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Effects of the Estrous Cycle and Early Pregnancy on Uterine Expression of Mx Protein in Sheep (*Ovis aries*)[1]

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ABSTRACT

Conceptuses of ruminant ungulates produce large amounts of a type I interferon, interferon-tau (IFNτ), which is the signal for maternal recognition of pregnancy. Induction of cellular Mx proteins is an important component of the response to type I interferon in the immune system, but Mx regulation and function have not been studied in the uterus. This study examined temporal and spatial alterations in ovine uterine Mx expression during the cycle and early pregnancy using immunohistochemistry, in situ hybridization, and Northern and slot-blot analysis. Sheep uterine endometrium expressed a single ~2.5-kilobase Mx mRNA transcript that was detectable at all stages of the estrous cycle and early pregnancy examined. In cyclic ewes, mRNA abundance in endometrium increased from Day 1 to peak levels at Day 13 and then declined to Day 15. In pregnant ewes, steady-state levels of Mx mRNA were first detected above the level in cyclic ewes at Day 13 postmating, were greater than 10-fold higher at Day 15, and remained elevated at Day 19. Expression of Mx mRNA in the myometrium did not change during the estrous cycle but increased ~23-fold between Days 11 and 15 of pregnancy. Immunohistochemical and in situ hybridization analysis revealed a similar temporal pattern of Mx expression. In cyclic ewes, Mx protein and mRNA were initially localized to the luminal epithelium at Days 1 and 3, increased from Days 5 to 13, especially in the shallow uterine glands, and then declined at Day 15. Pregnancy resulted in up-regulation of Mx expression in the luminal and glandular epithelium, stroma, and myometrium. Punctate Mx immunostaining and Mx mRNA concentrations were greatest when progesterone production was maximal during the estrous cycle and were strongly up-regulated by the conceptus across the entire uterine wall. It is suggested that a cascade of induction of Mx gene expression proceeds from the luminal epithelium to the outer longitudinal myometrium and that transcriptional activation of the promoter may involve both soluble cytokines (i.e., IFNτ) and steroid hormones (i.e., progesterone).

INTRODUCTION

All ungulate farm species have uterine-dependent ovarian cycles and, as such, the conceptus must signal its presence to the maternal system by altering endometrial gene expression to block the uterine luteolytic signal [1]. Pregnancy recognition in ruminants is accomplished by production of large amounts of a novel type I interferon (IFN), interferon-tau (IFNτ), by the mononuclear trophectoderm of the conceptus [2]. Production of IFNτ by the trophoblast occurs between Days 11 and 21 of early pregnancy in sheep and is minimally affected by classic inducers of IFN including virus and double-stranded RNA [2]. The total amount of IFNτ produced during early pregnancy can exceed 20×10^6 antiviral units (~200 μg) in a 24-h period [2].

In addition to its role in pregnancy recognition, IFNτ possesses potent antiviral, antiproliferative, and immunomodulatory activities similar to those of other type I IFNs [3–5]. IFN receptors are present at high concentrations on the uterine endometrium in sheep [6, 7], and endometrial type I IFN receptors can bind IFNτ and IFNα with similar avidity [8]. The second messenger system mediating effects of IFNτ on the endometrium is not well characterized, but it is thought to involve the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway characterized for other type I IFNs [9]. Proteins whose expression is activated by IFN in other systems have been shown to increase in response to pregnancy or IFNτ in ruminants, including 2′,5′ oligoadenylate synthetase [10], β2-microglobulin [11], IFN regulatory factors 1 and 2 [12], and ubiquitin cross-reactive protein [13]. Interestingly, granulocyte chemotactic protein-2 (GCP) was induced in bovine uterine cells by IFNτ but not by IFNα [14]. The effects of type I IFN on GCP expression have not been examined in other tissues. Therefore, IFNτ may activate pathways similar to those activated by type I IFN in other tissues, but novel IFNτ-induced proteins and signaling pathways may also exist. However, IFNτ-regulated genes are not well characterized in the uterus of ruminants.

One cellular response to type I IFNs demonstrated in many different species and cell types is induction of Mx protein(s) [15]. Mx proteins are type I IFN-induced antiviral proteins, expression of which is directly induced in response to viral infection and which are strongly up-regulated in response to type I IFN production during a viral infection [15]. To date the only known function for Mx protein is in the antiviral response [16]. However, Mx is a functional GTPase that shares sequence homology with a class of monomeric GTPases identified in diverse species with roles in intracellular protein and vesicle trafficking [17]. In all systems studied to date, Mx gene expression is regulated by type I IFNs or virus. However, relatively little is known about Mx regulation and function in the mammalian uterus.

An ovine uterine Mx cDNA was cloned, and Mx mRNA was shown to be expressed in the pregnant horn of unilaterally pregnant ewes and in ewes treated with IFNτ or IFNα

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[18]. However, authors of that study did not detect Mx mRNA expression during the estrous cycle. In addition, cell types in the endometrium that express Mx mRNA were not determined and Mx protein expression was not examined. The present study examined temporal and spatial (cell type) alterations in uterine Mx mRNA and protein expression in cyclic and early-pregnant ewes during the period of maternal recognition of pregnancy as a first step in defining the function of Mx in the uterus.

MATERIALS AND METHODS

Animal Procedures

All procedures involving animals were in accordance with The Guide for Care and Use of Agricultural Animals in Research and Teaching, were reviewed and approved by the Texas A&M Agricultural Animal Care and Use Committee, and are on file under Animal Use Protocol #5241.

Mature, crossbred Rambouillet ewes were monitored daily for estrous behavior using vasectomized rams. After two estrous cycles of normal duration (16–18 days), ewes were assigned randomly to cyclic or pregnant status (n = 3–5 ewes per day). Ewes assigned to the pregnant status were mated to intact rams at estrus (Day 0) and 12 and 24 h later. Uteri were collected from cyclic (Days 1, 3, 5, 7, 9, 11, 13, and 15 postestrus) and pregnant (Days 11, 13, 15, 17, 19 postmating) ewes at hysterectomy. All uteri (except those from Days 17 and 19 of pregnancy) were flushed with 20 ml sterile 154 mM NaCl, and pregnancy was confirmed by the presence of an apparently normal conceptus in uterine flushings (Days 11 and 15) or by the presence of a conceptus in uterine cross sections (Days 17 and 19). Caruncular and intercaruncular endometrium and myometrium from the uterine horn ipsilateral to the corpus luteum were separated and frozen for later RNA extraction. Uterine cross sections from the uterine horn ipsilateral to the corpus luteum were fixed for 24 h at room temperature in 4% paraformaldehyde, washed twice for 24 h in 70% ethanol, and embedded in TissuePrep (Fisher Scientific, Pittsburgh, PA) for immunohistochemical analyses. For in situ hybridization analysis, tissues were obtained from ewes at Days 1, 6, 11, 13, and 15 of the cycle and Days 11, 13, 15, 17, and 25 of pregnancy.

