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Regulation of Interferon-stimulated Gene (*ISG*)*12*, *ISG15*, and *MX1* and *MX2* by Conceptus Interferons (IFNTs) in Bovine Uterine Epithelial Cells

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ABSTRACT: Various endometrial genes in ruminant ungulates are regulated by conceptus interferon tau (IFNT). However, the effect of each IFNT isoform has not been carefully evaluated. In this study, the effects of 2 IFNT isoforms, paralogs found *in utero*, and interferon alpha (IFNA) on uterine epithelial and Mardin-Darby bovine kidney (MDBK) cells were evaluated. Expression vectors of the bovine interferon (*bIFNT*) genes *bIFNT1*, *bIFNTc1*, and *bIFNA* were constructed, and recombinant bIFNs (rbIFNs) were produced by 293 cells. Bovine uterine epithelial or MDBK cells were cultured in the presence or absence of increasing concentrations of each rbIFN for 24, 48, or 72 h. Transcript levels of the IFN-stimulated genes (*ISG*s) *ISG12*, *ISG15*, *MX1*, and *MX2* were analyzed using quantitative reverse transcription-polymerase chain reaction. These messenger RNAs were up-regulated by rbIFN in a time- and concentration-dependent manner. In the epithelial cells, the *ISG12* transcript level increased at 48 h after rbIFN treatment but slightly decreased at 72 h, whereas the transcript level of *ISG15* increased at 24 h and was maintained through 72 h. Expressions of *MX1* and *MX2* increased at 72 h after rbIFN treatment. *MX1* expression increased in all treatment groups, but *MX2* increased only by bIFNTc1. In MDBK cells, the expression of *ISG12* was increased by bIFNT1 and bIFNTc1 after 24 and 72 h; however, it was unchanged by rbIFNA. *ISG15* increased following the same pattern as that seen in uterine epithelial cells, and *MX1* showed a similar expression pattern. *MX2* expression was increased by bIFNTc1 treatment in uterine epithelial cells, and its expression was increased by both bIFNT1 and bIFNTc1 in MDBK cells. These results show that epithelial and MDBK cell responses to IFNs differ, suggesting that IFNs possess common functions, but may have acquired different functions following gene duplication. (**Key Words:** IFNT, Isoforms, ISG, MX, Uterus)

INTRODUCTION

Similar to interferon alpha (*IFNA*) gene family, interferon tau (*IFNT*), which encodes a trophoblast factor essential for the process of maternal recognition of pregnancy in ruminant ungulates, belongs to a gene family. Exhaustive experiments have been conducted to characterize the effects of IFNTs, but IFNT paralogs actually expressed *in utero* and their effects on uterine epithelial cells have not been carefully evaluated.

In mammalian females, the maintenance of corpus luteum function beyond the length of the normal estrous cycle and the continued secretion of steroid hormones are required to establish and maintain pregnancy. Pregnancy recognition signaling in domestic ruminants (Short, 1969) is a local phenomenon involving a conceptus factor that acts on the uterine endometrium to alter gene expression (Spencer and Bazer, 2004). During the peri-implantation period, the embryonic trophectoderm of the ruminant conceptus secretes the cytokine IFNT (Imakawa et al., 1987; Roberts et al., 1992), which acts on the uterine endometrium (Godkin et al., 1984) and attenuates endometrial production of the luteolysin prostaglandin F2α, in turn maintaining corpus luteum function (Spencer et al., 1996; Salamonsen et al., 1998). Secretion of ovine IFNT starts on d 8 to 9 of pregnancy (d 0 = day of estrus), peaks on d 16 to 17, and then declines rapidly (Godkin et al., 1982; Ashworth et al., 1989; Farin et al., 1989). Thus, IFNT expression is limited to the peri-attachment period, during which the blastocyst begins to elongate, while up to

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Table 1. Oligonucleotide primer sequences used for *IFN* cloning

GenBank Accession No.	Gene	Primer (5′-3′) forward and reverse	Length (bp)
M60903	*bIFNT1*	F:atggccttcgtgctctctct	585
		R:aagtgagttcagatctccac	
AF238613	*bIFNTc1*	F:atggcttttgtgctctctct	585
		R:agatgagttcagatctccac	
NM_001017411	*bIFNA*	F:atggccccagcctggtccttc	567
		R:gtcctttctcctgaaactctc	
NM_33197	*GAPDH*	F:cgagatccctccaaaatcaa	513
		R:ttctagacggcaggtcaggt	

approximately 20% of trophoblast cells form binucleate cells (Xie et al., 1996; Klisch et al., 2006) and attach to the uterine epithelium.

