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Characterizations of the bovine subtype Interferon-tau Genes† : Sequences of Genes and Biological Activity of Transcription Factors in JEG3 Cell

Min-Su Kim[1], Kwan-Sik Min[2], Hwan-Hoo Seong[1], Chan-Lan Kim[1], Dongkyo Kim[1], Kazuhiko Imakawa[3] and Sung Woo Kim[1*]

[1]Animal Genetic Resources Research Center, National Institute of Animal Science, RDA, Namwon 55717, Korea
[2]Animal Biotechnology, Hankyong National University, Kyonggi-do 17579, South Korea
[3]Laboratory of Animal Breeding, Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo 113-8657, Japan

ABSTRACT

Multiple interferon tau (IFNT) genes exist in bovine. An antiluteolytic substance secreted by the bovine conceptus and primarily responsible for maternal recognition of pregnancy is bovine trophoblast protein 1 (bIFNT1), a new type I interferon tau (IFNT) genes. The objectives of this research were to investigate whether multiple, distinct gene encode bIFNT1 and other type I bIFNT gene in the bovine genome and to examine expression of bIFNT1 and other bIFNTc1 mRNAs during conceptus development. These transcrips could be regulated through caudal-relatedhomeobox-2 (CDX2) and ETS2 and/or AP1 (JUN) expression, a transcription factor implicated in the control of cell differentiation in the trophectoderm. The presence of mRNAs encoded by bIFNT1 and type I bIFNTc1 genes were examined quantitatively via reverse transcription-polymerase chain reaction (RT-PCR) analysis of total cellular RNA (tcRNA) extracted from on day 17, 20 and 22 bovine conceptuses. The expression level of bIFNT1 was higher on day 17 transcripts were gradually weakly detectable on day 20 and 22. However, the other bIFNTc1 gene examined transcripts was highly expressed on day 20 and transcripts were weakly detectable on day 17 and 22 bovine conceptuses. Furthermore, human choriocarcinoma JEG3 was co-transfected with an -1kb-bIFNT1/c1-Luc constructs and several transcription factor expression plasmids. Compared to each -1kb-bIFNT1/c1-Luc increased when this constructs were co-transfected with, ETS2, AP1(JUN), CREBBP and/or CDX2. Also, bIFNTc1 gene was had very effect on activity by alone ETS2, and AP1 (JUN) expression factors in choriocarcinoma JEG3 cell. However, bIFNT1 gene expression of the upstream region was not identified. We demonstrated that the activities of bIFN genes are regulated by differential, tissue-specific and developmental competence during pregnancy.

(Keywords: bovine, IFNT, conceptus, transcription factor, JEG3)

INTRODUCTION

Interferon-tau (IFNT), produced by peri-implantation blastocysts, is a major protein implicated in the process of maternal recognition of pregnancy in ruminant ungulates (Godkin et al., 1982; Imakawa et al., 1987; Roberts et al., 1992). Extensive studies have been conducted on the implantation processes, during which the conceptus hatches from its zona pellucida and attaches to the uterine epithelial cells, resulting in placental formation. During the peri-implantation period of pregnancy in sheep, endometrial activity and their epithelial secretions are largely regulated by progesterone from the corpus luteum(CL) and cytokines/ hormones from the conceptus such as interferon tau (IFNT) (Spencer et al., 2004, 2007). IFNT, a major cytokine involved in the process of maternal recognition of pregnancy, is secreted into the uterine lumen by the mononuclear trophectoderm of the conceptus (Roberts et al., 1992; Imakawa et al., 2004). IFNT decreases endometrial oxytocin and estrogen receptors, which attenuates episodic prostaglandin F2a

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* Corresponding Author: Sung Woo Kim, Ph.D.
Phone: 82-63-620-3524; Fax: 82-63-620-3591
E-mail: sungwoo@korea.kr

(PGF2a) secretion, resulting in the prevention of luteolysis (Vallet et al., 1988; Spencer and Bazer et al., 1996). In the ovine, the maximum production of IFNT is attained on day 16. Expression of IFNT declines rapidly as the process of implantation proceeds, and by on day 22, when the ovine trophoblast is fully attached to the maternal endometrium, IFNT is no longer detected (Godkin et al., 1982; Imakawa et al., 1987; Guillomot et al., 1990).

Interferon-tau (IFNT) is one member of the Type I IFNs, an extensive grouping that includes the IFNA, B, and W (Roberts et al., 1997). IFNT gene was originated from a single duplication event from an IFNW gene about 36 million years ago in the mammalian lineage, leading to the present day pecoran ruminants, a suborder comprised of cattle, deer, giraffes and their relatives (Roberts et al., 1997; Roberts et al., 1998). It has been speculated that the initial duplication event that provided the primordial IFNT also disrupted the promoter element, which ultimately led to restricted trophoblast expression and to this novel role in maternal recognition of pregnancy (Roberts et al., 1997). The genes have continued to duplicate since then, and it has been estimated that there may be as many as ten IFNT in bovine, with all of them clustered within or in close proximity to the genetic locus that contains the other Type I IFN genes (Ryan et al., 1993). Exactly how many IFNT genes are expressed is unknown. Until this report, the sequences of only four closely similar bovine IFNT cDNA sequences had been deposited in GenBank (Imakawa et al., 1989; Stewart et al., 1990; Hansen et al., 1991). IFNT genes or cDNAs have been isolated and characterized for the ovine and bovine species, which exhibit a high degree of similarity within and among ruminants (Roberts et al., 1992; Nephew et al., 1993; Ryan and Womack et al., 1993; Alexenko et al., 2000; Ealy et al., 2001).