Polymerase Chain Reaction (PCR) Cloning of a Partial cDNA for Ovine Mx

Oligonucleotide primers (forward primer F1: 5′-GGAAATCAGTGAAGCCCAGATTG-3′ and reverse primer R1: 5′-AACCACAGCACTCCATTTGCAG-3′) were synthesized based on the published sequence for ovine (o) Mx [18] and used to PCR-amplify an 832-base pair (bp) fragment of the oMx gene (TA-oMx#1a) from Day 16 reverse-transcribed ovine endometrial total cellular RNA. The PCR product was cloned into the pCR-II cloning vector (InVitrogen, Carlsbad, CA) and sequenced to confirm identity.

RNA Isolation

Total cellular RNA (tcRNA) was isolated from frozen uterine tissues using the Trizol reagent (Life Technologies, Gaithersburg, MD). Amount and quality of the RNA were assessed by spectroscopy and by agarose/formaldehyde gel electrophoresis and UV illumination.

Complementary RNA Probes

For production of template for riboprobe synthesis, the oMx plasmid was linearized with *Xho* I or *Sac* II (antisense) and *Spe* I or *Bam*HI (sense) and gel purified using a Glassselect DNA isolation kit (5-prime 3-prime Inc., Boulder, CO). Homologous sense and antisense oMx cRNA probes used for Northern blot, slot-blot, and in situ hybridization analyses were synthesized using an in vitro transcription kit (Ambion, Austin, TX) and [α-^{32}P]UTP (Northern analysis) or [γ-^{35}S]ATP (in situ hybridization) according to manufacturer instructions. The linearized oMx cDNA (TA-oMx#1a) cloned into the PCR II vector (InVitrogen) was used with either SP6 (antisense) or T7 (sense) RNA polymerase (Ambion). Radiolabeled riboprobes were analyzed by 4% polyacrylamide/8 M urea gel electrophoresis and autoradiography and were column purified (STE Select-D(RF) spin columns; 5-prime 3-prime Inc.).

Northern Hybridization

Northern analysis was performed to determine the number and size of oMx transcripts present in endometrial total RNA. Denatured tcRNA (10 μg) was size fractionated on a 1.2% agarose gel containing formaldehyde and 3-[*N*-morpholino]propanesulfonic acid and transferred to a nylon membrane (Nytran plus; Schleicher & Schuell, Keene, NH) by capillary diffusion. The blot was baked (80°C) for 2 h, and RNA was cross-linked to the membrane using a UV transilluminator (Stratagene, La Jolla, CA). The Northern blot was then prehybridized in hybridization buffer (50% formamide, 50 mM Na_2PO_4 [pH 7.5], 5-strength SSC [single-strength SSC is 0.15 M sodium chloride and 0.015 M sodium citrate], 0.1% SDS, 1.0 mM EDTA, 0.5-strength Denhardt's, 200 μg/ml salmon testis DNA) for 4 h at 55°C. The radiolabeled antisense oMx cRNA probe was heat denatured at 95°C for 5 min and then hybridized to the blot for 16 h at 55°C. The blot was then washed three times in 0.1-strength SSC, 0.1% SDS for 20 min at 68°C. Hybridizing transcripts were visualized by autoradiography at −80°C and quantitated using an Instant Imager (Packard Imaging Co., Meridian, CT). For quantitating changes in Mx mRNA levels in endometrium (n = 3–5 ewes per day) and myometrium (n = 2–5 ewes per day) across days and between statuses, total RNA samples (4 μg RNA per ewe endometrium and 10 μg RNA per ewe myometrium) were immobilized on nylon membrane using a slot-blot apparatus (Schleicher & Schuell) and probed with a radiolabeled antisense Mx cRNA probe as described above. Signal intensity was quantified using an Instant Imager. Blots were stripped and reprobed with an actin antisense cRNA probe (Ambion) to correct for variations in amount of RNA loaded.

In Situ Hybridization

Cellular distribution of oMx mRNA in the uterus was examined by in situ hybridization analysis of paraformaldehyde-fixed, TissuePrep-embedded tissues. After sectioning (5 μM), tissue sections were deparaffinized in a xylene substitute (Hemo-De; Fisher Scientific) for 5 min and rehydrated through a graded series of ethanol baths (100%, 95%, 70%, and 50%; 2 min each). Sections were postfixed in freshly prepared 4% paraformaldehyde (in 0.1 M $NaPO_4$, pH 7.4) for 10 min and washed in 0.5-strength SSC for 5 min at room temperature (RT). RNA-free control sections were prepared by digesting with DNase-free RNase A (Sig-

ma Chemical Co., St. Louis, MO; 10 μg/ml in 500 mM NaCl, 10 mM Tris, pH 8) for 10 min at 37°C, and digestion was stopped by washing in 0.5-strength SSC for 5 min at RT. All tissue sections were then incubated with Proteinase K (Sigma; 20 μg/ml in 50 mM Tris, 5 mM EDTA, pH 8) for 10 min at RT and washed with 0.5-strength SSC for 10 min at RT. Sections were then covered with 100 μl hybridization buffer (50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl, 5 mM EDTA, 10 mM $NaPO_4$, 10% dextran sulfate, single-strength Denhardt's, 0.5 mg/ml yeast RNA, pH 8) and incubated in a covered box humidified with 4-strength SSC, 50% formamide for 3 h at 42°C. Labeled antisense and sense riboprobes were diluted with hybridization buffer to a concentration of 500 000 cpm/10 μl, denatured at 68°C for 5 min, and placed on ice immediately prior to use. Each slide contained two serial tissue sections, one hybridized with sense and the other with antisense probe. Hybridization buffer (10 μl) containing the appropriate probe was added to each 100 μl of prehybridization buffer. Sections were hybridized overnight at 55°C in a humidified box as described above and then washed in double-strength SSC containing 10 mM β-mercaptoethanol (βME)/1 mM EDTA for 10 min at RT, immersed in RNase A solution (15 μg/ml) for 30 min at RT, and washed in double-strength SSC containing 10 mM βME/1 mM EDTA for 10 min at RT. Sections were washed in 0.1-strength SSC containing 10 mM βME/1 mM EDTA for 2 h at 55°C and then in 0.5-strength SSC for 10 min at RT. Sections were dehydrated through a graded series of ethanol washes (50%, 70%, and 90%) each containing 0.3 M ammonium acetate, dried at room temperature, and exposed to x-ray film (X-OMAT AR; Eastman Kodak, Rochester, NY) overnight.