A number of IFN-stimulated genes (ISGs), including the *MX* genes, are expressed in the uterus (Spencer et al., 2007; 2008). These ISGs are hypothesized to regulate endometrial receptivity to implantation as well as survival, growth, and development of the conceptus. In fact, ISG and MX are up-regulated in the ovine uterus in response to IFNT (Charleston and Stewart, 1993; Ott et al., 1998; Johnson et al., 1999). During the course of our studies, a next-generation sequencer was used to survey transcripts found in bovine conceptuses during the peri-implantation period (unpublished observation). To our surprise, only 2 *IFNT* transcripts, *IFNT1* and *IFNTc1*, were found in the bovine uterus. However, uterine response to each of these IFNTs has not been executed.

The aim of this study was to characterize the response patterns of *ISG12*, *ISG15*, *MX1*, and *MX2* after exposure to bovine IFNs (bIFNs)-IFNT1, IFNTc1, and IFNA-in uterine epithelial and Mardin-Darby bovine kidney (MDBK) cells. We determined that the expression of *ISG12*, *ISG15*, *MX1*, and *MX2* transcripts in the bovine cells was influenced by recombinant bIFNs (rbIFNs) in a time- and concentration-dependent manner.

MATERIAL AND METHODS

Cell culture

Bovine uterine epithelial cells were kindly provided by Dr. K. Okuda, Okayama University. Briefly, cells were cultured in a growth medium composed of Dulbecco's modified Eagle medium (DMEM)/nutrient mixture Ham's F12 and RPMI medium 1640 (Sigma-Aldrich, St. Lois, MO, USA) containing 5% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA) and antibiotics (Sigma-Aldrich) at 37°C in a 5% CO_2 incubator. MDBK and human 293 cells (HTB36, ATCC) were grown in DMEM supplemented with 10% FBS (JRH Biosciences) and antibiotics at 37°C in 5% CO_2.

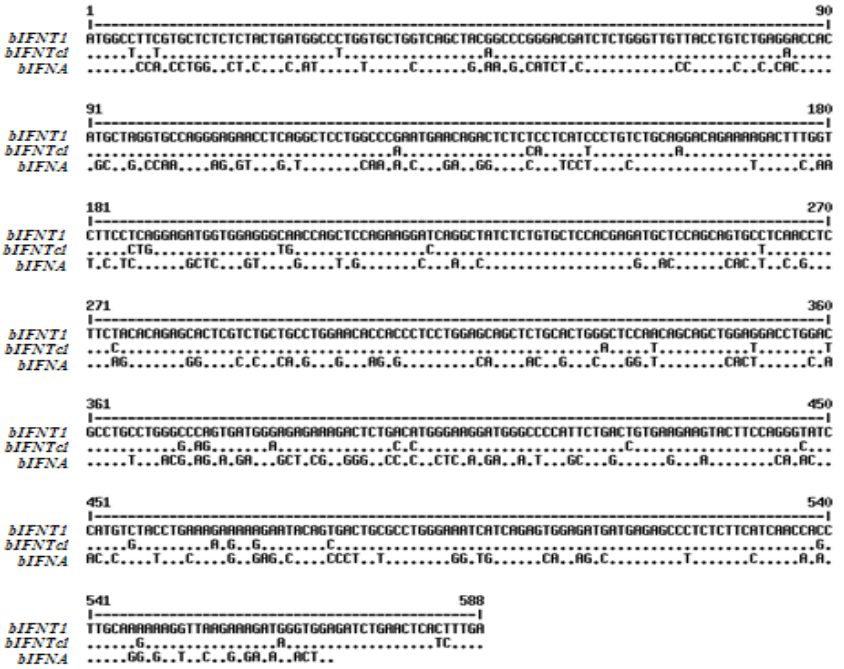
Construction of *bIFNs* subtype vectors for recombinant protein production

Full-length complementary DNA (cDNA) regions of *bIFNT1*, *bIFNTc1*, and *bIFNA* (GenBank accession Nos. *bIFNT1*: M60903; *bIFNTc1*: AF238613; and *bIFNA*:

Table 2. Primers for reverse transcription-polymerase chain reaction analysis

GenBank Accession No.	Gene	Primer (5′-3′) forward and reverse	Length (bp)
NM_001038050	*ISG12*	F:cttcaccagtgcaggaatca	195
		R:cccaaaaatttggacacgag	
NM_174366	*ISG15*	F:tgcagaactgcatctccatc	199
		R:ttcatgaggccgtattcctc	
AF069133	*ISG17*	F:catttgggtgtgagcatttg	159
		R:ttgctgatgggattgtgaaa	
NM_173940	*MX1*	F:gtccctgctaacgtggacat	155
		R:accaggtttctcaccacgtc	
NM_173941.2	*MX2*	F:gcagatcaaggcactcatca	168
		R:accaggtctggtttggtcag	
NM_177432.2	*IRF1*	F:gctgggacatcaacaaggat	163
		R:ctgctctggtccttcacctc	
AJ490936.1	*IRF2*	F:aaactgggccatccatacag	192
		R:ttagaaggccgctcagacat	
BC102948	*ACTB*	F:ctcttccagccttccttcct	178
		R:gggcagtgatctctttctgc	

A.



