Note that levels of Stat 1, Stat 2, IRF-9, and IRF-1 mRNAs were increased ($P < 0.05$) in endometrium from ewes receiving intrauterine injections of IFNτ. In contrast, IRF-2 mRNA levels were not affected by treatment ($P > 0.10$).

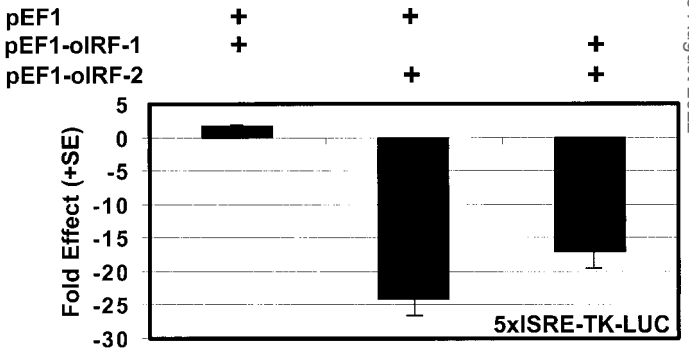

FIG. 9. Effects of ovine IRF-1 and ovine IRF-2 on activity of a promoter containing ISRE in transient transfection assays. Ovine S cells were transiently cotransfected with the 5xISRE-TK-LUC reporter construct and pEF1, pEF-1-oIRF-1, and/or pEF1-oIRF-2 as indicated. Luciferase and β-galactosidase activities were determined at 24 h after transfection. β-Galactosidase activity was used to normalize luciferase activity data. Data are presented as the mean fold-effect from four independent experiments with SEM.