Presently, multiple ovine and bovine IFNT are transcribed during early pregnancy and encode proteins that can possess different biological activities (Ealy et al., 1998; Winkelman et al., 1999). In ruminant ungulates, large amount of an anti-luteolytic substance secreted by the bovine conceptus and primarily responsible for maternal recognition of pregnancy is bovine trophoblast protein-1 (bIFNT1), a new type I (bIFNTc1, bIFNTc2, and bIFNTc3) genes. The gene of bIFNT1 has greater sequence homology with bIFNTc2 (98% nucleotide identity; 99% amino acid identity) and bIFNTc3 (97% nucleotide identity; 98% amino acid identity) than bIFNTc1 (93% nucleotide identity; 89% amino acid identity). In the bIFNT1, other bIFNT genes like bIFNTc1, bIFNTc2, and bIFNTc3 whose coding regions are >90% identical to that of bIFNT1, the homologies of their 5'-upstream regions (between -1000 to +51 base pair, bp) are approximately 90%. The remaining 10% sequences with specific nucleotides may be responsible for the different degrees of expression levels which were observed for bIFNT1 and bIFNTc1 or bIFNTc2 and/or bIFNTc3. It was determined on the basis of homology in nucleotide sequence that IFNT1, bIFNTc2, and bIFNTc3 represented the same gene (Gene Bank Database). Multiple interferon (IFNT) genes exist in cattle, but it has remained unclear how many are expressed, the extent of their variation, and whether different genes exhibit similar patterns of expression and code for proteins with similar biological activities. However, molecular mechanisms of these bIFNT genes have not been characterized because of their temporal expressions. To understand the molecular mechanisms responsible for different degrees of bIFNT gene transcriptions, four bIFNT genes, bIFNT1, new type I bIFNTc1 genes were examined using transient transfection analyses. The objectives of were (1) To identify the multiple bIFNT mRNA isoforms present in the peri-implantation bovine conceptus and to compare these sequences; (2) To examine the expression of transcription factors in human choriocarcinoma JEG3. This sequence information may provide insight into the features that regulate bIFNT expression and function during early pregnancy in ruminants and aid in the assessment of bIFNT evolution across species.

MATERIALS and METHODS

1. Animals and Tissue Preparation

All animal procedures in the present study were approved by the Committee for Experimental Animals at Zen-noh Embryo Transfer (ET) Center and the University of Tokyo. Estrous synchronization, super-ovulation and ET processes were performed as previously described (Ideta et al., 2007). Seven-day embryos (Day 0 = day of estrus) were collected from superovulated cattle. Twelve embryos derived from the superovulation were transferred nonsurgically into the uterine horn of three Holstein heifers (n = 4, each), ipslateral to the CL on the day 7 of the estrous cycle. Elongated conceptuses

were collected nonsurgically by uterine flushing on day 17, 20, or 22. Conceptuses in the uterine flushing media were obtained by centrifugation at 1,000 rpm for 5min, snap-frozen and transferred to Laboratory of Animal Breeding at University of Tokyo and Animal Genetic Resources Research Center.

2. DNA isolation

Genomic DNA was isolated from the bovine trophoblast using Genomic DNA purification kit (Promega, Madison, WI) according to the protocol provided by the manufacturer. The quality and integrity of the genomic DNA was determined using agarose gel (1%) electrophoresis and visualization under UV light after staining with ethidium bromide. The purity and quantity of genomic DNA were checked using the spectrophotometric reading at OD260 and OD280.

3. Polymerase chain reaction (PCR)

The genomic DNA isolated from one animal of bovine was used as template for amplifying the IFNT genes. A pair of degenerate primers
(viz., IFNT1/start F-5'-AGGTACCACTGATACCAAAGCTGAAAC-3' and IFNT1/stop R-5'-AGCTAGCCTGCTGGGCTGGCTGAGATGG-3') (viz., IFNTc1/start F-5'-AGGTACCAATGATGCTAAAGCTGAAAC-3' and IFNTc1/stop R-5'-AGCTAGCCTGCTGGGCTGGCTGAGATGG-3') (viz., IFNTc2/start F-5'-AGGTACCACTGATGCCAAAGCTGAAAC-3' and IFNTc2/stop R-5'-AGCTAGCCTGCTGGGCTGGCTGAGATGG-3') (viz., IFNTc3/start F-5'-AGGTACCACTGATGCCAAAGCTGAAAC-3' and IFNTc3/stop R-5'-AGCTAGCCTGCTGGGCTGGCTGAGATGG-3') designed on the basis of IFNT sequences of bovine (Genebank Accession Number bIFNT1; M60903: bIFNTc1; AF238613: bIFNTc2; AF238612: bIFNTc3; AF238611) were used. PCR amplification was carried out in a total volume of 25μl of reaction mixture containing 100ng of genomic DNA, 1X PCR buffer [100mM Tris-HCl (pH 8.8 at 25°C), 500mM KCl and 0.8% Nonidet P40], 1.5mM $MgCl_2$, 200μM dNTPs, 2μM of each primer and 1.0unit of Taq DNA polymerase. A negative control with no template DNA was also included. The PCR protocol involved an initial denaturation at 95°C for 2min followed by 30cycles of denaturation (95°C for 30s), annealing (60°C for 30s) and extension (72°C for 1min) proceeded by one cycle of final extension (72°C for 10min). The PCR product was checked by agarose gel (1%) electrophoresis in 1xTAE buffer after staining with ethidium bromide.

4. Cloning of amplified fragment

The PCR product was cloned into pGEM T-easy vector (Promega, Madison, WI) following the manufacturers instructions. Positive recombinant clones were identified using blue and white screening. Further the presence of the insert was confirmed by restriction digestion Kpn I with Nhe I and using plasmid PCR.

