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Endometrial ISG17 mRNA and a Related mRNA Are Induced by Interferon-tau and Localized to Glandular Epithelial and Stromal Cells from Pregnant Cows

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The interferon stimulated gene product, ISG17, conjugates to bovine uterine proteins in response to conceptus-derived interferon (IFN)-τ. The objectives of the present experiments were to examine induction of ISG17 (0.65 kb) and a related 2.5 kb mRNA in response to IFN-τ and pregnancy using Northern blotting procedures, and to determine cell types in the endometrium that expressed ISG17 mRNA using *in situ* hybridization. RNA was isolated from endometrial explants or from bovine endometrial (BEND) cells cultured in the absence (control) or presence of 25 n*M* recombinant (r) boIFN-τ for 0, 3, 6, 12, 24, or 48 h. The major ISG17 0.65 kb mRNA and a minor 2.5 kb mRNA were induced ($p < 0.05$) after 6 h (explants) or 3 h (BEND cells) treatment with rboIFN-τ. Both mRNAs were present in endometrium from day 18 pregnant cows, but were absent in endometrium from nonpregnant cows. The ISG17 mRNA was localized to stromal and glandular epithelial cells on d 18 of pregnancy. The 2.5 kb mRNA may encode a novel ISG17 homolog, or a unique polyISG17 repeat that is similar in structure to the polyubiquitin genes. Because ISG17 mRNA is induced in stromal and glandular epithelial cells, it could be assumed that ISG17 has a role in regulating intracellular proteins in both cell types.

Key Words: Interferon; ubiquitin; uterus; pregnancy.

Introduction

ISG15 was first identified as a 15-kDa product of an interferon (IFN) stimulated gene (ISG) in mouse Ehrlich ascites tumor cells *(1)*. Later, it was shown to be induced by type I IFNs in human (hu) lung carcinoma cells (A-549), huDaudi cells, and MDBK cells *(2–6)*. The appearance of ISG15 following culture with IFN closely paralleled acquisition of an antiviral state. Human ISG15 may be released in response to type I (i.e., α, β, ω, τ) IFN to regulate secretion of IFN-γ by T- and B-lymphocytes *(7)*, potentially serving as a switch between the two IFN classes. Human ISG15, through inducing release of IFN-γ from T-cells, augments natural killer (NK) cell proliferation, and activates monocytes and macrophages *(8)*. Also, serum ISG15 concentrations are elevated in healthy women treated with single or multiple doses of IFN-β *(9)*. Other than these experiments, little else is known regarding the extracellular function of ISG15 and potential interaction with neighboring cells.

Amino acid *(4,10)*, cDNA *(5)* and gene *(11)* sequences have been reported for ISG15. Two domains of ISG15 had 29–31% sequence identity with ubiquitin. This prompted investigators to also call this gene product ubiquitin cross reactive protein or UCRP *(4)*. The most notable similarity in structure between ISG15 and ubiquitin was conservation of the carboxy-terminal Leu-Arg-Gly-Gly residues that function in the ligation of ubiquitin *(12,13)* and ISG15 *(4,14)* to targeted intracellular proteins.

Affinity-purified antiserum against ISG15 does not recognize ubiquitin *(14)*. Using this antiserum it was shown that ISG15 and its conjugates were localized in a punctate cytoskeletal pattern that was similar to that observed for intermediate filament-associated proteins (i.e., cytokeratin and vimentin) in the cytoplasm in many human tissues *(15,16)*. Loeb and Haas *(16)* hypothesized that one function of ISG15 might be to serve as a trans-acting binding factor for directing the association of otherwise soluble target proteins to these fibers. Also, using immunocytochemical approaches, it has recently been reported that ISG15 is absent in nonpregnant tissue, but accumulates along with its conjugates in decidualized stromal cells in response to pregnancy *(17)*. Because ISG15 and its conjugates are present in many tissues and cell lines and are associated

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with the cytoskeletal network, some functions may be universal. The roles of ISG15 and its conjugates are unknown. Also, it is not known if ISG15 undergoes polymerization in a manner similar to ubiquitin. Finally, the fate and identity of proteins conjugated to ISG15 remains to be determined.

Ubiquitin is an 8-kDa cytosolic "stress" protein that is critical to many biological processes *(18,19)* such as regulation of the cell cycle *(20)*, modification of receptors *(21)*, termination of signal transduction *(22)*, and activation of transcription factors *(23)*. The classical role of ubiquitin entails induction of targeted proteolysis. Ubiquitin covalently attaches to proteins and then forms polymers which targets the complex to the proteasome where ubiquitin is recycled and the coupled protein is degraded.

The bovine (bo) uterus produces a 17-kDa protein in response to conceptus-derived IFN-τ on days 15–26 of pregnancy *(24)*. This protein was called UCRP because it retained enough antigenic similarity to cross-react with antiserum against ubiquitin on Western blots *(25)*. The boUCRP cDNA was isolated after screening a bovine endometrial cDNA library using antiserum against ubiquitin *(26)*. The boUCRP cDNA encoded a 17-kDa protein that shared only 31% inferred amino acid sequence identity with a tandem ubiquitin repeat, but retained several functional amino acids with ubiquitin *(26)*. For example, the C-terminal Leu-Arg-Gly-Gly amino acids of ubiquitin conjugate to targeted proteins *(12,13)* and are retained in boUCRP. Conjugation of boUCRP to cytosolic endometrial proteins is induced by pregnancy and IFN-τ *(27)*. Like ubiquitin, boUCRP may form a monomer with targeted proteins, or it may form polymers in which it is sequentially added to targeted proteins through covalent attachment at Lys residues.

Three ubiquitin genes have been described: UbA, UbB, and UbC *(28)*. UbA mRNA (0.65 kb) encodes ubiquitin fused with a ribosomal protein *(29–32)*. UbB mRNA (1.2 kb) encodes a polyubiquitin protein that is cleaved posttranslationally to yield ubiquitin monomers. The UbC gene exhibits polymorphism (>2.6 kb mRNA) and encodes a seven to nine repeat polyubiquitin protein *(32)* that also is cleaved posttranslationally to yield ubiquitin monomers.

The boUCRP gene encodes a single 17-kDa protein and was recently renamed ISG17 *(33)* to follow nomenclature originally developed in the mouse *(1)* and human *(11)*. Transcription of the 0.65 kb mRNA encoding ISG17 is induced by pregnancy *(34)*. However, it is unknown which cell types in the bovine endometrium express ISG17 mRNA. Transcription of the ISG17 mRNA also is induced in response to IFN-τ *(34)*, but the timing of induction has not been studied. Dot blot hybridization of total cellular bovine endometrial RNA revealed that ubiquitin mRNAs are up-regulated in response to pregnancy, but not by IFN-τ *(34)*. It is not known which ubiquitin genes are up-regulated in response to pregnancy.