B.

DNA / Protein	*bIFNT1*	*bIFNTc1*	*bIFNA*
bIFNT1		93.2%	64.2%
bIFNTc1	89.7%		65.4%
bIFNA	47.6%	48.7%	

Figure 1. Comparison of the nucleotide sequences of bovine interferon tau (*bIFNT*), *bIFNT1* and *bIFNTc1*, and *bIFNA* genes. (A) Alignments were done using those of GenBank accession Nos. *bIFNT1*: M60903; *bIFNTc1*: AF23861; and *bIFNA*: NM-001017411. Alignment of nucleotides represents the open reading frame. (B) Homology of nucleotide and amino acid sequences of bIFNT1, bIFNTc1, and bIFNA. bIFNT nucleotide and amino acid sequences are highly conserved.

NM_001017411) were amplified via polymerase chain reaction (PCR) using specific primers (Table 1). Each of these products was inserted into mammalian expression vector pcDNA3.1/v5-His-TOPO (Invitrogen, Carlsbad, CA, USA). Each vector was completely sequenced to rule out the possibility of PCR errors.

Production and purification of recombinant bIFNs

The plasmid was transfected into 293 cells using HilyMax transfection reagent (Dojin Chemicals, Kumamoto, Japan) following the manufacturer's instructions. After 48 h, the cells were subjected to selection for stably transfected cells using 400 mg/ml G418. Selection was continued until monolayer colonies formed. The transfectants were transferred and maintained in DMEM supplemented with 10% FBS and 400 mg/ml G418. After the transfectants were grown to 90% confluence, the medium was discarded, and fresh DMEM medium containing 1.5% FBS, 400 mg/ml G418, and 15 mM sodium butyrate was added. One day later, the culture medium was collected. Target proteins from the culture medium were purified using His-tagged protein purification reagent (Medical and Biological Laboratories, Nagoya, Japan) according to the manufacturer's protocol.

Western blot analysis

Western blot analysis was performed using the culture medium and purified recombinant protein. Protein concentrations were determined using a protein assay dye reagent concentrate (Bio-Rad Laboratories, Hercules, CA, USA). Cell lysates (10 μg) were loaded into each lane and separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA) and treated with rabbit anti-bIFNT antibody (Protein Purify, Isesaki, Japan) and rabbit anti-His-probe antibody