5. Sequencing and sequence analysis of IFNT subtype genes

The positive clones as well as the direct PCR product were sequenced using an ABI PRISM automatic sequencer (ABI-PRISM, Foster City, CA) using standard cycle conditions by Sanger's dideoxy chain termination method with standard sequencing primers (viz., M13F and M13R) and the primers employed for amplification, respectively. The sequences were subjected to BLAST analysis (www.ncbi.nlm.nih.gov/BLAST). The nucleotides as well as deduced amino acid sequences of bIFNT were aligned with that of ruminant ungulate species available in the GenBank database using the Clustal method of MegAlign Program of Lasergene Software (DNASTAR). A phylogram was also constructed to analyze the evolutionary significance.

5. RNA extraction and RT-PCR

Total RNAs from on day 17, 20 and 22 conceptuses tissues (80 to 100ng) were extracted using Isogen Reagent (Nippon Gene, Tokyo, Japan) according to the protocol provided by the manufacturer (Nagaoka et al., 2003). The RNA extracted from each sample then underwent reverse transcription (RT) into cDNA using oligo (dT) 12-18 primers and SuperScript II (Gibco BRL Life Technologies, Rockville, MD) according to the protocol suggested by the manufacturer, and these RT products were used as templates for PCR analysis. The PCR mixture consisted of 1μl of RT product, 1μl of 10X PCR buffer, 0.4μl each of forward and reverse primers (10 pM), 0.2 μl of dNTP mixture (10 mM), 0.3μl of $MgCl_2$ (50 mM), 6.6μl of ddH_2O, and 0.1μl of Taq DNA polymerase (5U/μl; Invitrogen, Carlsbad, CA). The PCR was performed under the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 1min, 57°C for 1min, and 72°C for 1min. The PCR product was analyzed by electrophoresis on 1% agarose gel stained with ethidium bromide. The cDNA fragment was extracted from agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Tokyo, Japan) and then cloned into pGEM T-easy vector (Promega, Madison, WI), and nucleotide

sequences were determined by DNA sequencing (ABI-PRISM, Foster City, CA). β-actin mRNA was used as an internal control.

6. Construction of rec-IFNT subtype vectors

The upstream regions of bIFNT1, bIFNTc1, bIFNTc2, and bIFNTc3 (GenBank accession numbers bIFNT1; M60903: bIFNTc1; AF238613: bIFNTc2; AF238612:bIFNTc3; AF238611, respectively) -1000 to +51 bp were amplified through PCR with specific primers. Each of these products was inserted into the Kpn I and Nhe I site of pGL3 basic vector (Promega, Madison, WI). Renilla luciferase pRL-TK vector (Promega), driven by herpes simplex virus-thymidine kinase (HSV-TK) promoter, was used to normalize the transfection efficiency (Imakawa et al., 2006). Amounts of reporter construct relative to that of the internal control vector pRL-TK were 20:1. CDX2, ETS2, AP1 and CREBBP expression plasmids were described previously (Imakawa et al., 2006). All expression vectors, pSG5 (Agilent)-based constructs, were driven by the SV40 promoter/enhancer. These plasmid constructs were each confirmed to have expected nucleotide sequences by dideoxy sequencing.

7. Cell culture

Human choriocarcinoma JEG3 cells (HTB36, American Type Tissue Collection, ATCC, Rockville, MD) were grown in DME medium supplemented with 10% FBS (JRH Biosciences) and antibiotics (Invitrogen) at 37°C in air with 5% CO_2.