Liquid film emulsion autoradiography was performed by covering each slide in Kodak NBT-2 liquid photographic emulsion (1:1 in distilled H_2O). Slides were then dried and stored with desiccant in a light-tight box at 4°C for 8 wk. Slides were developed using Kodak D19 developer (1:1 in distilled H_2O) for 4 min at 15°C, rinsed in distilled water for 30 sec at 15°C, and fixed in Kodak Fixer for 5 min at 15°C. Slides were then rinsed twice in distilled water for 5 min, lightly counterstained with Harris modified hematoxylin in acetic acid (Fisher Scientific), dehydrated through a graded series of ethanol baths into Hemo-De, and coverslipped with Permount (Fisher Scientific). Photomicrographs were taken with a Zeiss Photomicroscope (Carl Zeiss, New York, NY) and Kodak Technical-Pan black and white film.

Immunohistochemistry

Localization of oMx protein was determined using a monoclonal antibody directed against the amino terminus of human MxA (1319.35.126; kindly supplied by M. Horisberger; Novartis, Basel, Switzerland) and a Super ABC Mouse/Rat Kit (Biomeda, Foster City, CA). Paraformaldehyde-fixed, paraffin-embedded uterine tissues were sectioned (5 μm), affixed to silane-treated slides, deparaffinized twice in Hemo-De for 5 min, and rehydrated through a series of graded alcohols (2 min each). Endogenous peroxidase activity was quenched by incubating sections in 3% peroxide in methanol for 5 min. Sections were then rinsed in water, equilibrated in PBS (pH 7.4) for 5 min, digested with pronase E (0.5 mg/ml PBS; Sigma) for 8 min at 37°C, and rinsed with water to stop digestion. The remainder of the procedure was as described in the Super ABC kit. The chromogen used to detect peroxidase activity was 3–3′ diaminobenzadine tetrahydrochloride (DAB; Sigma). Sections were counterstained lightly with Harris modified hematoxylin (Fisher Scientific). The primary antibody was used at 5 μg/ml in PBS containing 1% BSA for 16 h at 4°C, and mouse IgG (5 μg/ml) was used to control for primary antibody. Additional controls included no primary antibody and no secondary antibody. Photomicrographs were recorded on Kodak Elite II 100 color film.

Data Analysis

For immunohistochemical analysis, distribution and intensity of staining were evaluated independently by two individuals and were scored as absent (−), present above background (+), low (++), moderate (+++), or abundant (++++). Quantitative data from slot-blot analysis of endometrial and myometrial total RNA were analyzed using General Linear Models procedures of the Statistical Analysis System [19]. The mathematical model included main effects of Status (cyclic vs. pregnant) and Day (1, 3, 5, 7, 9, 11, 13, 15 cyclic and 11, 13, 15, 17, 19 pregnant) and their interaction. Error terms used in the tests of significance were according to the expectation of the mean square for error. Regression analysis was used to characterize changes in Mx mRNA abundance across Day within Status. Actin hybridization signal was used as a covariate in the analysis to adjust for variations in RNA loading. Data presented are adjusted least-squares means and standard errors.

RESULTS

Northern Blot Hybridization

A partial cDNA for oMx was cloned from reverse-transcribed Day 16 ovine endometrial RNA by PCR using primers based on the published sequence for oMx [18]. The cDNA (TA-oMx#1a) was 97% identical to the oMx sequence published by Charleston and Stewart [18]. This partial oMx cDNA was used in Northern blot, slot-blot, and in situ hybridization analysis to characterize temporal and spatial alterations in uterine Mx expression during the estrous cycle and early pregnancy. An antisense riboprobe synthesized from the 832-bp oMx cDNA recognized a transcript of approximately 2.5 kilobases (kb) in endometrial (Fig. 1) and myometrial tcRNA from Day 15 cyclic and pregnant ewes, which agrees with results of Charleston and Stewart [18]. Sense riboprobes showed no significant hybridization (data not shown).

Slot-Blot Analysis

Results from slot-blot analysis of endometrial (4 μg/ewe) and myometrial (10 μg/ewe) tcRNA isolated from cyclic and pregnant ewes are shown in Figure 2. For endometrium, steady-state concentrations of Mx mRNA were higher in pregnant than in cyclic ewes ($p < 0.01$; Fig. 2A). Mx mRNA expression was detected during the estrous cycle, and an effect of Day ($p < 0.02$) was detected, expression being lowest at Days 1 and 15 and highest between Days 11 and 13 of the estrous cycle. For pregnant ewes, endometrial Mx mRNA concentrations increased ($p < 0.01$) from Day 11 to maximal concentrations between Days 15 and 19 (Fig. 2A). Elevation of Mx mRNA above concentrations in cyclic ewes was first detectable on Day 13 of pregnancy. Overall, Mx mRNA concentrations increased 25-fold between Days 11 and 15 of early pregnancy and remained high through Day 19 of pregnancy. For myometrium, concentrations of Mx mRNA did not differ dur-

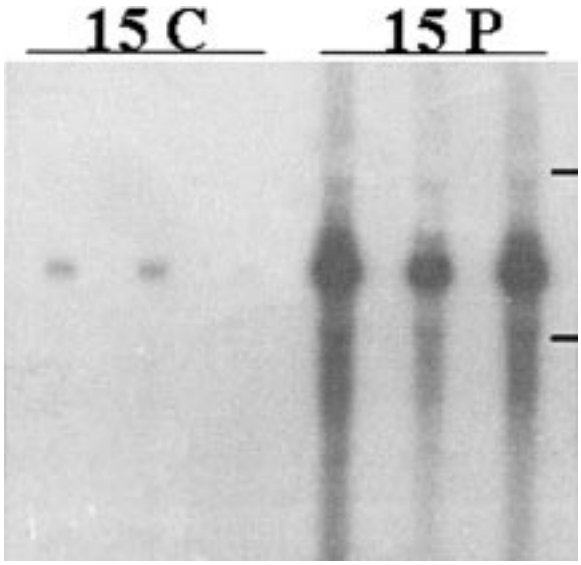

FIG. 1. Northern blot analysis of tcRNA (10 μg) isolated from endometrium collected at Day 15 of the estrous cycle (15 C) or Day 15 of pregnancy (15 P) (n = 3 ewes per day). Relative migration of 28S and 18S ribosomal RNA is indicated by the black bars. Specific mRNA hybridization was detected at ~2.5 kb in endometrium RNA isolated from both cyclic and pregnant ewes at Day 15.

ing the estrous cycle ($p > 0.1$; Fig. 2B). Overall, pregnant ewes had higher concentrations of Mx mRNA than cyclic ewes ($p < 0.01$). Elevation of myometrial Mx mRNA above concentrations in cyclic ewes was first detected at Day 15 of pregnancy and continued on Days 17 and 19 ($p < 0.01$; quadratic). Myometrial Mx mRNA concentrations increased approximately 23-fold between Days 11 of 15 of pregnancy. Since endometrial and myometrial Mx expression were determined in independent experiments using different amounts of RNA and different preparations of radiolabeled antisense Mx cRNA probes, no comparisons should be made of relative hybridization intensity between the two tissues.