To our knowledge, only three publications exist that describe induction of ISG15 *(1,5)* and ISG17 *(34)* mRNAs by type I IFNs. In these experiments, only a single time of induction of mRNA was determined, and data were not quantitative. Recently, we have identified a novel, 2.5 kb mRNA that hybridizes with the ISG17 cDNA probe and is induced in the endometrium in response to IFN-τ and pregnancy. Objectives of the present experiments were to:

1. study temporal induction of ISG17 and the 2.5 kb mRNAs in response to IFN-τ in cultured endometrial explants and in a primary bovine endometrial cell (BEND) line;
2. identify which ubiquitin gene was up-regulated in response to pregnancy; and
3. determine cell types in the endometrium that expressed ISG17 mRNA using *in situ* hybridization.

Results

Figure 1 shows Northern blot hybridization of endometrial RNA using either a ISG17 (A), or ubiquitin (B) cDNA probe. The ISG17 cDNA probe does not hybridize with ubiquitin mRNAs (Figs. 1A and B). Endometrial explants were collected from three d 14 nonpregnant cows and cultured in the absence or presence of recombinant (r) boIFN-τ for times specified. Signals on Northern blots were scanned using densitometry. The 0.65 kb ISG17 transcript was induced ($p < 0.05$) after 6 h treatment with recombinant (r) boIFN-τ, continued to increase through 24 h, and then declined to levels that remained higher than controls at 48 h (Fig. 1C). A larger, 2.5 kb mRNA also hybridized with the ISG17 probe (Fig. 1A) and was induced following culture with rboIFN-τ (Fig. 1D). The temporal pattern of expression of the 2.5 kb transcript in response to rboIFN-τ was similar to that observed for the ISG17 mRNA, with the exception that levels of expression were lower, and by 48 h, the 2.5 kb mRNA declined to levels that were not different from controls. Endometrial explants that were not treated with rboIFN-τ did not express the ISG17 mRNA or the 2.5 kb mRNA. Induction of ISG17 mRNA was half-maximal by ~6 h, whereas induction of the 2.5 kb transcript did not reach half-maximal levels until ~7.5 h after treatment with rboIFN-τ. Both mRNAs reached maximal levels following 24 h culture with rboIFN-τ.

The ubiquitin cDNA probe hybridized with UbB and UbC transcripts, but did not hybridize with ISG17 or the larger ISG17-related 2.5 kb transcript (Figs. 1A and B). Recombinant boIFN-τ had no effect on expression of UbB or UbC mRNAs. When blots were quantitated using densitometry, ubiquitin mRNAs changed over time ($p < 0.05$) in culture with an up-regulation from 0 to 1.5 h, followed by a decline from 1.5 h to 48 (Figs. 1E and F).

The decline in the ISG17 and 2.5 kb mRNAs from 24 to 48 h, and ubiquitin mRNAs from 1.5 h to 48 h was not caused by technical error or degradation of RNA. Ribosomal RNA (18S) did not change in endometrial explants