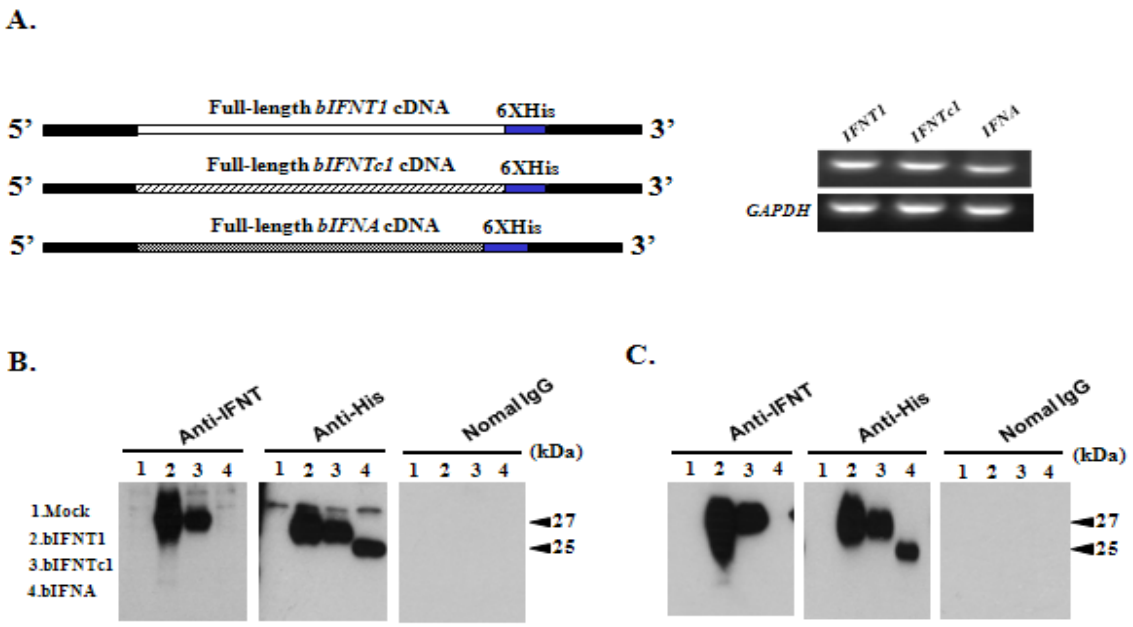


Figure 2. Production and analysis of recombinant bIFNs. (A) Structures of *bIFNT1*, *bIFNTc1*, and *bIFNA* complementary DNA (cDNA) pcDNA3.1/v5-His TOPO TA constructs. Messenger RNA (mRNA) was detected in 293 cells that had been stably transfected with the *IFN* cDNA constructs. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA was used as the internal control. (B) Detection of recombinant bIFNT1, bIFNTc1, and bIFNA. Bands of 27 and 25 kDa were detected in the culture medium of 293 cells that had been stably transfected with *bIFNT1*, *bIFNTc1*, and *bIFNA* cDNA constructs. Recombinant bIFNT1, bIFNTc1, and bIFNA levels were detected with anti-bIFNT antibody and anti-His antibody but not with normal immunoglobulin G (IgG). (C) Detection of recombinant bIFNT1, bIFNTc1, and bIFNA. The 27 and 25 kDa bands were detected in the His-purified recombinant protein from the culture medium of 293 cells that had been stably transfected with *bIFNT1*, *bIFNTc1*, or *bIFNA* cDNA constructs. Recombinant bIFNT1, bIFNTc1, and bIFNA levels were detected with anti-bIFNT antibody and anti-His antibody but not with normal IgG.

(Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted to 1:1000 with phosphate-buffered saline. The proteins were detected using secondary antibody conjugated with horseradish peroxidase and an enhanced chemiluminescence western blotting detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Normal rabbit immunoglobulin G (IgG; Sigma-Aldrich) was used as a negative control.

rbIFN treatment on uterine epithelial and MDBK cells

Bovine uterine epithelial cells and MDBK cells were treated with the rbIFNs (1, 5, and 10 μg/ml) in 3 replicates, each to determine their response to different concentrations of each protein. Cells were isolated at 24, 48, and 72 h after treatment for the evaluation of response patterns of *ISG12*, *ISG15*, *MX1*, and *MX2* messenger RNAs (mRNAs).

RNA extraction and reverse transcription (RT)-PCR

Total RNAs were extracted from each sample using Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol (Nagaoka et al., 2003). After extraction, the RNA was subjected to reverse transcription (RT) into cDNA using oligo (dT) 12 to 18 primers and SuperScript II (Gibco BRL Life Technologies, Rockville, MD, USA) according to the protocol suggested by the manufacturer. The cDNA molecules were used as templates for PCR analysis. Levels of *ISG12*, *ISG15*, *MX1*, and *MX2* were examined via PCR using specific primers (Table 2). The PCR mixture consisted of 1 μl RT product, 1 μl 10×PCR buffer, 0.4 μl each forward and reverse primers (10 pM), 0.2 μl deoxyribonucleotide triphosphate mixture (10 mM), 0.3 μl $MgCl_2$ (50 mM), 6.6 μl double-distilled H_2O, and 0.1 μl *Taq* DNA polymerase (5 U/μl; Invitrogen). The PCR was performed under the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min. The PCR product was analyzed via electrophoresis on 1% agarose gel stained with ethidium bromide. β-actin (*ACTB*) mRNA was used as an internal control.

Statistical analysis

RT-PCR data were analyzed using a Chemi-imager with AlphaEase software (Alpha Innotech Corp., San Leandro, CA, USA). Cells were the experimental unit and the fold changes of dependent variables were tested against concentration, time, and concentration×time. When significant concentration×time interaction was detected, variables were analyzed within the time. Data are presented as least squares means±standard error of the mean of