In Situ Hybridization

Representative photomicrographs from in situ hybridization analysis of uterine tissues obtained on Days 6, 11, 13, and 15 of the estrous cycle (Fig. 3) and on Days 11, 13, 17, and 25 of pregnancy (Fig. 4) showed status-specific (pregnant vs. cyclic) and cell type-specific hybridization on all days examined, whereas sense probes exhibited a diffuse, nonspecific hybridization pattern (Fig. 4H).

Relative hybridization intensity of Mx mRNA in cyclic and early-pregnant ewes is summarized in Table 1. For cyclic ewes, specific hybridization was detected, primarily over the luminal epithelium between Days 1 and 11 postestrus. Hybridization increased in the shallow glandular epithelium by Day 13 before decreasing at Day 15 (Fig. 3). Hybridization in the deep glands, stroma, and myometrium was not above background. A weak hybridization signal was detected in luminal but not glandular epithelium at Days 1 (not shown) and 15 (Fig. 3H) of the estrous cycle.

Pregnancy resulted in a marked increase in Mx mRNA expression (Fig. 4). Increased hybridization signal was first detected consistently on Day 13. Maximal Mx mRNA expression was observed on Day 17 of pregnancy in luminal epithelium and was greater than that observed on any day of the estrous cycle. Interestingly, expression was also detected in shallow and deep glands and throughout the stroma (Fig. 4) and myometrium (not shown). Stromal and myometrial Mx expression were not detected by in situ hybridization analysis in uterine tissues from cyclic ewes. Mx expression continued to be detected on Day 25 of pregnancy (Fig. 4I), well beyond the period of maximal production of oIFNτ (Days 15–17). On Day 25 of pregnancy, Mx expression was confined primarily to luminal and shallow glandular epithelium with minimal expression in stroma and myometrium. There was no detectable difference in the pattern of Mx expression between caruncular and intercaruncular endometrium.

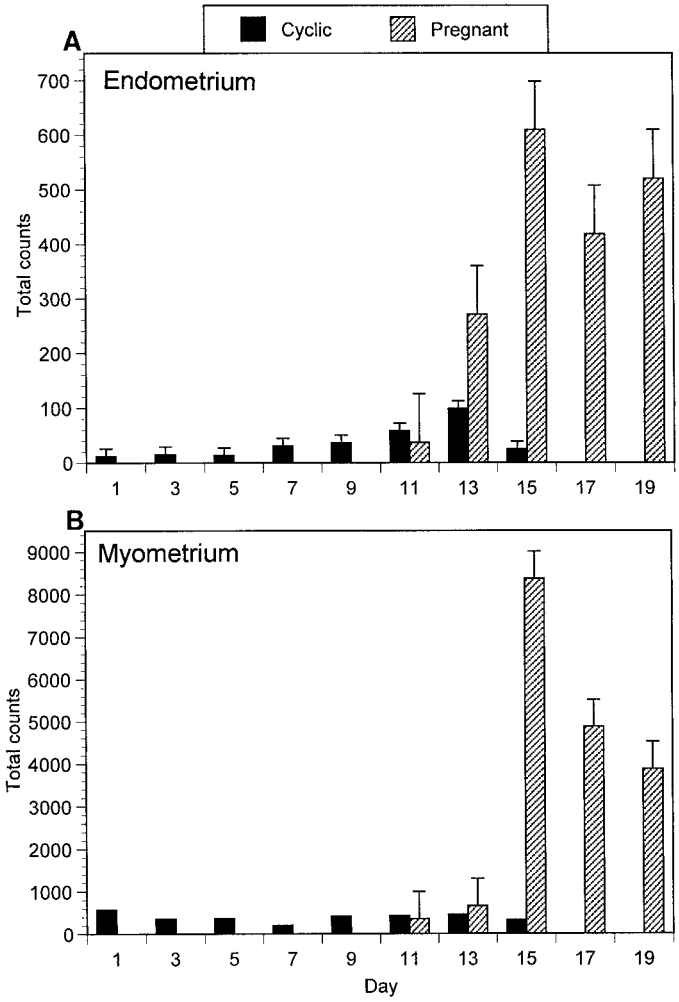

FIG. 2. Slot-blot analysis of Mx mRNA abundance in RNA isolated from endometrium (**A**) and myometrium (**B**) at Days 1–15 of the estrous cycle and 11–19 of pregnancy. In endometrium from cyclic ewes, Mx mRNA concentrations increased from Day 1 to maximal concentrations on Day 13 and then declined to Day 15 postestrus ($p < 0.02$; cubic). In endometrium from pregnant ewes, Mx mRNA concentrations increased from Day 11 to maximal concentrations on Day 15 and remained elevated at Day 19 postmating ($p < 0.01$; quadratic). At Day 15, concentrations of Mx mRNA were 10-fold greater in pregnant than in cyclic endometrium. In myometrium from cyclic ewes (**B**), concentrations of Mx mRNA did not differ during the estrous cycle. During pregnancy, Mx mRNA increased in the myometrium after Day 13 to maximal concentrations on Day 15 and then declined to Day 19 ($p < 0.01$).

Immunohistochemistry

Representative photomicrographs depicting immunohistochemical analyses of uterine tissues from cyclic and preg-