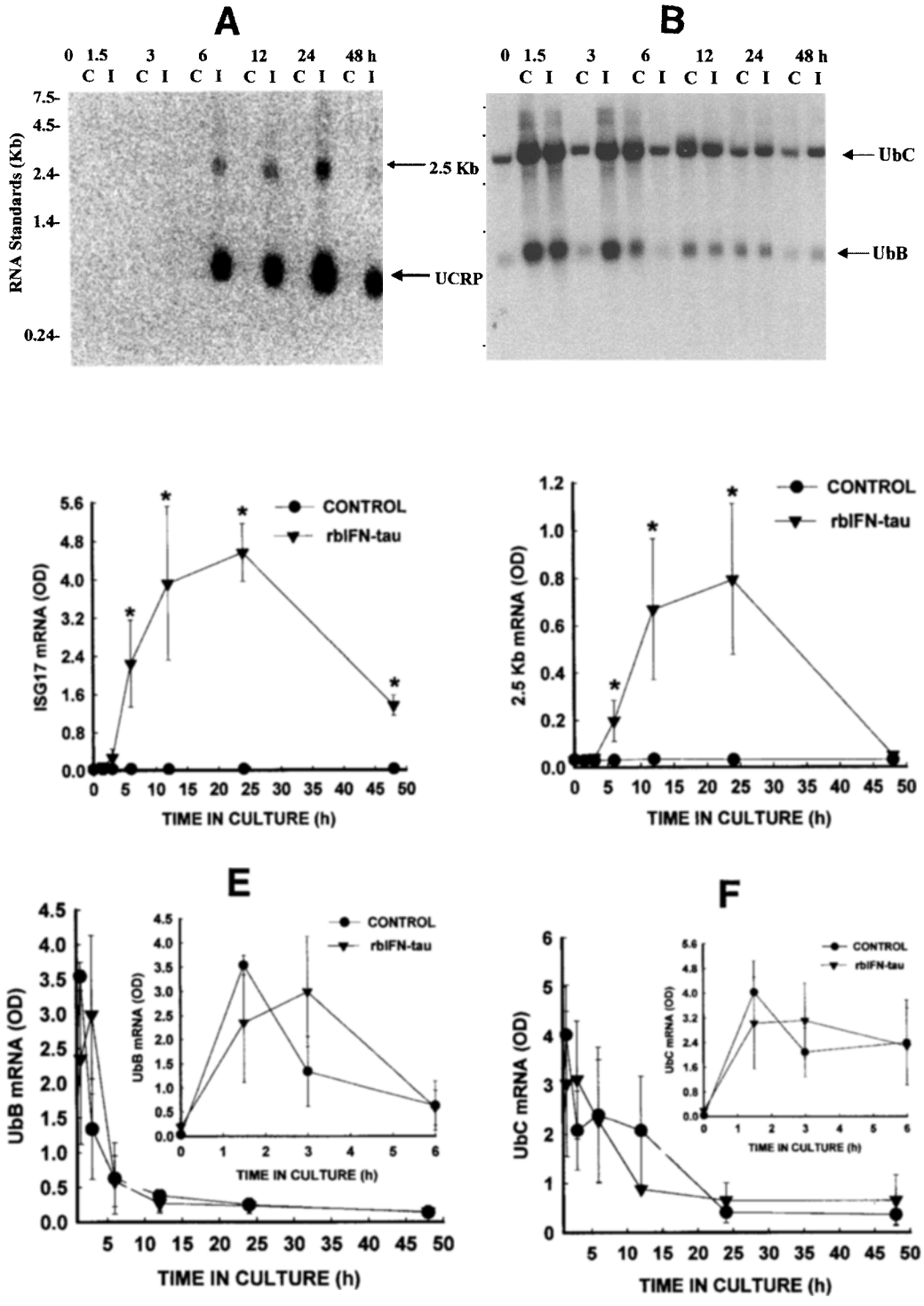

Fig. 1. Effect of rboIFN-τ (25 n*M*) on endometrial mRNAs in cultured endometrial explants. Interferon-τ induced ISG17 mRNA and a larger-related mRNA (Panel A), but had no effect on ubiquitin mRNAs (UbB and UbC; Panel B). The 2.5 kb transcript that hybridized to the ISG17 probe was different in size when compared with ubiquitin mRNAs and did not hybridize with the ubiquitin cDNA probe. Recombinant boIFN-τ induced ISG17 (Panel C) and the 2.5 kb (Panel D) mRNAs (means differ from controls when designated with an *; $p < 0.05$). Ubiquitin mRNAs were not affected by IFN-τ, but increased ($p < 0.05$) from 0 to 1.5 h and then decreased ($p < 0.05$) as a function of time in culture.

cultured with IFN-τ or over time in culture. Ribosomal RNA OD means pooled across IFN-τ treatment were 5.0 ± 0.9, 4.4 ± 0.6, 4.2 ± 0.2, 3.8 ± 0.5, 4.5 ± 0.6, 4.2 ± 0.7, 4.5 ± 0.6 OD (0, 1.5, 3, 6, 12, 24, 48 h, respectively).

Induction of ISG17 and its related mRNA was slightly different when culturing BEND cells with rboIFN-τ (Fig. 2). The ISG17 mRNA and its related mRNA were induced within 3 h following treatment with rboIFN-τ. The

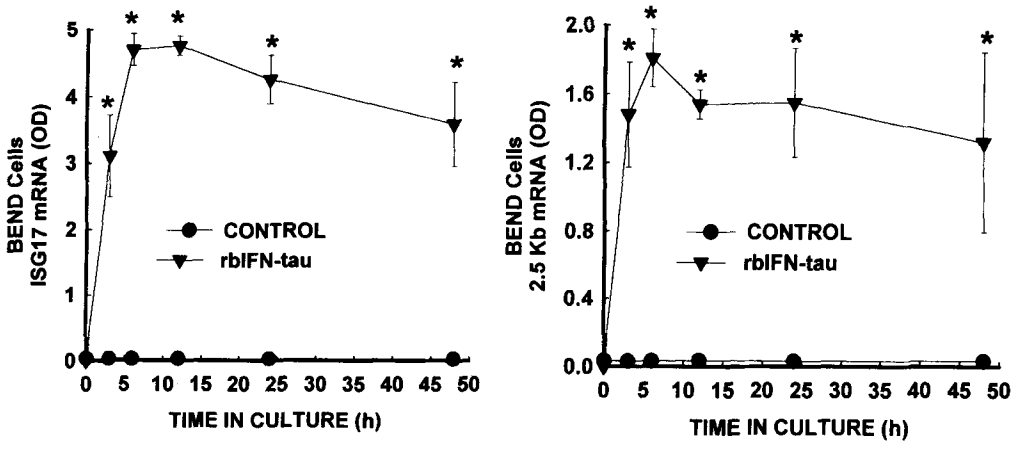

Fig. 2. Induction of ISG17 mRNA and its related 2.5 kb mRNA in BEND cells cultured with 25 n*M* rboIFN-τ. Northern blots were scanned in ISG17 mRNA (0.65 kb) and 2.5 kb regions using densitometry and analyzed. Values represent mean optical density ± standard error. Means differ ($p < 0.05$) when compared with controls when designated with an *.

induction of these mRNAs by rboIFN-τ was 3 h quicker in BEND cells when compared with endometrial explants. In BEND cells, the ISG17 mRNA and its related mRNA continued to accumulate from 3 to 6 h and then remained elevated from 6 to 48 h following treatment with rboIFN-τ. This pattern is in contrast with induction of maximal levels by 24 h and a decline in mRNAs from 24 to 48 following treatment with rboIFN-τ in explant culture. Neither ubiquitin mRNAs, nor 18S ribosomal RNA changed in response to IFN-τ or over time in culture in BEND cells (Table 1).

Shown in Fig. 3 are representative Northern blots of endometrial RNA extracted from nonpregnant and pregnant cows when hybridized with either the ISG17 or ubiquitin cDNA probes. In each d 18 pregnant cow, both ISG17 and the 2.5 kb transcript were present. Total cellular RNA from endometrium from five day 18 nonpregnant cows had no signal for ISG17 or the related transcript. Quantitation of these data revealed that pregnancy induced ($p < 0.05$) transcription of both ISG17 and the related 2.5 kb mRNA when compared with controls (Fig. 3C).

Ubiquitin mRNAs also were expressed in higher ($p < 0.05$) amounts in endometrial tissues from pregnant when compared with nonpregnant cows (Fig. 3D). The induction of ubiquitin mRNA during early pregnancy in the cow was not due to the effect of IFN-τ (Fig. 1). Quantitation of Northern blots using densitometry revealed that expression of the UbB mRNA did not differ between pregnant and nonpregnant cows (Fig. 3D). In contrast, UbC mRNAs were up-regulated significantly in endometrium collected from pregnant when compared with nonpregnant cows (Fig. 3D). Because UbC mRNAs exhibited heterogeneity in expression (Fig. 3B), optical density values for each UbC mRNA within sample were combined for analysis.

Using *in situ* hybridization, actin mRNA was localized primarily to stromal cells and did not differ in endometrium from nonpregnant and pregnant cows (Fig. 4). Ubiquitin mRNAs also were localized to stromal cells, but were more prevalent in endometrial sections from pregnant than nonpregnant cows. This is in agreement with Northern blot data for the UbC mRNA shown in Fig. 3D. *In situ* hybridization using ISG17 antisense RNA probes revealed that ISG17 mRNA was not present in endometrial tissues from nonpregnant cows (Fig. 5). However, it was localized not only to glandular epithelium, but also to stromal regions of sections obtained from three d 18 pregnant cows.