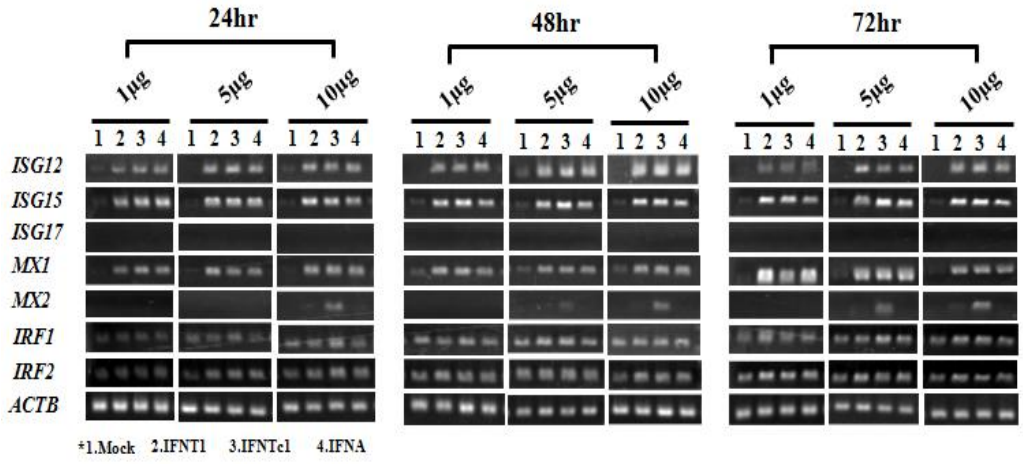


Figure 3. Reverse transcription-polymerase chain reaction (RT-PCR) showing the levels of response factor (IFN-stimulated gene [*ISG*] *12*, *ISG15*, *ISG17*, *MX1*, *MX2*, *IRF1*, *IRF2*) mRNAs in uterine epithelial cells treated with recombinant bIFNT1, bIFNTc1, or bIFNA proteins. The changes in mRNAs isolated from uterine epithelial cells were determined at 24, 48, and 72 h after initiation of the *in vitro* culture. The figure represents an ethidium bromide-stained gel of the amplified products. β-actin (*ACTB*) was used as the internal control. Lane 1: Mock; Lane 2: bIFNT1; Lane 3: bIFNTc1; Lane 4: bIFNA.

relative fold change from 0 h levels using the critical threshold method.

RESULTS

Structural analysis of *bIFNT1*, *bIFNTc1*, and *bIFNA* and production of recombinant proteins

Comparison of the nucleotide sequences of the *bIFN* genes *bIFNT1*, *bIFNTc1*, and *bIFNA* is shown in Figure 1A. The homology search revealed that the nucleotide sequence of *bIFNT1* had high homology with that of *bIFNTc1* (93%). However, the sequence showed low homology with *bIFNA*