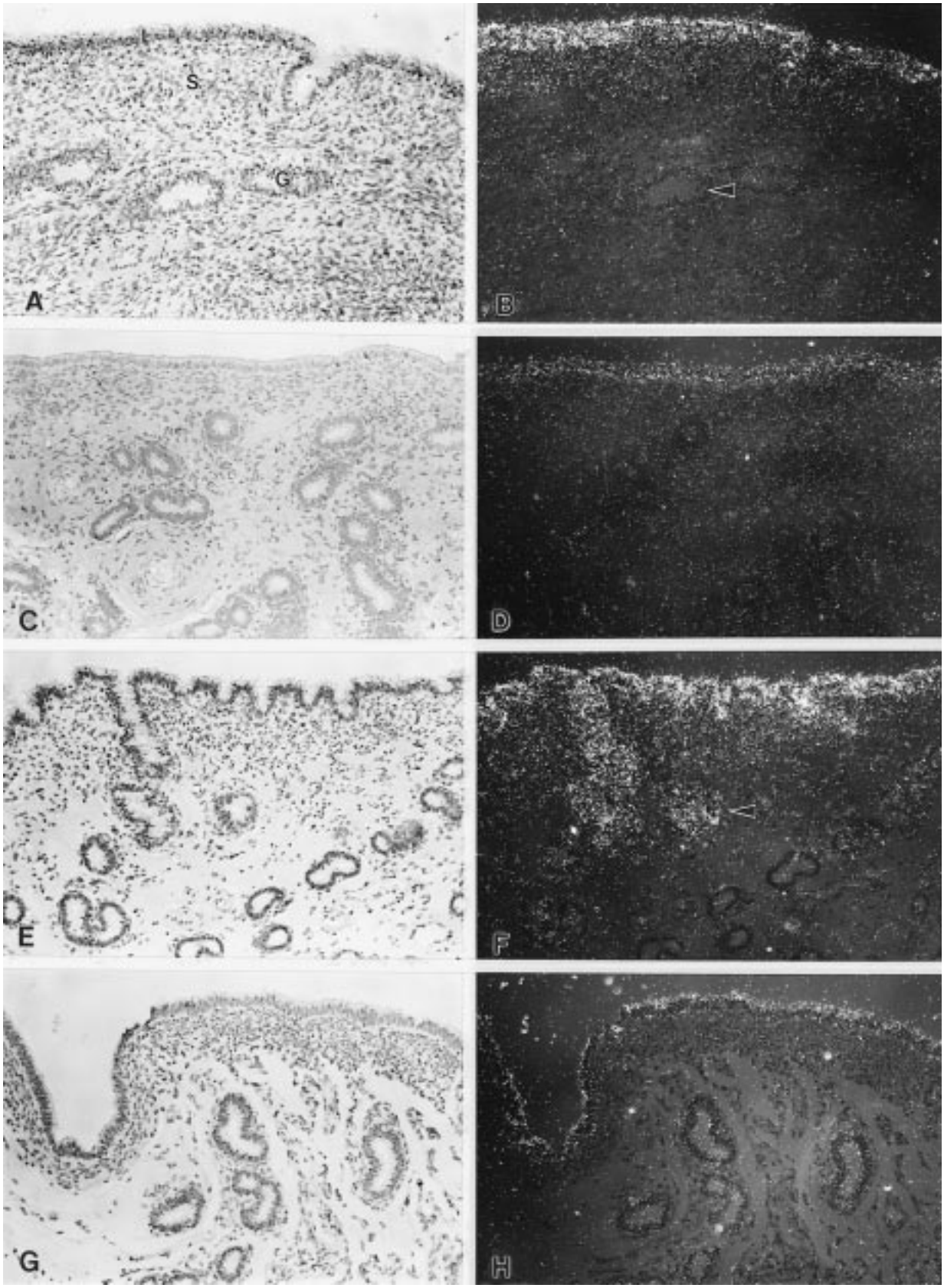

FIG. 3. Representative in situ hybridization analysis of uterine cross sections at Days 6 (**A, B**), 11 (**C, D**), 13 (**E, F**), and 15 (**G, H**) of the estrous cycle under brightfield (**A, C, E, G**) and darkfield (**B, D, F, H**) illumination. See Figure 4H for representative background (sense) signal intensity. At Day 6, 11, and 15 postestrus, specific hybridization was localized primarily to the luminal epithelium (L), and was not above background in the stroma (S) or uterine glands (G; see arrow in **B**). At Day 13, hybridization intensity was increased in the shallow glands (see arrow in **F**) and in the shallow stroma. Overall, hybridization intensity increased from Day 1 to Day 13 and then decreased to Day 15 (×180; reproduced at 90%).

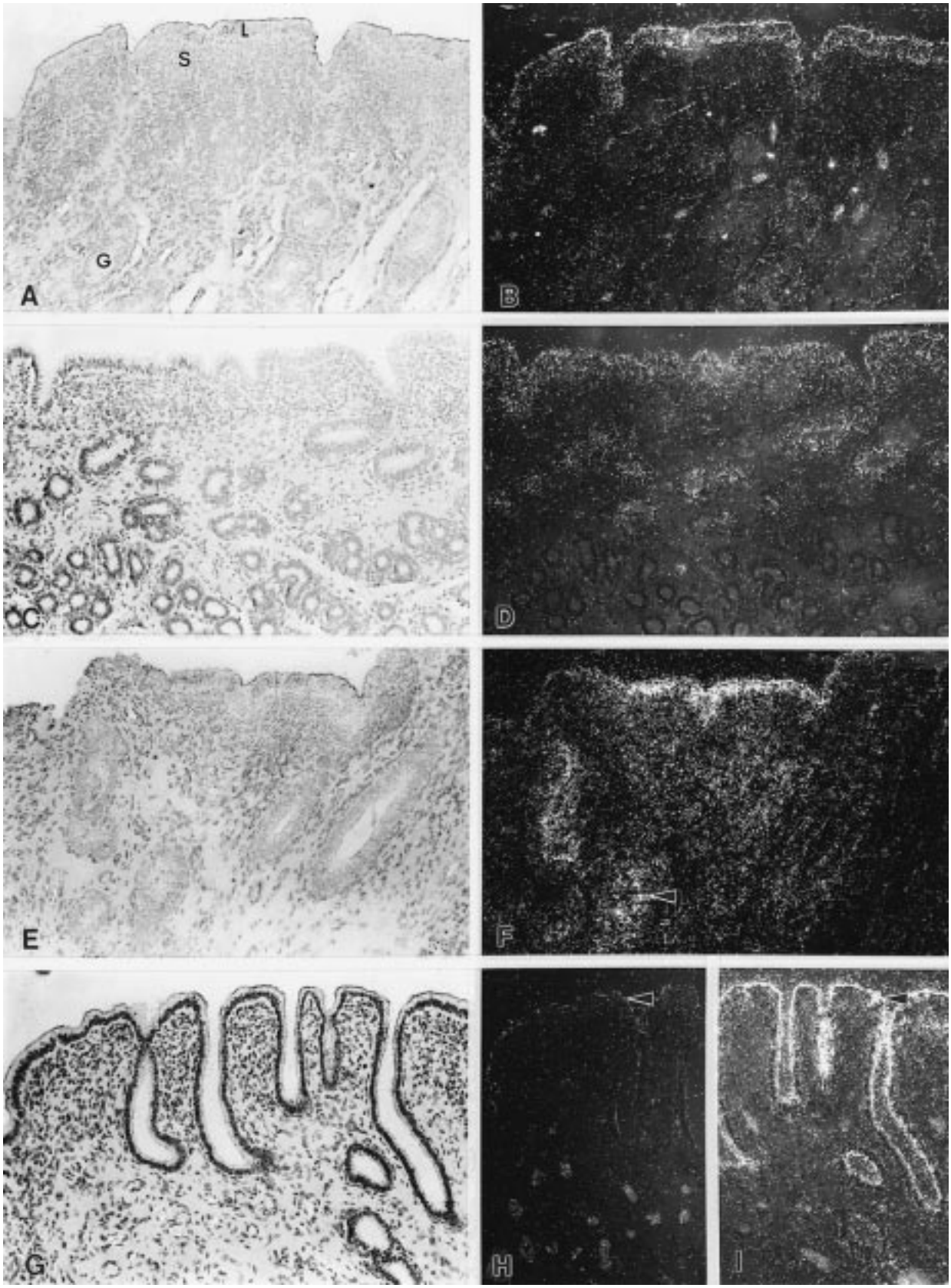

FIG. 4. Representative in situ hybridization analysis of uterine cross sections at Days 11 (**A, B**), 13 (**C, D**), 17 (**E, F**), and 25 (**G–I**) of pregnancy under brightfield (**A, C, E, G**) and darkfield (**B, D, F, H, I**) illumination. **H** shows representative background (sense) hybridization signal (compare hybridization intensity at arrows in **H** and **I**). Overall hybridization intensity increased from Day 11 to Day 17 and declined to Day 25. Relative hybridization intensity was consistently detected above that in cyclic ewes at Day 13 of pregnancy. By Day 17 of pregnancy, Mx mRNA abundance was strongly elevated in the luminal epithelium (L), shallow glands (G; see arrow in **F**), stroma (S), and myometrium (not shown; ×180; reproduced at 90%).