Discussion

The conceptus is recognized by the uterus between the 15th and 17th day of pregnancy in cattle *(35,36)*. IFN-τ is secreted maximally from the conceptus during this period

Table 1
Ubiquitin and 18 S Ribosomal mRNAs in Cultured BEND cells ($n = 3$ replicates) as a Function of Time in Culture[a]

	Time in Culture (h)					
mRNA	0	3	6	12	24	48
UbB	2.6 ± 0.2	1.9 ± 0.4	2.5 ± 0.4	2.6 ± 0.2	3.4 ± 0.4	3.0 ± 0.3
UbC	6.3 ± 0.8	6.6 ± 1.2	8.3 ± 0.2	5.4 ± 2.8	8.0 ± 0.2	4.9 ± 2.7
18 S	4.6 ± 0.6	4.1 ± 0.5	4.4 ± 0.1	4.3 ± 0.1	4.1 ± 0.2	4.4 ± 0.3

[a]Values represent the mean optical density ± standard error.

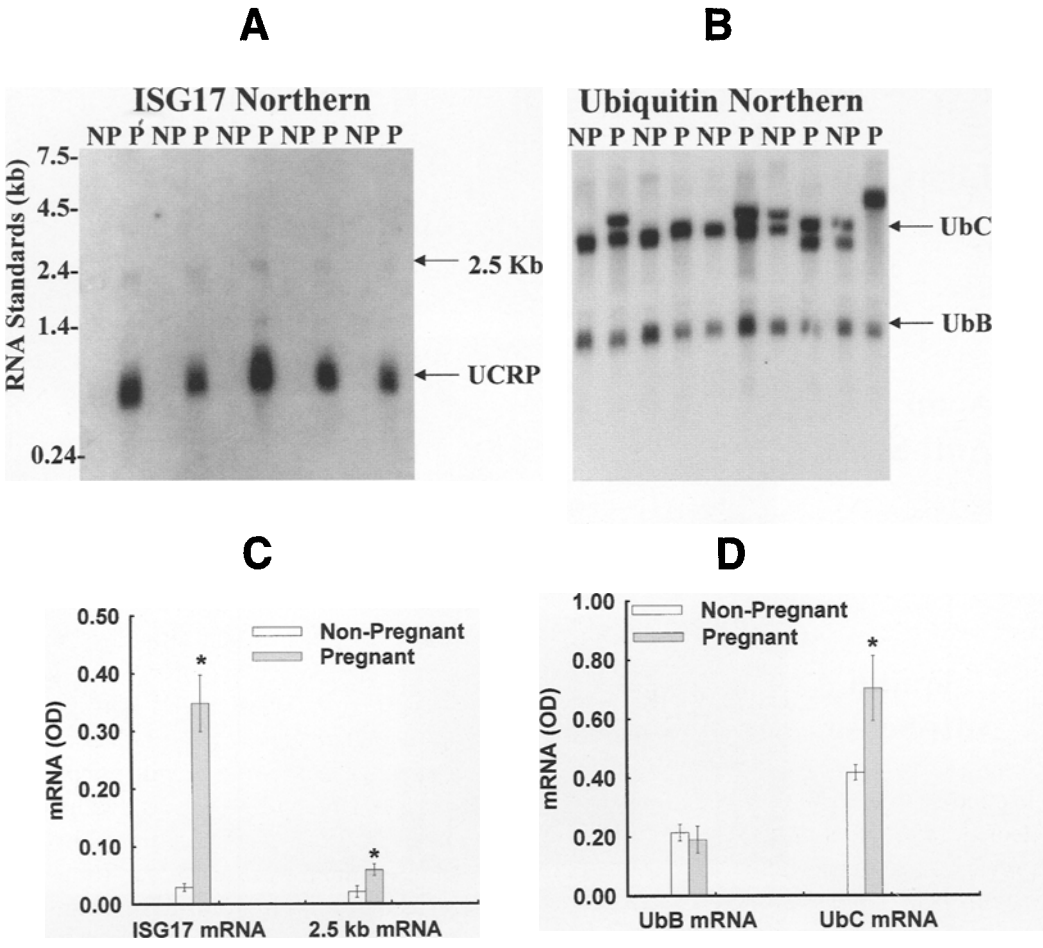

Fig. 3. Northern blot analysis of ISG17 (Panel A) and Ubiquitin (UbC and UbB; Panel B) mRNAs in endometrium collected from five day 18 pregnant (P) or nonpregnant (NP) cows. Signals for the ISG17 and 2.5 kb mRNAs (Panel C), and the ubiquitin mRNAs (Panel D) were quantitated using densitometry and analyzed. Means designated with an * differ ($p < 0.05$) within mRNA type.

and acts to attenuate secretion of $PGF_2\alpha$ *(35)*. In cows, basal secretion of $PGF_2\alpha$ remains unchanged during pregnancy, while luteolytic pulses are attenuated *(35)*. Therefore, the corpus luteum is maintained.

Bovine ISG17 is induced in the endometrium by IFN-τ *(25)*. It also conjugates to endometrial proteins in response to IFN-τ and pregnancy *(27)*. The regulation of these proteins by ISG17 may be integral to establishing early pregnancy in ruminants. Bovine ISG17 is encoded by a single gene *(33)* that is transcribed as a 0.65 kb mRNA *(34)*. The ISG17 gene contains a promoter that is followed by an mRNA cap site, translational start site, a small intron, and then coding and noncoding sequences. Nothing is unique about the ISG17 gene that might indicate complex processing. The gene encodes a single ISG17 molecule, unlike ubiquitin genes that encode several ubiquitin repeats or ubiquitin fusion proteins.

In the present experiments, two distinct endometrial mRNAs that hybridized to the ISG17 probe were induced by rboIFN-τ in endometrial explants and BEND cells. A 0.65 kb transcript was the dominant mRNA and encodes ISG17. A second, minor 2.5 kb transcript, also was evident on Northern blots of RNA from endometrial explant tissues after induction with rboIFN-τ. Both ISG17 mRNA and the 2.5 kb mRNA were present in endometrium from pregnant cows, but were absent in endometrium from nonpregnant cows.

The larger ISG17-related mRNA is induced in a manner that is temporally similar to the ISG17 mRNA in response to rboIFN-τ. Both transcripts are induced within 6 h following culture with rboIFN-τ in cultured endometrial explants. Also, both transcripts decline significantly from 24 to 48 h following culture with rboIFN-τ. The decline in these mRNAs is not due to inherent instability of mRNA under explant culture conditions because 18 S ribosomal RNA remained constant throughout time in culture. Rather, it is more plausible that the decline in mRNA in explant culture was due to depletion of rboIFN-τ that was added at 0 h.