8. Transient transfection, Luciferase assay

JEG3 cell were each cultured in the medium described in

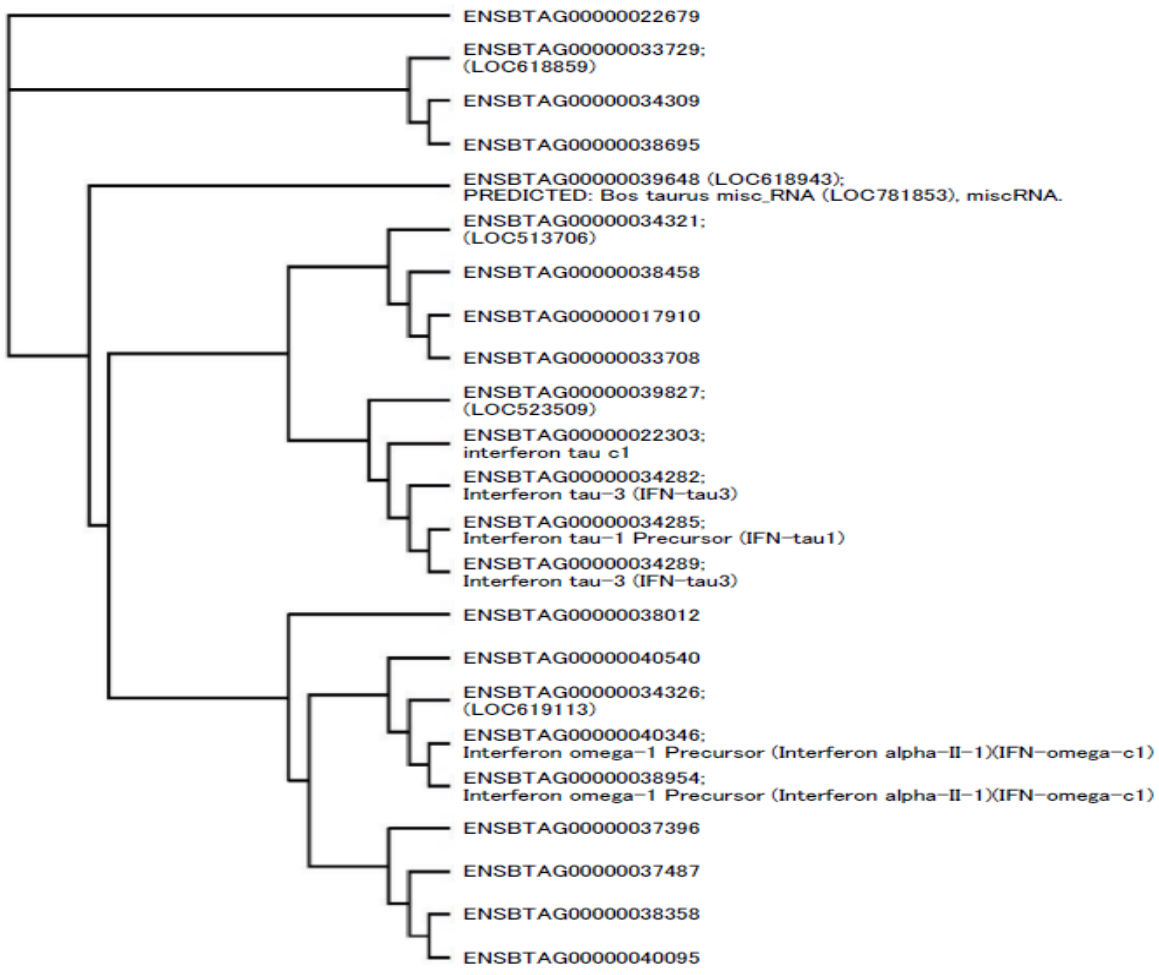

Fig. 1. The dendrogram of bovine IFN subfamilies by phylogenetic leneage. The IFN genes in each subfamily were located on chromosome 8 and the following gene(s) have been identified as a putative paralogous family within species using Taxonomy Level Gene Identifier.

Table 1. Homology of nucleotide and amino acid sequences of bovine *IFNT1*, *c1*, *c2*, and *c3*

Protein/DNA	*bIFNT1*	*bIFNTc1*	*bIFNTc2*	*bIFNTc3*
bIFNT1		93.2%	98.9%	97.8%
bIFNTc1	89.7%		93.6%	92.8%
bIFNTc2	99.3%	93.2%		98.7%
bIFNTc3	98.3%	92.9%	98.3%	

IFNT nucleotide and amino acid sequences are highly conserved.

the cell culture section, and plated on a 24-well plate for subsequent transfection (Imakawa et al., 2006). At 60–80% confluence, transient transfection was performed using HilyMax reagents (Dojin Chemicals, Kumamoto, Japan) according to the manufacturer's protocol. In brief, 2mg total plasmid DNAs including bIFNT-reporter (1.5 mg), expression plasmids (total of 0.5 mg) and 4ml HilyMax were prepared in 30ml DMEM with no supplements (plasmid mixture). Amounts of total plasmids for each transfection were adjusted with the inclusion of pSG5 only (empty vector). After 15min, plated cells were overlaid with the plasmid mixture and incubated at 37°C for 48hr under 5% CO_2 in air. Forty-eight hours after transfection, the cells were lysed by the addition of 100ml Passive Lysis Buffer (Promega). Luciferase assay was performed using Dual-Luciferase Reporter Assay System as described previously (Imakawa et al., 2006).

9. Statistical analysis

The results of luciferase assays were expressed as means ±SEM. Differences in fold activation (luciferase activity) were examined by ANOVA, followed by multiple comparison tests of Fisher's LSD.

RESULTS

1. Phylogenetic analysis

The dendrograms constructed on the basis of either the nucleotide or deduced amino acid sequences of IFN gene of all the cattle available in the GenBank showed almost the similar groupings. The IFN genes in each subfamily were located on bovine chromosome 8 by genomic analysis (Fig 1). Including only bIFNT variants, distinct classifications were formed and bIFNT1 and c1 gene were grouped samely.