TABLE 1. Distribution and relative abundance of Mx mRNA and protein in the ovine uterus during the estrous cycle and early pregnancy.

		Mx mRNA abundance[a]						Mx Protein Staining Intensity[a]						
		Epithelium[b]			Stroma[c]			Epithelium[b]			Stroma[c]		
Status	Day	LE	SG	DG	SC	SS	MYO	Status	Day	LE	SG	DG	SC	SS	MYO
Cyclic	1	+/−	+/−	−	−	−	−	Cyclic	1	+	+	−	−	−	−
	6	++	+	−	+	−	−		3	+	+	−	−	−	−
	11	++	+	−	+	−	−		5	++	+	−	+	−	−
	13	++/+++	++	−	++	−	−		7	++	+	−	+	−	−
	15	+	+/−	−	−	−	−		9	++	+	−	+	−	−
Pregnant	11	++	++	+	++	−	−		11	++	++	−	++	−	−
	13	+++	+++	+	++	+	+		13	++	++	−	++	−	−
	15	+++	+++	++	++	+	++		15	+	+	−	−	−	−
	17	++++	++++	+++	+++	++	++	Pregnant	11	++	++	+/−	++	−	−
	25	++	++	+	++	+	+		13	++/+++	++	+	++/+++	−	+
									15	+++	+++	++	+++	+	++
									17	++++	++++	+++	++++	++	+++
									19	++++	++++	++++	++++	+++	++++

[a] Mx mRNA abundance and Mx protein staining intensity were evaluated visually as absent (−), present above background (+), low (++), moderate (+++), or abundant (++++) for endometrial tissue and myometrial tissues obtained from cyclic (n = 3 per day) and pregnant (n = 5 per day) ewes.

[b] Legends: LE, luminal epithelium; SG, shallow glandular epithelium; DG, deep glandular epithelium.

[c] SC, stratum compactum; SS, stratum spongiosum; MYO, myometrium.

nant ewes, using a mouse monoclonal antibody against human MxA, are shown in Figures 5 and 6. Overall, the pattern of Mx immunostaining was consistent with the results obtained from in situ hybridization analysis. For cyclic ewes, immunoreactive Mx expression was low on Day 1 (Fig. 5A) of the cycle, was maximal between Days 5 (Fig. 5B) and 13 (Fig. 5F), and declined by Day 15 (Fig. 5G) of the estrous cycle. During diestrus, punctate Mx staining was moderately abundant and confined primarily in the luminal and shallow glandular epithelium. Numerous immunoreactive immune cells were scattered throughout the stroma and occasionally around the deep uterine glands (Fig. 5H). In general, Mx immunostaining intensity was low to undetectable in the stroma and myometrium in cyclic ewes. There was no detectable Mx immunostaining in the mononuclear trophoblast at Days 17 and 19 of pregnancy (Fig. 6, C and D) and only an occasional positive binucleate giant cell (Fig. 6D).

In pregnant ewes, Day 13 was the first day that Mx immunostaining intensity was consistently greater than observed in cyclic ewes, particularly in the shallow glands and stroma. By Day 15 of pregnancy (Fig. 6B), Mx staining increased in luminal epithelium, stroma, and myometrium. Punctate Mx staining of tissues from pregnant ewes was abundant in epithelium and reached maximum values on Day 19 (Fig. 6E), by which time the trophoblast had eroded much of the luminal epithelium (Fig. 6D). However, similar Mx staining was observed in adjacent areas of intact luminal epithelium (Fig. 6E).

Interestingly, Mx staining increased dramatically in the myometrium between Days 13 (Fig. 6F) and 19 (Fig. 6I) of pregnancy. During this same period, Mx staining intensity also increased in immune cells present throughout the stroma and at the interface between the stroma and myometrium (Fig. 6I). Myometrial Mx immunostaining was first evident in the inner circular myometrium and then progressed outward so that by Day 19, both the inner circular and outer longitudinal myometrium (data not shown) strongly expressed Mx protein.

DISCUSSION

This study was an examination of both Mx mRNA and protein localization in the uterine epithelium, stroma, and myometrium during the estrous cycle and early pregnancy in sheep. Two interesting findings from the present study are that Mx expression in the endometrial epithelium is apparently regulated during the estrous cycle and that the conceptus induces Mx expression in the uterine epithelium, stroma, and myometrium. Type I IFN was shown previously to induce Mx expression in the murine [20] and ovine [18] uterus. In the former study, Mx mRNA was expressed constitutively and was IFN inducible in the uterine endometrium and myometrium, but changes over the estrous cycle or early pregnancy were not examined nor was Mx protein localized [20]. In the latter study, endometrial Mx mRNA abundance was measured during early pregnancy and in response to type I IFN in sheep. In that study, Mx mRNA expression was not detected during the estrous cycle, nor was the spatial pattern of Mx protein or mRNA expression characterized in the cyclic or pregnant uterus [18].

In situ and immunohistochemical analyses in the present study revealed that uterine luminal and glandular epithelia exhibited the most abundant Mx expression. However, during early pregnancy, the stroma and myometrium also expressed relatively high amounts of Mx mRNA and protein.