In BEND cells, a more rapid induction of ISG17 and its related mRNA was observed within 3 h following addition of rboIFN-τ. Also, ISG17 and its related mRNA remained elevated from 3 to 48 following culture with rboIFN-τ, whereas in endometrial explants these mRNAs declined from 24 to 48 h. The more rapid response to rboIFN-τ and

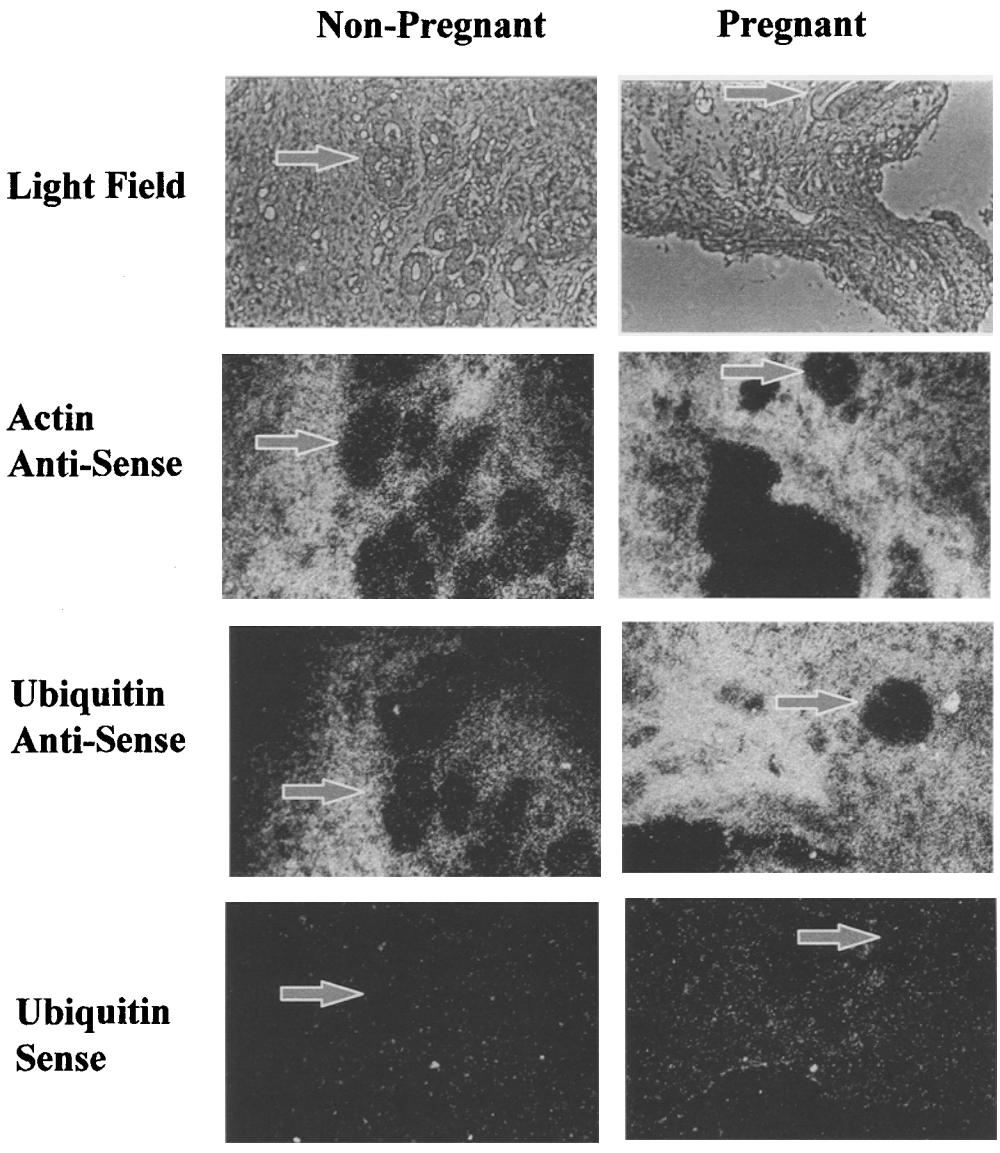

Fig. 4. Representative *in situ* hybridization of actin and ubiquitin mRNA in endometrium sectioned from day 18 nonpregnant ($n = 3$) or pregnant ($n = 3$) cows. Hybridization using ubiquitin and actin (not shown) sense riboprobes yielded little signal and served as appropriate negative controls. Arrows identify glands in each serial section within pregnancy status.

the continued expression of these mRNAs in BEND cells, when compared with endometrial explants, can be explained by a more highly enriched epithelial cell and potentially IFN-τ receptor population.

The 2.5 kb mRNA is not the UbC mRNA. It is about 3.8 times the size of the ISG17 0.65 kb transcript and may encode an ISG17 polymer. Some ubiquitin genes have this organization and encode polyubiquitin "pre"-proteins that are cleaved posttranslationally into ubiquitin monomers *(19)*. To our knowledge, no ubiquitin gene has been described that is processed through RNA splicing or generation of intact mRNAs that encode ubiquitin monomers. The possibility that a gene encoding polyISG17 exists also seems unlikely because only one major gene encoding ISG17 has been identified after extensively screening a bovine genomic library and after examining several bovine genomic Southern blots using the ISG17 cDNA as a probe *(33)*. Alternatively, the larger transcript may share limited nucleotide sequence identity with ISG17 and be a member of the growing ubiquitin homolog family *(37)*, or it might be a unique ISG17 fusion protein.