2. Structural analysis of bIFNT subtypes.

The 4 types of oligonucleotides were designed from the sequence analysis of bIFNT equivalent genes in cattle. Among ten clones detected and sequenced, 4 different subtype of nucleotide sequences were detected (Fig. 2). One clone was distinct, which was amplified from the latter set of oligonucleotides, and the remaining clones amplified from the former set of oligonucleotides had two kinds of nucleotide sequences: two clones shared nucleotides and the remaining four clones had the same sequence. For nucleotide sequences rep resenting three subtypes of nucleotides, that had been designated as bIFNT1, c1, c2 and c3, similarity in DNA sequence between bIFNT1 and c1, bIFNT1 and c2, bIFNT1 and c3, bIFNTc1 and c2, bIFNTc1 and c3, bIFNTc2 and c3 are 93%, 98%, 97%, 93% , 92% and 98%, respectively (Table 1). There are only 40 nucleotide differences between bIFNT1 and c1 sequences. The sequences of IFNT1, c2, and c3 seem to be homologous IFNT in bovine as they have high homology. When these IFNTc2 and c3, were compared to the ones in other ruminant species, they were closer to that of IFNT1. Whether this is IFNT or other IFN related gene such as IFNW remains to be determined. In addition, a region of the open reading frame (ORF) of IFNT1, c1, c2, and c3 was compared in bovine (Fig. 2). The open-reading frame (ORF) of 595 nucleotides coded for a 195 aa polypeptide that contained a 23 aa signal peptide followed by a 172 aa mature protein (Fig. 3). It was determined on the basis of high homology (> 97%) in nucleotide sequence that IFNT1, c2, and c3 represented the same gene. Furthermore, the upstream regions of these four genes are compared of the IFNT genes. All sequences were very similar, and that of c2, c3 was identical with that of the previously reported sequence for bIFNT1.

```
        +68                                                                                                  +157
bIFNT 1  ATGGCCTTCGTGCTCTCTCTACTGATGGCCCTGGTGCTGGTCAGCTACGGCCCGGGACGATCTCTGGGTTGTTACCTGTCTGAGGACCAC
bIFNTc1  .....T..T.....................T.................A.....................................A.....
bIFNTc2  ..............................................................................................
bIFNTc3  ..............................................................................................

        +158                                                                                                 +247
bIFNT 1  ATGCTAGGTGCCAGGGAGAACCTCAGGCTCCTGGCCCGAATGAACAGACTCTCTCCTCATCCCTGTCTGCAGGACAGAAAAGACTTTGGT
bIFNTc1  .....................................A..............CA.....T..........A.....................
bIFNTc2  ..............................................................................................
bIFNTc3  ..............................................................................................

        +248                                                                                                 +337
bIFNT 1  CTTCCTCAGGAGATGGTGGAGGGCAACCAGCTCCAGAAGGATCAGGCTATCTCTGTGCTCCACGAGATGCTCCAGCAGTGCCTCAACCTC
bIFNTc1  .....CTG..............TG.................C.....................................T........
bIFNTc2  ...........................................................T..................T........
bIFNTc3  ........................G.....................................................T........

        +338                                                                                                 +427
bIFNT 1  TTCTACACAGAGCACTCGTCTGCTGCCTGGAACACCACCCTCCTGGAGCAGCTCTGCACTGGGCTCCAACAGCAGCTGGAGGACCTGGAC
bIFNTc1  ...C.....................................................A.....T...........T.........T
bIFNTc2  ..............................................................................................
bIFNTc3  ...C...T..................T.....................................................................

        +428                                                                                                 +517
bIFNT 1  GCCTGCCTGGGCCCAGTGATGGGAGAGAAAGACTCTGACATGGGAAGGATGGGCCCCATTCTGACTGTGAAGAAGTACTTCCAGGGTATC
bIFNTc1  ...........G.AG.......A..............C.C.........................C.....................C...
bIFNTc2  ..............................................................................C...
bIFNTc3  ............................G.....................................................T.AC...

        +518                                                                                                 +607
bIFNT 1  CATGTCTACCTGAAAGAAAAAGAATACAGTGACTGCGCCTGGGAAATCATCAGAGTGGAGATGATGAGAGCCCTCTCTTCATCAACCACC
bIFNTc1  .....G.........A.G..G........C.....................................................G.
bIFNTc2  ...................................................A..............................
bIFNTc3  ...................G.....................................................................

        +608                                          +655
bIFNT 1  TTGCAAAAAAGGTTAAGAAAGATGGGTGGAGATCTGAACTCACTTTGA
bIFNTc1  ......G..............A.................TC....
bIFNTc2  ..............................................
bIFNTc3  ..............................................
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Fig. 2. Comparison of nucleotide sequence of bIFNT1 and related type bIFNT genes at ORF domain. Multiple alignments of all known members of the bovine IFNT subtype. Nucleotide sequence of the ORF region of the bovine IFNT subtype. Alignment of nucleotides represents the open-reading frame only. Nucleotide residues of various bIFNT1 and related type bIFNT genes differed from those of bIFNT1.

3. Examination of upstream regions by bIFNT subtype

The wild-type 5'-upstream sequences of bIFNT1, c1, c2, and c3 genes were examined for the degrees of transactivation using a transient transfection method into JEG cell and luciferase activities were measured (Fig. 4). In these data sets, similarly to its expression in vivo, the reporter plasmid of the upstream region of the bIFNT1, c2, and c3 genes exhibited high luciferase activity whereas the c1 gene had very low activity, less than of that expressed by bIFNT1, c2, and c3 (Fig. 4).

4. Effects of AP1, ETS2, CREBBP and/or CDX2 on bIFNT subtype genes transcription in JEG3 cell

To examine the responsiveness of bIFNT1, c1, c2, and c3-Luc constructs to transcription factor AP1 (JUN), ETS2, CREBBP and/or CDX2 the bIFNT1, c1, c2, and c3-Luc

```
          -23                          +1                          +27
bIFNT 1   MAFVLSLLMALVLVSYGPGRSLGCYLSEDHMLGARENLRLLARMNRLSPH
bIFNTc1   ..............S...........N............Q.....T.
bIFNTc2   ..................................................
bIFNTc3   ..................................................

          +28                                                   +77
bIFNT 1   PCLQDRKDFGLPQEMVEGNQLQKDQAISVLHEMLQQCLNLFYTEHSSAAW
bIFNTc1   S..........W.....D................F...H........
bIFNTc2   ...................................F............
bIFNTc3   ................S..................F...HI.......

          +78                                                  +127
bIFNT 1   NTTLLEQLCTGLQQQLEDLDACLGPVMGEKDSDMGRMGPILTVKKYFQGI
bIFNTc1   ...........H...D.......Q..E....AL..............
bIFNTc2   ..................................................
bIFNTc3   ..................................................HD.