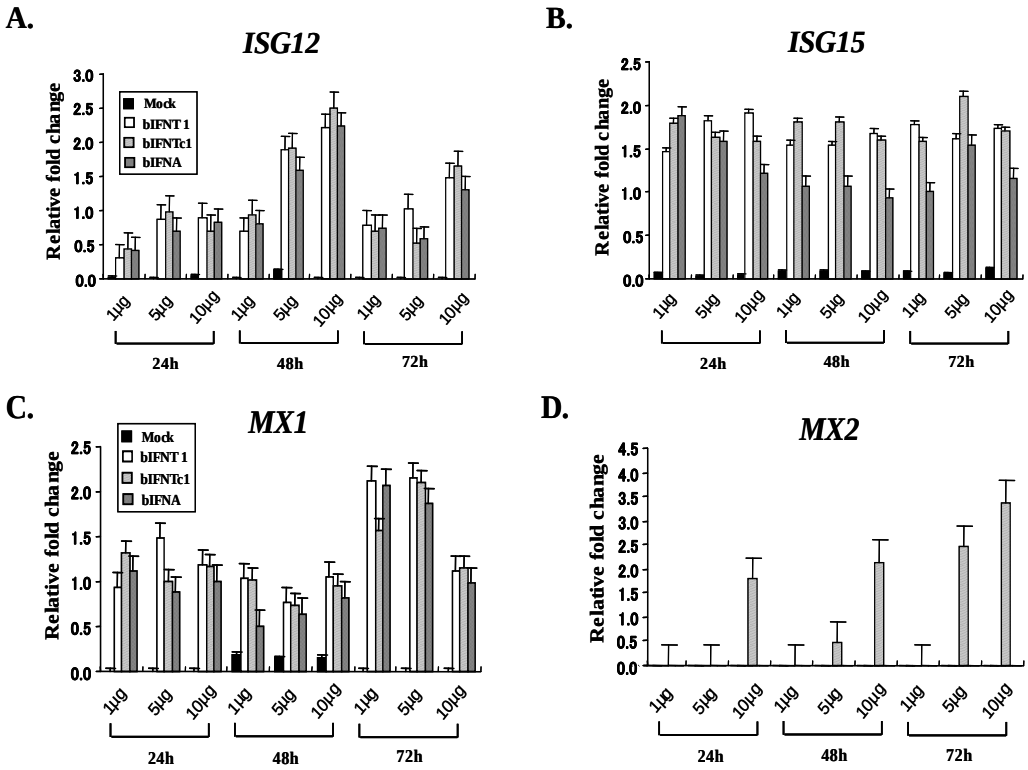


Figure 4. Levels of responsive factor mRNA affected by recombinant bIFNT1, bIFNTc1, and bIFNA in uterine epithelial cells. Using quantitative RT-PCR, steady-state mRNA levels for *ISG12* (upper left), *ISG15* (upper right), *MX1* (lower left), and *MX2* (lower right) in uterine epithelial cells were examined at 24, 48, and 72 h after the initiation of *in vitro* culture. Bars represent the average fold changes from 0 h levels using the critical threshold method. Error bars represent treatment standard error of the mean.

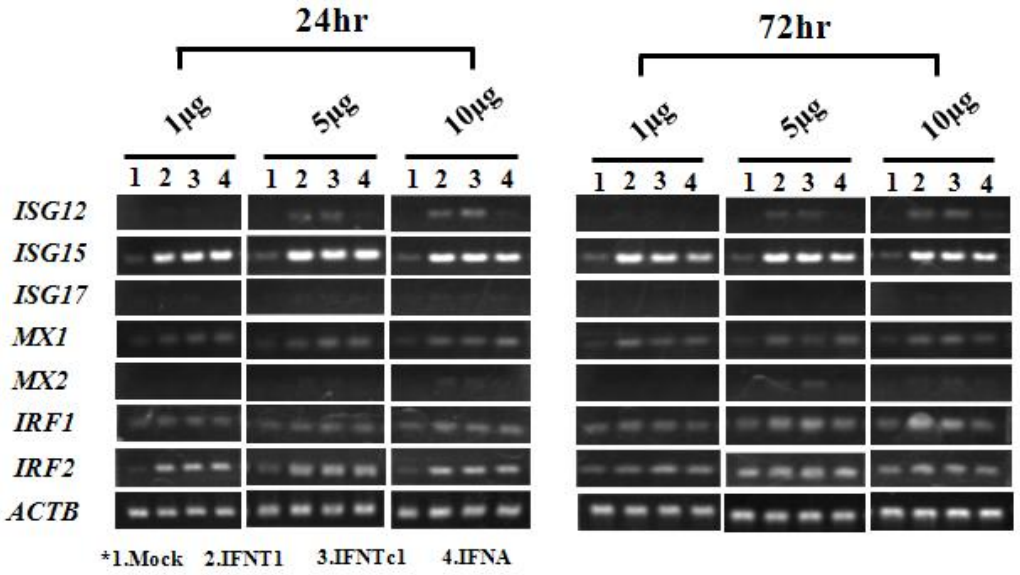


Figure 5. RT-PCR showing the abundance of response factor (*ISG12*, *ISG15*, *ISG17*, *MX1*, *MX2*, *IRF1*, *IRF2*) mRNAs with recombinant bIFNT1, bIFNTc1, and bIFNA proteins in MDBK cells. Changes in response factor mRNA levels were determined with RNAs isolated from Mardin-Darby bovine kidney (MDBK) cells at 24 and 72 h after the initiation of *in vitro* culture. The figure represents ethidium bromide-stained gels of the amplified products. *ACTB* was used as an internal control. Lane 1: Mock; Lane 2: bIFNT1; Lane 3: bIFNTc1; Lane 4: bIFNA.

cDNA (64%; Figure 1B).

Three expression vectors (pcDNA3.1-*bIFNT1*, pcDNA3.1-*bIFNTc1*, and pcDNA3.1-*bIFNA*) were transfected into 293 cells (Figure 2A), from which culture media were collected and purified via a His-tagged protein purification method. The supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Specific antibodies for IFNT and His were used to detect recombinant proteins produced by the 3 expression vectors via western blot (Figure 2B, C). rbIFNT1 and rbIFNTc1 showed single bands with molecular weights of 27 kDa. However, the band was a slightly lower at 25 kDa for bIFNA owing to shortening by 6 amino acids. No band was detected in the fresh medium or with normal IgG (Figure 2B, C). The 27 kDa band represented the approximate molecular weight predicted from the *rbIFNT1* and *rbIFNTc1* cDNA sequences, including the carbohydrate chains.