Genes encoding a single ubiquitin monomer that is fused at the C-terminal to one of two ribosomal proteins have been described *(29–32)*. These ubiquitin fusion proteins assist in the synthesis of new proteins *(31)*. The N-terminal ubiquitin moiety acts as a chaperone to protect the N-terminal of its ligand, facilitates ribosome biogenesis *(31)*, and targets cytosolic translocation *(38)*. The ribosomal protein portion of the fusion partner becomes incorporated into a ribosomal subunit. The ribosome is then released and either floats freely in the cytoplasm to translate cellular proteins, or is embedded in the endoplasmic reticulum to translate

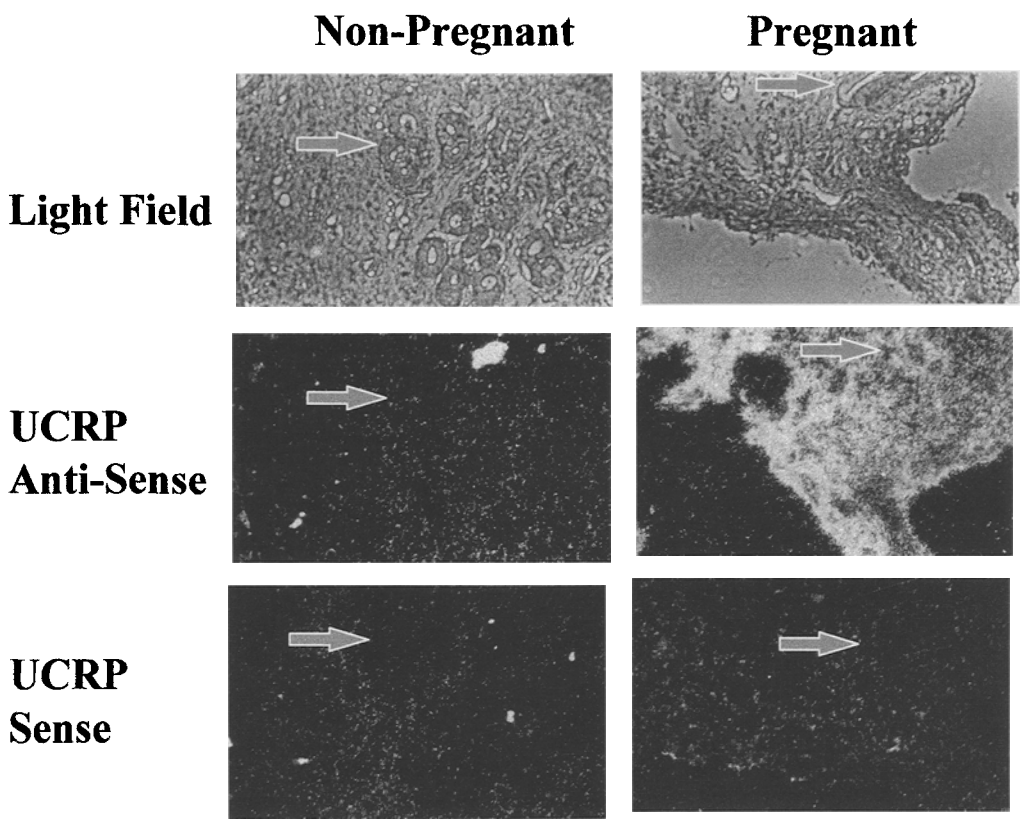

Fig. 5. *In situ* hybridization of ISG17 mRNA in endometrial tissue sections. Notice the extensive hybridization signal in the tissue section representing d 18 of pregnancy when using the ISG17 antisense riboprobe. This intense hybridization signal was present in sections derived from all three d 18 pregnant cows, and was absent in all three d 18 nonpregnant cows. The hybridization signal in sections from pregnant cows was localized in both glandular epithelium and stromal cells. Arrows identify glands in each serial section within pregnancy status.

proteins that are to be packaged and secreted. Ubiquitin then enters the cytoplasmic pool where it can conjugate to and regulate intracellular proteins. The 2.5 kb mRNA described in the present experiments might encode a similar fusion protein with similar function in endometrial protein synthesis during early pregnancy. However, in ruminants, we have never identified a UbA mRNA in endometrium using polyubiquitin cDNA as a probe. The 2.5 kb mRNA that hybridizes to the ISG17 probe might encode an ISG17-fusion protein, but the size of this transcript is much larger than the UbA mRNA.

The 2.5 kb ISG17 mRNA is not the same size as known ubiquitin mRNAs. It also is not detected using the ubiquitin cDNA probe on Northern blots. Thus, it is either a product of a complex ISG17 gene that has not been described (i.e., like UbB or UbC), or is a related gene that encodes a protein that might be similar, but much larger in size than ISG17. In either case, transcription of the gene encoding the larger transcript is induced by rboIFN-τ and presence of the conceptus.

The UbB and UbC mRNAs were not up-regulated in response to rboIFN-τ in explant or BEND cell culture. However, both ubiquitin mRNAs increased in cultured endometrial explants within 1.5 h regardless of rboIFN-τ treatment and then declined. This pattern of induction was probably due to the "stress" of removal of tissue from the in vivo environment and placement into an in vitro environment, that typifies a classical heat shock response *(39)*. Ubiquitin has long been recognized as a member of the heat shock family *(40)*. In primary BEND cells that are kept in a stable environment, ubiquitin mRNAs were not affected by time in culture. Because the ubiquitin mRNAs were not up-regulated by rboIFN-τ, the higher amount of UbC mRNA in endometrium from pregnant cows was not due to conceptus-derived IFN-τ. A more likely explanation is that some other conceptus secretory product, or conceptus-induced maternal product up-regulated the UbC gene. Oddly, the UbB mRNA was not affected by pregnancy status. This result may be interpreted to mean that the promoters regulating these genes are different. For example, the UbC gene promoter contains response elements that are activated by the conceptus, whereas the UbB gene promoter does not. Because ubiquitin has been shown to form polymers and target proteins to degradation *(18,19)*, we suspect that a subset of endometrial proteins are targeted to degradation in response to the up-regulation of the UbC gene during pregnancy in ruminants.

In situ hybridization using a ubiquitin antisense riboprobe revealed that ubiquitin mRNAs were up-regulated in

endometrial tissues from pregnant when compared with nonpregnant cows. Expression of the UbB transcript, as determined by Northern blot, did not differ in endometrium collected from d 18 pregnant and nonpregnant cows. However, UbC mRNAs were up-regulated in endometrium collected from d 18 pregnant when compared with nonpregnant cows.

ISG17 mRNA was localized to glandular and stromal tissues of endometrial tissue sections prepared from d 18 pregnant cows, but was not found in sections from nonpregnant cows. The widespread localization of ISG17 in endometrial cell types on day 18 of pregnancy was unexpected and may reflect either an IFN-τ concentration gradient that develops from the luminal epithelium to the deep stromal and glandular epithelium, or cell to cell communication that is induced in response to IFN-τ. Because both ISG17 and the 2.5 kb mRNA were detected using the ISG17 riboprobe, it may be necessary to determine if the expression of these two mRNAs differs between luminal, glandular, and stromal regions of the endometrium. Likewise, the function of ISG17 and the related protein might be examined in a cell-specific experimental approach.