          +128                                        +172
bIFNT 1   HVYLKEKEYSDCAWEIIRVEMMRALSSSTTLQKRLRKMGGDLNSL
bIFNTc1   .....K.....................S..E....I......S
bIFNTc2   ................M...........................
bIFNTc3   ..................................................
```

Fig. 3. Comparison of amino acid sequence of bIFNT1 and related type bIFNTc1, bIFNTc2, bIFNTc3 genes. Alignment of amino acid sequence represents the open-reading frame only. Amino acid sequence residues of various bIFNT1 and related type bIFNT differ from those of bIFNT1. Signal peptide sequences (aa-23 to -1) are italicized.

reporter plasmid with each or combination of transcription factor, was cotransfected into JEG3 cells, and luciferase activities were measured (Fig. 5). When JEG3 cells were cotransfected with the bIFNT1, c1, c2, and c3-Luc constructs and AP1(JUN), ETS2, CREBBP and/or CDX2 expression plasmid, Luc activity was enhanced approximately 6-fold more than twice of those cotransfected with bIFNT1, c1, c2, c3-Luc constructs and PSG5 empty plasmid (Con) (Fig. 5). However, expression patterns of these luciferase activities were differed among INFTs. The degree of transcriptional activation of the bIFNT1 gene was similar to that c2 and c3 genes by a combination of AP1 (JUN), ETS2, and/or CDX2 expression plasmid. Whereas bIFNTc1 gene was decreased almost 50% compared to that bIFNT1, c2, and c3 genes, CDX2 expression vector along with bIFNTc1-Luc could not increase the transcriptional activaty. Although, CDX2 expression vector decreased of the luciferase activaty of the bIFNTcl－Luc, cotransfected with the bIFNTc1-Luc construct and AP1 (JUN) or/and ETS2 expression plasmid, Luc activity was enhanced approximately 3-fold more than the bIFNT1, c2, and c3-Luc. Furthermore, CDX2 factor had no effect on activity of bIFNTc1 gene than the bIFNT1, c2, and c3 genes in JEG3 cells.

5. Levels of expression of bIFNT subtype genes mRNA

The regions of these two genes are compared of the IFNT gene in Fig. 2. The open-reading frame (ORF) of 595 nucleotides coded. There are only 40 nucleotide differences between bIFNT1 and c1 sequences. For nucleotide sequences rep resenting three subtypes of nucleotides that had been

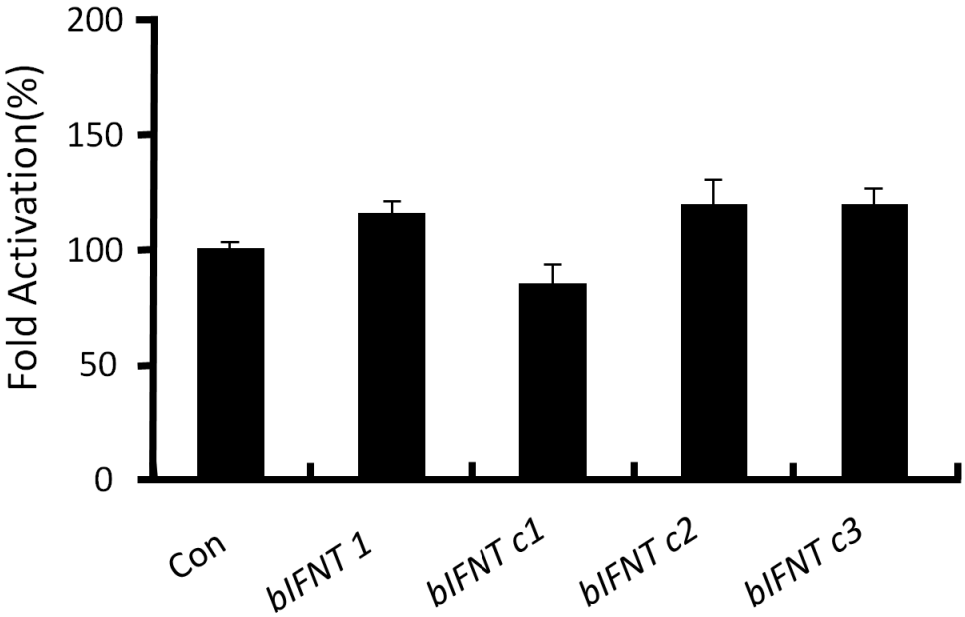

Fig. 4. Different activities of luciferase reporter gene with upstream region of the bIFNT1, bIFNTc1 and bIFNTc2 and bIFNTc3 in human choriocarcinoma JEG3 cells. Luciferase reporter plasmids (-1000-Luc) containing the upstream region (-1000 to +51) of the wild type bIFNT1 and bIFNT c1 and bIFNTc2 and bIFNTc3 genes were transfected into human choriocarcinoma (JEG3) cell. Data were expressed as relative luciferase activity to that of the bIFNT1-reporter and related type bIFNT c1 and bIFNTc2 and bIFNTc3-reporter constructs without any expression plasmid. Luciferase activities are expressed relative to that of the control (pGL3-Control vecter) and the data are shown as means±SEM with four independent replications.