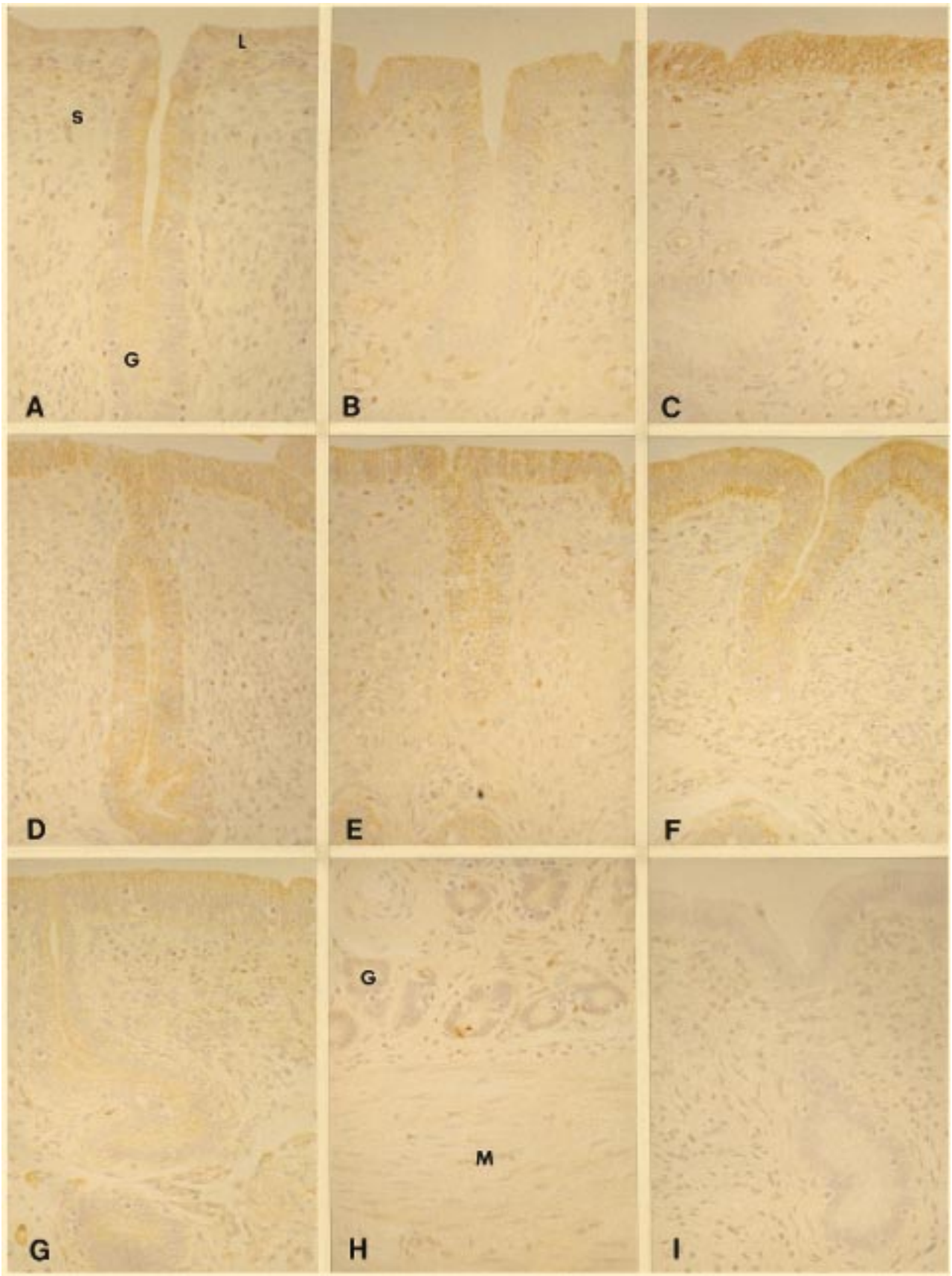

FIG. 5. Representative immunohistochemical localization of Mx in uterine endometrium of cyclic ewes collected at Days 1, 5, 7, 9, 11, 13, and 15 (**A–G**, respectively) postestrus. **H** shows representative staining intensity for Mx in the deep glands (G) and myometrium (M) that did not change throughout the estrous cycle. **I** shows representative background staining for both cyclic (Fig. 5) and pregnant (Fig. 6) tissues. Overall, staining intensity increased in the luminal epithelium (L) and shallow glands (G), but not in the stroma (S) or myometrium (M), from Days 1 to 13 and decreased by Day 15 postestrus (×275; reproduced at 93%).