It is concluded that the ISG17 mRNA and its related 2.5 kb mRNA are induced by rboIFN-τ in cultured endometrial explants and BEND cells. The ubiquitin mRNAs are not upregulated by IFN-τ. However, the ISG mRNAs, in addition to the UbC transcript are induced or up-regulated by pregnancy. A role for the ISGs in modulating or targeting proteins to degradation should be considered, although this is speculative because proteins conjugated to the ISGs have not been identified. The up-regulation of ubiquitin in endometrial tissues in the present experiment and as previously reported *(34)*, is intriguing in context of known function of ubiquitin in determining cellular fate of targeted proteins.

Methods

Animals

Experiments were approved by the University of Wyoming Animal Care and Use Committee. Cows were treated with prostaglandin $F_2\alpha$ (PGF: Lutalyse: Upjohn Co. Kalamazoo, MI) and observed for estrus. Day of estrus was defined as day 0 of the ~21 day estrous cycle. Pregnancy was verified by the presence of a conceptus.

Induction of ISG17 mRNA by rboIFN-τ

Cows ($n = 3$) were slaughtered on day 14 of the estrous cycle. Endometrial explants were dissected from the uterine lining and cultured (0.5 g/5 mL) in serum-free Eagle's minimal-essential medium (MEM; Sigma Chemical Co.) at 37°C in an atmosphere of 50% O_2: 45% N_2: 5% CO_2 as described previously *(25)*. Endometrium was cultured in the absence (control) or presence of 25 n*M* rboIFN-τ (5×10^7 IU/mg; from R.M. Roberts, University of Missouri) for 1.5, 3, 6, 12, 24, and 48 h, collected and stored at –80°C.

A primary bovine endometrial cell line called BEND cells also was cultured (2×10^6 cells/T-150 flask) in the absence or presence of 25 n*M* rboIFN-τ for 0, 3, 6, 12, 24, and 48 h. Culture conditions and use of BEND cells to examine induction of ISG17 protein have previously been described *(41)*. After culture, cells were scraped from flasks and extracted to yield total cellular RNA as described previously *(34)*.

Induction of ISG17 mRNA by Pregnancy

Endometrium was collected from day 18 nonpregnant ($n = 5$) and pregnant ($n = 5$) cows as previously described *(34)*. D 18 of pregnancy represents a time during which release of IFN-τ by the conceptus *(35,36)* and uterine production of ISG17 *(25)* is maximal.

Northern Blot

Complementary cDNA probes were generated through random prime reactions (Life Technologies Inc., Grand Island, NY). Bovine ISG17 *(26)* and ubiquitin *(34,42)* cDNA's, and the 18 S ribosomal DECA template (Ambion, Austin TX) were labeled using 50 μCi [α-^{32}P]dCTP (New England Nuclear, Boston, MA) and Klenow enzyme.

One hundred milligrams of homogenized endometrial tissue explants were extracted for total cellular RNA using Tri Reagent (Molecular Research Inc., Cincinnati, OH). Homogenates were incubated for 5 min at 20°C, mixed with 0.2 mL chloroform, and then incubated for 15 min. Protein and DNA was separated by centrifugation (12,000*g*, 15 min, 4°C) and discarded. RNA (aqueous phase) was precipitated using isopropanol (10 min, 20°C) and then pelleted by centrifugation (12,000*g*, 12 min, 4°C). Pellets were washed with cold 75% ethanol, dried, and resuspended in diethylpyrocarbonate-treated (DEPC) water.

For Northern blot analysis, total cellular RNA was loaded (10 μg/lane) onto 1.5% agarose gels containing 8% formaldehyde. Gels were electrophoresed (0.2 *M* MOPS, 0.05 *M* sodium acetate, 0.01 *M* EDTA, pH 7.0) and transferred to 0.2 μm nylon membranes (ICN Biomedicals Inc., Costa Mesa, CA) by capillary method in 10X SSC (1.5 *M* NaCl, 0.15 *M* $C_6H_5O_7Na_3$, 24 h, 20°C). Blots were baked (2 h, 80°C) and then prehybridized (3 h, 42°C; 50% formamide, 5X SSC, 0.05 *M* $NaPO_4$, 5X Denhardt's solution, 0.1% SDS, 0.1 mg/mL salmon sperm DNA). Blots were hybridized (18 h, 42°C), washed (3 × 15 min: 2X SSC/0.1% SDS, 1X SSC/0.1% SDS, 0.1X SSC/0.1% SDS; 42°C), and placed on Sterling X-ray film (BioWorld, Cleveland, OH) (24 h). Signals on blots were quantitated using densitometry *(34)*.

Statistical Analysis

Time-course data were analyzed using analysis of variance procedures. The model included IFN, animal, time, and two-way interactions. Time and IFN were tested by using respective interactions with animal as the error term. Effect of pregnancy was tested using analysis of variance

procedures. When main effects were significant ($p < 0.05$), pairwise comparisons were made using t-test.

In Situ Hybridization

Bovine endometrial tissues from d 18 of the estrous cycle ($n = 3$) or pregnancy ($n = 3$) were removed (1/8 inch square piece) and fixed in Histochoice (Amresco, Solon, OH). Endometrial tissues were dehydrated in a series of ethanol solutions (50%, 70%, 95%, 95%, and absolute), cleared in xylene, and embedded in paraffin blocks. Serial sections (6 μm thickness) were floated on distilled water containing 0.02% diethylpyrocarbonate (50°C) and placed onto subbed microscope slides (1% gelatin, 0.01% chromium potassium sulfate). Just prior to *in situ* hybridization, slides were deparaffinized and rehydrated in DEPC water.

Rehydrated sections were post-fixed in 4% paraformaldehyde for 30 min (20°C), followed by 3 × 5 min rinses in DEPC phosphate buffered saline and 1 rinse in DEPC water (20°C). Slides were placed in 0.1 *M* HCl for 15 min, then rinsed in DEPC water (20°C). To each section, 5 μL of prehybridization buffer (50% deionized formamide; 0.1% denatured salmon sperm DNA; 0.3 *M* NaCl/0.03 *M* sodium citrate [2X SSC]; 0.02% polyvinylpyrrolidone; 0.02% BSA; 0.02% ficoll 400; 10% dextran sulfate; 10 m*M* dithiothreitol) was applied and slides were incubated at 42°C for 4 h. Labeled (^{35}S) sense and anti-sense ISG17 *(26)*, ubiquitin *(34, 42)*, and γ-actin (kindly provided by Dr. L. Kedes, University of Southern California School of Medicine), riboprobes were diluted in prehybridization buffer and each probe was added in 5 μL aliquots of 500,000 cpm's to bovine endometrial sections. After an overnight incubation at 42°C, sections were rinsed (3 × 5 min: 2X SSC, 1X SSC, 0.1X SSC; 42°C). Under total darkness, slides were dipped in photographic emulsion (Kodak NTB-2) at 42°C and allowed to expose for 5 d at 2°C. Autoradiograms were developed. Sections were stained with 0.5% Giemsa, and mounted under Permount for phase contrast microscopic evaluation.