designated as bIFNT1 and c1, similarity in DNA sequence between bIFNT1 and c1 are 93%, respectively (Table 1). For assay levels expression of of bIFNT1 and c1 mRNA in bovine day 17, 20, and 22 bovine trophoblasts, oligonucleotides identical sequence primer used for bIFNT1 and c1 mRNAs levels analysis (364bp; bIFNT1/c1-F; 5'-CAGAAAAGACTTTGGTCTTCC-3'; bIFNT1/c1-R; 5'-AGAGAGGGCTCTCATCATCTC-3') (Table 2) (Fig. 6A). Relative amounts of bIFNT1 and c1 mRNAs on day 17, 20, and 22 bovine trophoblasts. RNA extracted from frozen bovine conceptuses (n=3 each), was analyzed for the presence of these transcripts using RT-PCR. Agarose gel electrophoresis of the amplicon expected to reveal an amplification of a fragment of approximately 364 bp (Fig. 6B). bIFNT1 and c1 were found on day 17, 20, and 22 bovine conceptuses, however, expression patterns of these mRNA different (Fig. 6C). The size of the amplicon was further confirmed by the nucleotide sequencing. A total of thirty prospective recombinant colonies that contained the 364 bp insert were sequenced. Sequence analyzed of bIFNT1 and related type c1 genes by *Escherichia coli* cells (n=3 each). In order to characterize the difference on the levels of expression of bIFNT1 and c1, the IFNT mRNA in the nucleotides region of bIFNT1 and c1, the IFNT were analyzed. The levels of expression of bIFNT1 was shown higher on day 17 and transcripts were shown gradually lower, weakly detectable on day 20 and 22. However, the other bIFNTc1 gene transcripts were expressed highly on day 20 and expressions of transcripts became weaker on day 17 and 22 bovine conceptuses (Fig. 6C).

Table 2. Oligonucleotides sequence used for *bIFNT*-ORF assay

Accession Gene Bank	Primer(5'-3') Forword and Reverse	Length(bp)
bIFNT1 (M60903)	F: cagaaaagactttggtcttcc	364
bIFNTc1 (AF238613)	R: agagagggctctcatcatctc	

*Locations of oligonucleotides are identical to those in *bIFNT1* and *bIFNTc1*-ORF domain.

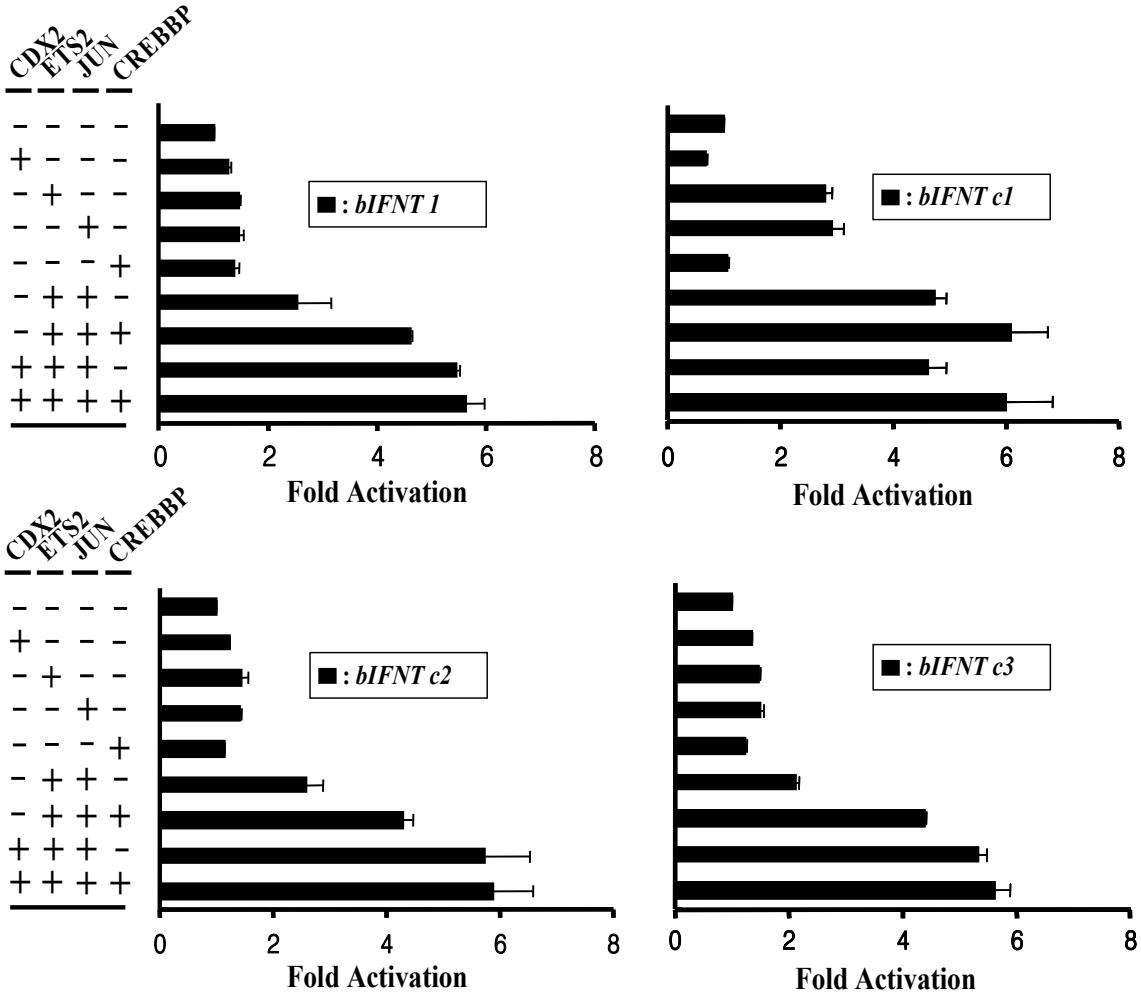

Fig. 5. Transativation assay of promoter/enhancer region of bIFNT1, bIFNT c1, bIFNTc2 and bIFNTc3 genes. Relationships of upstream of respective genes on the CDX2, ETS2, AP1 (JUN) and/or co-activator CREBBP were tested by the expression level of luciferase. Luciferase-reporter plasmids (-1000-Luc) containing the upstream region (-1000 to +51) were co-transfected into JEG3 cell with combination of CDX2, ETS2, JUN and/or CREBBP. The data are shown as means±SEM with four independent replications.

DISCUSSION

The data from the present study demonstrate that bIFNT is encoded by multiple, distinct genes in bovine. At present, there are 4 novel sequences that we have been working to identify different bIFNT sequences and expression levels. To date, none of the four full-length bIFNT genes isolated and three genes have the same nucleotide sequence (GenBank Database). Such multiplicity is also found among other type 1 IFN, such as the IFNA but the latter is involved in protecting host cells from viral pathogens and might be expected to be evolving at a high rate (Robers et al., 1997). The functional significance of multiple genes and proteins for bIFNT remains to be elucidated. As proposed by this study, multiple genes may produce slightly different products that differ in biological activity and possibly play different roles at the level of the feto-maternal interface. There are well-established differences in functional properties among other proteins also produced in large amounts and encoded by multiple genes, such as actin and human IFNA (Fyrberg et al., 1980; Cleveland et al., 1980; Cheetham et al., 1990).