Up-regulation of *ISG12* and *ISG15* mRNAs by rbIFNs in uterine epithelial cells

To investigate responsiveness to rbIFNs in uterine epithelial cells, *ISG*, *MX*, and interferon regulatory factor gene (*IRF*) transcripts were examined after rbIFN treatment. A concentration- and time-dependent response was recorded with 1, 5, and 10 μg/ml at 24, 48, and 72 h. Recombinant proteins used in this study affected *ISG12* and *ISG15* transcripts but not that of *ISG17* (Figure 3). Likewise, these treatments had some effects on *MX1* and *MX2* transcripts but not on *IRF* transcripts (Figure 3). Therefore, *ISG17* and *IRF* were excluded from subsequent quantitative studies. All rbIFNs significantly up-regulated *ISG12* and *ISG15* mRNAs after 24, 48, and 72 h of incubation. *ISG12* mRNA was remarkably increased at 48 h. After that, it decreased to the same level as that measured at 24 h (Figure 4A). Stimulation by rbIFNs also significantly increased the expression of *ISG15* mRNA. In particular, the *ISG15* mRNA level was increased at 24 h after treatment and was maintained through 72 h (Figure 4B), and this effect was not dose dependent. In the rbIFNA treatment, the expression levels of *ISG15* mRNA were significantly decreased at 48 h.

Changes in *MX1* and *MX2* mRNAs by rbIFN stimulation in uterine epithelial cells

rbIFNs significantly up-regulated the level of *MX1* mRNA at 24, 48, and 72 h after treatment (Figure 4C). The up-regulation of *MX1* was mild in the first 24 h post-induction with rbIFNT1, rbIFNTc1, and rbIFNA (1 and 10 μg/ml), then slightly decreased at 48 h, and was increased at 72 h. The *MX2* expression levels gradually increased and peaked at 72 h after treatment with 10 μg/ml rbIFNTc1 and showed a high increase only at 72 h (Figure 4D). *MX2* mRNA was not affected by rbIFNT1 or rbIFNA in the uterine epithelial cells. Thus, the patterns of *MX1* and *MX2* responses to rbIFNs were different.

Up-regulation of *ISG12* and *ISG15* mRNAs by rbIFNs in bovine MDBK cells

rbIFNs significantly up-regulated *ISG12* and *ISG15*

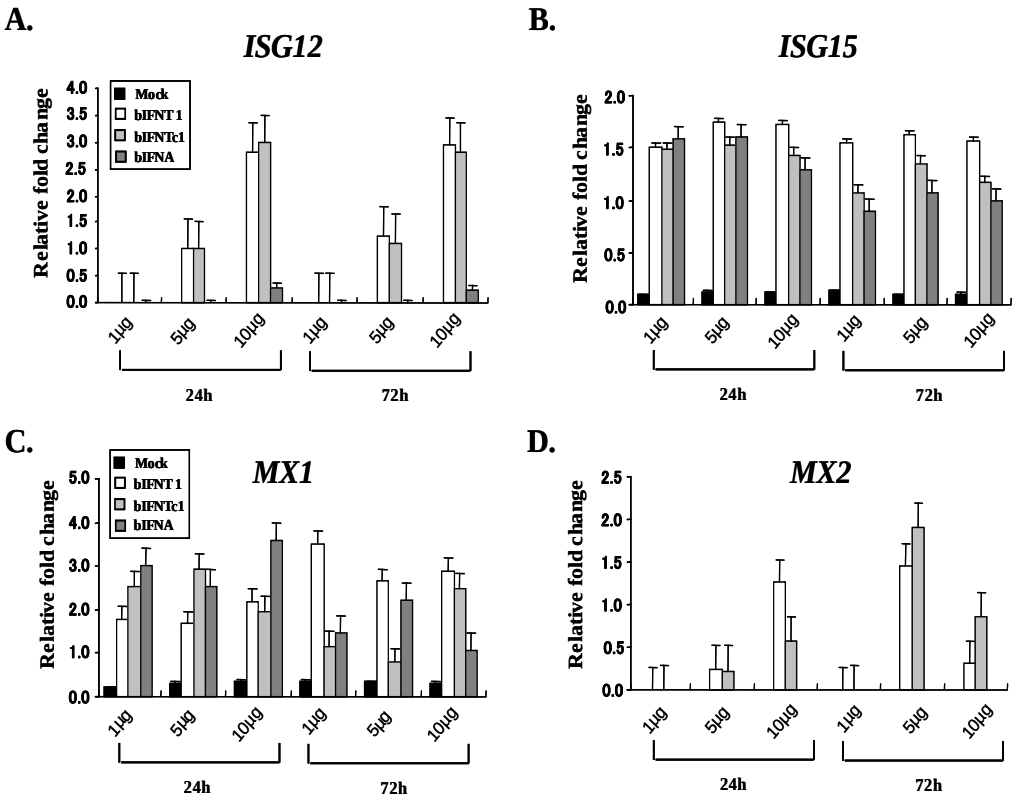


Figure 6. Levels of response factor mRNA with recombinant bIFNT1, bIFNTc1, and bIFNA treatment in MDBK cells. Using quantitative RT-PCR, mRNA levels for *ISG12* (upper left), *ISG15* (upper right), *MX1* (lower left), and *MX2* (lower right) in MDBK cells were examined at 24 and 72 h after the initiation of *in vitro* cultures. Bars represent the average fold changes from 0 h levels using the critical threshold method. Error bars represent standard error of the mean.