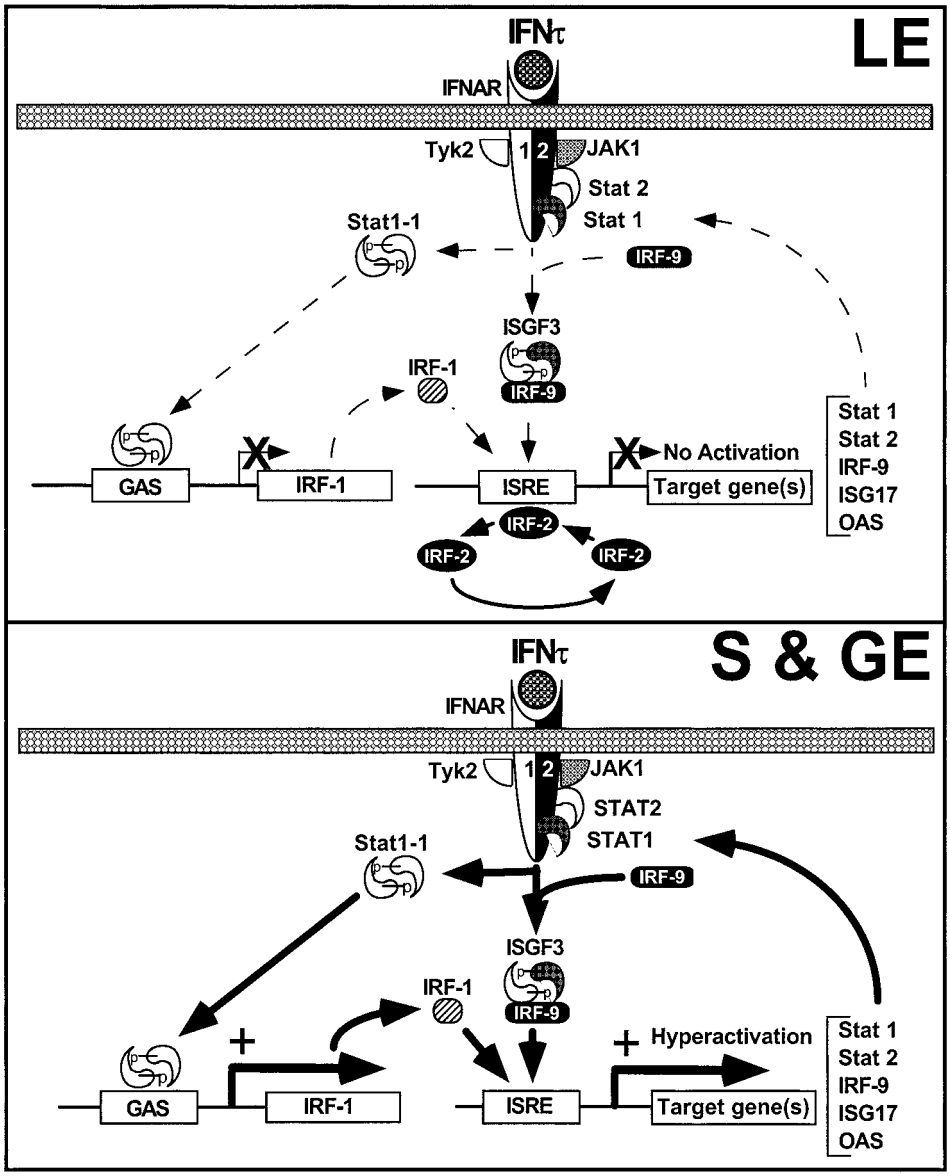

FIG. 10. Model for IFNτ signaling in the endometrial epithelium and stroma of the ovine uterus. The IFNτ, produced in large amounts by the developing conceptus, binds to the type I IFN receptor (IFNAR) present on cells of the ovine endometrium. In cells of the S and middle to deep GE (**bottom**), IFNτ-mediated association of the IFNAR subunits facilitates the cross-phosphorylation and activation of two janus kinases, Tyk2 and JAK1, which, in turn, phosphorylate the receptor and create a docking site for Stat 2. Then, Stat 2 is phosphorylated, thus creating a docking site for Stat 1, which is then phosphorylated as well. Both Stat 1 and Stat 2 are then released from the receptor and can form two transcription factor complexes. The ISGF3 is formed by association of the Stat1–2 heterodimer with IRF-9 in the cytoplasm, which translocate to the nucleus and transactivate genes containing an ISRE(s), such as Stat 1, Stat 2, IRF-9, ISG17, and OAS. The GAF is formed by binding of Stat 1 into homodimers, which translocate to the nucleus and transactivate genes containing a GAS element(s), such as IRF-1. The IRF-1 can also bind and transactivate ISRE-containing genes. The simultaneous induction of Stat2 and IRF-9 gene expression by IFNτ appears to shift transcription factor formation from GAF toward predominantly ISGF3. Therefore, IFNτ activation of the JAK-Stat signal transduction pathway allows for constant formation of ISGF3 and GAF transcription factor complexes and hyperactivation of ISG expression. In the cells of the LE and sGE, IFNτ is prevented from activating ISGs in the LE and sGE by IRF-2 (**top**). The IRF-2, a potent and stable repressor present in the nucleus, increases during early pregnancy in LE and sGE. The continual presence of IRF-2 inhibits ISRE-containing target genes through direct ISRE binding and coactivator repulsion.

pregnancy and intrauterine IFNτ. Transient transfection assays revealed that ovine IRF-2 strongly inhibited transcriptional activity of an ISRE-driven reporter construct, which supports the results of Fleming et al. [52], who found that ovine IRF-2 inhibited reporter constructs driven by closely related IRF-binding elements or IRF-Es in transient transfection assays.

Collectively, available in vitro and in vivo results support the hypothesis that IRF-2 prevents the induction of ISG expression in ovine LE and sGE in response to IFNτ (Fig. 10). The IRF-2, a unique member of the IRF family of transcription factors, is a potent repressor of IFNα/β-induced gene transcription [33, 34, 54–57]. Both IRF-2 and ISGF3 bind to ISREs [58]. Unlike ISGF3 and IRF-1, IRF-2 is a stable nuclear protein and is expressed in many cell types [55]. Recently, it was demonstrated that IRF-2 competes with IRF-1 for ISRE binding, and that IRF-1 and IRF-2 formed a heterocomplex wherein IRF-2 decreased transactivation by IRF-1 [35]. The IRF-2 contains a repression domain [59] and has a rapid on-off rate for ISRE binding [34]. Senger et al. [35] provided strong evidence that IRF-2 represses transcription by inhibiting recruitment of coactivators into enhanceosomes by transcription factors. Hida et al. [34] proposed that IRF-2 continuously interacts with ISRE elements in promoter regions of ISGs to inhibit ISGF3 transcription factor complex induction of ISGs. Although the IRF-1 gene is not directly attenuated by IRF-2, IRF-1 gene expression did not increase in the endometrial LE and sGE during early pregnancy in the present study. Increases in IRF-1 gene transcription in several cell lines requires induction of the Stat 1 gene and formation of Stat 1 dimers, that is, Stat 1 homodimers or GAF, or Stat 1–2 heterodimers [10, 11, 24, 25]. Therefore, the lack of IRF-1 induction by IFNτ in the ovine uterine endometrial LE may be explained by the lack of detectable increases in Stat 1 and Stat 2 gene expression in these cells. Another possibility is that IFNτ activates other signal transduction pathways that are unrelated to the JAK-Stat pathway, such as nuclear factor-kappa B by stimulation of phosphatidylinositol 3-kinase and Akt [60], but that are involved in IFNτ regulation of LE gene expression. These signaling pathways are involved in other type I IFN effects on gene expression and warrant further investigation. A plausible hypothesis is that activation of non-JAK-Stat pathways by

IFNτ and the consequent lack of ISG induction in the LE relate to the higher levels of basal prostaglandin $F_{2\alpha}$ secretion in early pregnant ewes [7, 8].

In the present study, an increase in IRF-1 expression was detected in the endometrium from ewes on Days 11–15 of the estrous cycle. The precise cellular and molecular mechanism regulating this event is not known, but it correlates with a decline in overall endometrial IRF-2 gene expression in the present study as well as with increases in ERα and OTR gene expression that are specific to the endometrial LE and sGE [4–6]. The ovine ERα and bovine OTR genes contain several IRF-Es [52, 61]. Recently, Fleming et al. [52] found that the promoter/enhancer region of the ovine ERα gene contains two functional IRF-Es and one ISRE. Furthermore, overexpression of ovine IRF-2 inhibited transcriptional activity of several regions of the ovine ERα promoter containing a functional IRF-E or ISRE. Therefore, the overall ratio of IRF-1 to IRF-2 protein in endometrial LE and sGE may regulate ERα and, perhaps, OTR expression during development of the endometrial luteolytic mechanism in cyclic ewes and during pregnancy recognition in pregnant ewes.

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