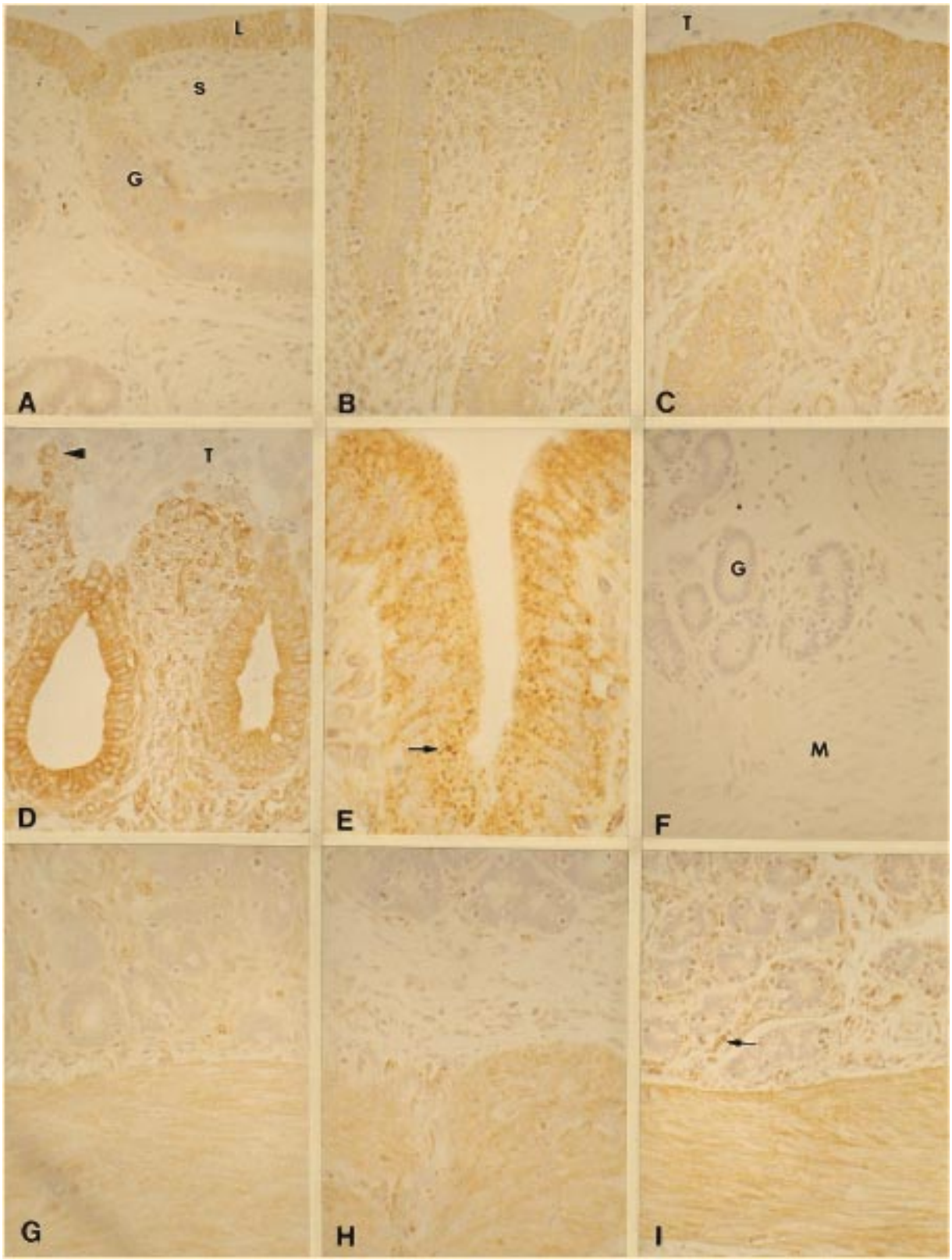

FIG. 6. Representative immunohistochemical localization of Mx in uterine endometrium of pregnant ewes collected at Days 11, 15, 17, and 19 of pregnancy (**A–D**, respectively; ×275). **E** shows higher magnification (×690) of luminal epithelium (L) at Day 19 to illustrate the abundant punctate Mx immunoreactivity (see arrow, **E**). **F–I**, respectively, illustrate the increased Mx immunoreactivity in the deep glands (G) and myometrium (M) from Days 13, 15, 17, and 19 of pregnancy. Increased immunoreactive Mx expression was detected in immune cells near the interface between the endometrium and myometrium (see arrow, **I**). Note the absence of Mx immunoreactivity in the trophoblast (T) at Days 17 (**C**) and 19 (**D**) except for the occasional positive binucleate giant cell (see arrowhead in **D**). Also, note the strong increase in Mx immunoreactivity in the luminal epithelium, stroma, shallow and deep glands, and myometrium between Days 11 and 19 postmating. Reproduced at 88%.

A cascade response was observed during early pregnancy with respect to the order and intensity of Mx mRNA expression in tissues composing the uterine wall. Expression in the luminal epithelium increased first, followed by that in glandular epithelium, stroma, and myometrium (see Fig. 4). High Mx expression in luminal epithelium is consistent with the hypothesis that the conceptus acts locally, via production of IFNτ, to alter uterine gene expression [2]. However, two possibilities may account for increased Mx expression in deeper tissues. First, conceptus-produced IFNτ may have access to all uterine cell types down to the myometrium and be directly responsible for the increase in Mx expression in those tissues. Second, IFNτ could activate Mx expression in luminal epithelium, which then activates Mx expression in the stroma and myometrium through paracrine mechanisms. These paracrine mechanisms could involve soluble factors produced by the luminal and shallow glandular epithelium that traverse the basement membrane, or migratory immune cells, which then secrete cytokines to activate Mx in deeper tissues. Consistent with the latter suggestion, numerous unidentified immune cells were evident in pregnant ewes throughout the stroma, particularly near the interface between the endometrium and myometrium (Fig. 6H). If the epithelium does produce such mediators of interferon (''interferon-medins,'') they may serve functions related to the establishment and maintenance of pregnancy such as stimulating secretion, modulating the endometrial mucosal immune system, and/or ensuring myometrial quiescence to support embryo attachment and growth and development of the fetus and placenta. At present, however, the only confirmed function for Mx is in the antiviral response.

The observation that Mx expression was modulated in the endometrial epithelium during the estrous cycle is novel and was unexpected. Charleston and Stewart were unable to detect Mx mRNA in endometrium during the estrous cycle [18]. In addition, Mx expression is thought to be tightly regulated by type I IFNs (α, β, ω, and τ) in the immune system [15]. Therefore, it was surprising to determine that Mx was expressed in luminal and shallow glandular epithelium throughout the estrous cycle. However, the uterine endometrium is a mucosal tissue and, as first line of defense against invading pathogens, supports a dynamic resident population of lymphocytes. Many of these cells are situated within or just beneath the luminal epithelium and may respond to viral pathogens [21] with the production of type I IFN. In a previous study, apparently constitutive expression of mRNA Mx was detected in rat uterine endometrium (although stage of the estrous cycle was not determined); and in control rats in an experiment examining regulation of Mx by exogenous IFN, it was noted that Mx was expressed in the duodenum and uterine endometrium [20]. Currently, acute responses of resident endometrial immune cell populations to the periimplantation conceptus and to intrauterine injection of recombinant ovine IFNτ are being examined.

Pattern of Mx expression in the cyclic ovine uterus was not constitutive but was regulated in the absence of conceptus-produced IFNτ. Mx expression was greatest in the luminal epithelium and shallow glands during the time when these tissues are expressing progesterone receptors and are exposed to locally high concentrations of progesterone. At Day 1 (low progesterone) and at Day 15 (low progesterone receptors) of the cycle, immunoreactive Mx and Mx mRNA were reduced. Mx expression may be influenced by progesterone in the cyclic endometrium and may play a role in preparing the uterus to support the developing conceptus.

On the basis of the limited structural homology of Mx with members of the GTPase superfamily of proteins, and its cellular localization in the luminal and glandular epithelium of the uterus during early pregnancy, we hypothesize that Mx plays a role in the processes of intracellular protein or vesicle trafficking in the uterus. The prototype members of this superfamily, the dynamins, are involved in endocytosis of clathrin-coated vesicles [22]. Although the homology between Mx and dynamin I outside the GTPase domains is limited, purified Mx does form homo-oligomers in solution, presumably through interactions between putative leucine zipper motifs [23]. In addition, Mx shares a short sequence of 10 amino acids with the dynamins that is thought to be involved in self-assembly [23]. The role that Mx plays in the stroma and myometrium remains to be determined. In the myometrium, Mx may alter contractility by altering the sensitivity of the myometrium to Ca^{2+} as demonstrated for other members of the GTPase superfamily [24]. Because Mx is a GTPase, its activity may be modified by upstream or downstream signaling mechanisms. To date, however, the only firm evidence for a role for Mx comes from studies of its involvement in the antiviral response [23], a function that requires GTPase activity [16,25]. While a role for Mx as an antiviral protein in the uterus remains a clear possibility, it is likely that Mx serves additional roles in the uterus.

In conclusion, the early ovine conceptus affects the entire uterine wall, resulting in changes in uterine Mx expression in diverse cell types including luminal and glandular epithelium, stroma, immune cells, and myometrium. Mononuclear trophoblast cells, which produce IFNτ, did not express Mx. The observation that Mx concentrations in the uterine endometrial epithelium are regulated during the estrous cycle has not been previously shown in any species and supports the hypothesis that steroid hormones may interact with IFNτ to regulate uterine Mx expression. Whether stimulation of Mx in these cell types represents direct effects of the conceptus through its secretory products, e.g., IFNτ, or represents a combination of direct and indirect responses of the uterine wall to the conceptus, remains to be determined.

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