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References

1. Farrell, P. J., Broeze, R. J., and Lengyel, P. (1979). *Nature* **279,** 523–525.
2. Haas, A. L., Ahrens, P., Bright, P. M., and Ankel, H. (1987). *J. Biol. Chem.* **262,** 11,315–11,323.
3. Knight, Jr., E. and Cordova, B. (1991). *J. Immunol.* **146,** 2280–2284.
4. Loeb, K. R. and Haas, A. L. (1992). *J. Biol. Chem.* **267,** 7806–7813.
5. Blomstrom, D. C., Fahey, D., Kutny, R., Korant, B. D., and Knight, E., Jr. (1986). *J. Biol. Chem.* **261,** 8811–8816.
6. Korant, B. D., Blomstrom, D. C., Jonak, G. J., and Knight, E., Jr. (1984). *J. Biol. Chem.* **259,** 14,835–14,839.
7. Recht, M., Borden, and E. C., Knight, E. Jr. (1991). *J. Immunol.* **147,** 2617–2623.
8. D'Cunha, J. D., Knight, Jr., E., Haas, A. L., Truitt, R. L., and Borden, E. C. (1996). *Proc. Natl. Acad. Sci. USA* **93,** 211–215.
9. D'Cunha, J. D., Ramanujam, S., Wagner, R. J., Witt, P. L., Knight, Jr., E., and Borden, E. C. (1996). *J. Immunol.* **157,** 4100–4108.
10. Knight, E. Jr., Fahey, D., Cordova, B., Hollman, M., Kutny, T., Reich, N., and Blomstrom, D. C. (1988). *J. Biol. Chem.* **263,** 4520–4522.
11. Reich, N., Evans, D., Levy, D., Fahey, D., Knight, E., Jr., and Darnell, J. E., Jr. (1987). *Proc. Natl. Acad. Sci. USA* **84,** 6394–6398.
12. Ecker, D. J., Butt, T. R., Marsh, J., Sternberg, E. J., Margolis, N., Monia, B. P., Jonnalagadda, S., Khan, M. I., Weber, P. L., Mueller, L., and Crooke, S. T. (1987). *J. Biol. Chem.* **262,** 14,213–14,221.
13. Wilkinson, K. D. and Audhya, T. K. (1981). *J. Biol. Chem.* **256,** 9235–9241.
14. Narasimhan, J., Potter. J. L., and Haas, A. L. (1996). *J Biol Chem* **271,** 324–330.
15. Lowe, J., Mcdermott, H., Loeb, K., Landon, M., Haas, A. L., and Mayer, R. J. (1995). *J. Pathology* **177,** 163–169.
16. Loeb, K. R. and Haas, A. L. (1994). *Molec Cell Biol* **14,** 8408–8419.
17. Bebington, C., Bell, S., Doherty, F., Fazleabas, A., and Fleming, S. (1999). *Biol Reprod* In Press.
18. Finley, D. and Chau, V. (1991). *Annul. Rev. Cell. Biol. 7,* 25–69.
19. Hershko, A. and Ciechanover, A. (1992). *Annul. Rev. Biochem.* **61,** 761–807.
20. Finley, D., Ciechanover, A., and Varshavsky, A. (1984). *Cell* **37,** 43–55.
21. Hicke, L. (1997). *FASEB* **11,** 1215–1226.
22. Kim, T. K. and Maniatis, T. (1996). *Science* **273,** 1717–1719.
23. Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. (1994). *Cell* **78,** 773–785.
24. Naivar, K. A., Ward, S. K., Austin, K. J., Moore, D. W., and Hansen, T. R. (1995). *Biol. Reprod.* **52,** 848–854.
25. Austin, K. J., Ward, S. K., Teixeira, M. G., Dean, V. C., Moore, D. W., and Hansen, T. R. (1996). *Biol. Reprod.* **54,** 600–606.
26. Austin, K. J., Pru, J. K., and Hansen, T. R. (1996). *Endocrine* **5,** 1–7.
27. Johnson, G. A., Austin, K. J., Van Kirk, E. A., and Hansen, T. R. (1998). *Biol. Reprod.* **58,** 898–904.
28. Jentsch, S., Seufert, W., and Hauser, H. P. (1991). *Biochim Biophys Acta* **1089,** 127–139.
29. Baker, R. T. and Board, P. G. (1991). *Nucleic Acids Res.* **19,** 1035–1040.
30. Redman, K. L. and Burris, G. W. (1996). *Biochem. J.* **315,** 315–321.
31. Finley, D., Bartel, B., and Varshavsky, A. (1989). *Nature* **338,** 394–401.
32. Baker, R. T. and Board, P. G. (1989). *Am. J. Hum. Genet.* **44,** 534–542.
33. Perry, D. J., Austin, K. J., and Hansen, T. R. (1999). *Molecular Endocrinology* (In Press).
34. Hansen, T. R., Austin, K. J., and Johnson, G. A. (1997). *Endocrinology* **138,** 5079–5082.
35. Thatcher, W. W., Meyer, M. D. and Danet-Desnoyers, G. (1995). *J. Reprod. Fertil.* **49,** 15–28.
36. Roberts, R. M., Cross, J. C., and Leaman, D. W. (1990). *Endocrine Reviews* **13,** 432–452.
37. Haas, A. L. and Siepmann, T. J. (1997). *FASEB* **11,** 1257–1268.

38. Johnsson, N. and Varshavsky, A. (1994). *EMBO J.* **13,** 2686–2698.
39. Haas, A. L. and Bright, P. M. (1987). *J. Biol. Chem.* **262,** 345–351.
40. Bond, U. and Schlesinger, M. J. (1985). *Mol. Cell. Biol.* **5,** 949–956.
41. Staggs K. L., Austin, K. A., Johnson, G. A., Teixeira, M. G., Talbott, C. T., Dooley, V. A., and Hansen, T. R. (1998). *Biol Reprod* **59,** 293–297.
42. Murdoch, W. J., Austin, K. J., and Hansen, T. R. (1996). *Endocrinology* **137,** 4526–4529.