mRNAs at 24 and 72 h after treatment in bovine MDBK cells (Figure 5), and the expression was dramatically increased in the rbIFNT1 and rbIFNc1 treatment groups. *ISG12* mRNA expression was unchanged by rbIFNA treatment (Figure 6A), but *ISG15* mRNA expression was significantly increased at 24 and 72 h after rbIFNA, rbIFNT1, or rbIFNTc1 treatment (Figure 6B). Thus, the mRNA expression pattern for *ISG15* was similar in uterine epithelial cells and MDBK cells. However, *ISG12* mRNA demonstrated a slightly different expression pattern after rbIFNA treatment.

Changes in *MX1* and *MX2* mRNAs by rbIFN stimulation in MDBK cells

MX1 mRNA expression increased at 24 h after rbIFNT1, rbIFNTc1, and rbIFNA treatment. However, it decreased slightly at 72 h after rbIFNA treatment. By contrast, *MX1* mRNA expression increased slightly at 72 h after rbIFNTc1 exposure (Figure 6C). *MX2* expression gradually increased after rbIFNT1 or rbIFNTc1 treatment, but it was unchanged by rbIFNA (Figure 6D). These patterns did not differ between uterine epithelial cells and MDBK cells.

DISCUSSION

IFNT from a blastocyst (d 15 to 16 after breeding in cattle) clearly alters endometrial gene expression, allowing the establishment of pregnancy in ruminant ungulates (Spencer and Bazer, 2004). To our knowledge, this is the first study to examine the effect of IFNT paralogs, of which transcripts have been found in the bovine uterus on endometrial epithelial cell responses. The examination of recombinant IFNs in this study revealed that IFNs have common functions as well as functions unique to each paralog. This observation further suggested that endometrial responses to IFNT must be re-evaluated using each IFNT isoform as well as with a combination of IFNT isoforms. Likewise, *in vivo* IFNT infusion study requires re-evaluation based on the dose and kinds of IFNT isoforms, which may affect the outcome.

Until recently, IFNT was thought to act exclusively in a paracrine manner in the endometrium, causing endometrial responses in pregnant sheep and cattle (Vallet et al., 1991; Charleston and Stewart, 1993; Ott et al., 1998; Spencer et al., 1998; Johnson et al., 2000). ISGs are induced systemically within days of blastocyst signaling in ewes

(Yankey et al., 2001). In addition, Oliveira and coworkers (2008) showed that ISG expression occurs in extrauterine tissues during early pregnancy, possibly owing to endocrine IFNT release from the uterine vein. These studies do not completely prove that IFNT could escape from the uterus and initiate a peripheral antiviral response. Regardless of proof of direct or indirect effects of IFNT in the circulation, the degree of response observed in *ISGs* and *MXs* in this study suggested that ISGs escaped from the uterus into the circulation.

Charleston and Stewart (1993) first showed that *MX* was strongly up-regulated in ovine endometrium during early pregnancy. Later, *MX* was shown to be expressed in the ovine uterus during the estrous cycle (Ott et al., 1998). The roles of ISG17 and MX in the ovine endometrium are unknown, but their temporal and spatial patterns of expression provide insight into their physiological relevance. One interpretation of these results from primates, rodents, ruminants, and pigs is that *ISG17* and *MX* are part of a universal uterine response to fetal/placental development. In support of a general role for *MX* in early pregnancy, its expression has been shown in the uterine endometria of sheep (Ott et al., 1998), cattle, pigs, and horses (Hicks et al., 2003), and rodents (Chang et al., 1990). The regulation and immediate functional implications of these various expression patterns may be independent but are almost certainly coordinated to favor conceptus development and survival.

In summary, the results of this study indicate that rapid communication occurs between the conceptus and the uterine endometrium at approximately the time of maternal recognition of pregnancy in cattle. These changes result in gene expression that can be measured in uterine epithelial cells and possibly in the circulation. Uterine epithelial *ISG12*, *ISG15*, *MX1*, *MX2* were activated, whereas *IRF1* and *IRF2* were not. The required adaptor molecules and transcription factors were mostly up-regulated in a time- and concentration-dependent manner. These results led us to conclude that bovine uterine epithelial cells respond to rbIFNs differently, which warrants further investigation.

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