On the basis of phylogenetic analysis (Fig. 1 and Table 1), the sequences fall into main groups. Group 5 appears to be the most ancient, but overall similarities are high. It is generally

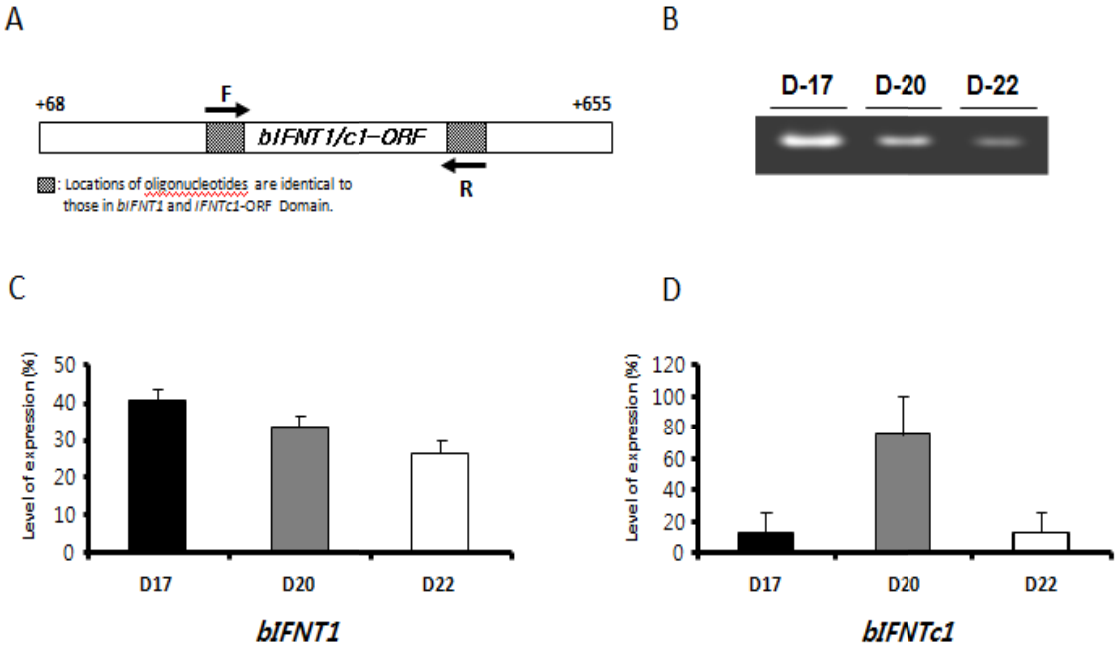

Fig. 6. Relative mRNA expression patterns of bIFNT1 and bIFNTc1 in bovine conceptuses. A: Oligonucleotides identical sequence primer used for bIFNT1 and bIFNTc1 mRNAs levels assay. Locations of oligonucleotides are identical to those in bIFNT1 and bIFNT c1-ORF domain regions. B: Relative amounts of bIFNT1 mRNAs in on day 17, 20, and 22 bovine trophoblasts. RNAs extracted from frozen bovine conceptuses were analyzed for the presence of these transcripts using RT-PCR. C and D: Changes in bIFNT1 and related type bIFNTc1 genes mRNAs levels were determined in mRNAs isolated from on day 17, 20 and 22 conceptuses with three independent replications.

accepted that the ancestors of the bovine (cattle and their relatives) and caprinae (sheep and goats) diverged about 20 million years ago (Miyamoto et al., 1993). This time is, however, long compared with the years since first domestication of cattle >10,000 yr ago (Diamond et al., 1997) and particularly during the short period (-200 yr) that selection was being used to develop the modern dairy and beef breeds. The bIFNT genes are believed to have evolved from a common ancestral IFNW by a duplication event that occurred 36 million years ago and maintained among the ungulate ruminants without any change in the size of the gene (Roberts et al., 1997). Like its progenitor IFNW, the 585bp ORF of the IFNT gene encodes for a 195 amino acid pre-protein of which the first 23 residues is the signal peptide that is cleaved off to yield a mature protein of 172 amino acids (Roberts et al., 1997). Therefore, even though the expressed bIFNT represent a rapidly evolving group of genes, it is likely that the majority of them are represented in all bos taurus breeds. Their uniformity contrasts with the diversity of the ovine IFNT, which differ as much as 10% in nucleotide sequence (Ealy et al., 1998). It will be of interest to determine whether, in cattle, a more ancient set of pseudogenes exists that can form the link to the IFNT of sheep.

Nuclease protection was used to estimate the relative amounts of different classes of transcript in pooled samples of RNA during the period of bIFNT genes expression (Ealy et al., 2001). The data confirm observations of others that the zenith of bIFNT production occurs around on day 17 (Bartol et al., 1985; Helmer et al., 1988; Farin et al., 1990). They also indicate that different genes, or at least different classes of genes, are most likely transcribed at comparable rates during the period between on day 14 and 25. Such differences are consistent with observations that multiple, distinct bIFNT1 cDNA have also been isolated, that in vitro translation of bovine conceptus mRNA yielded several isoelectric variants and that several isoforms of bIFNT genes were identified by amino acid sequencing. At least two of the genes isolated in the current study appear to encode isoforms of bIFNT secreted by the bovine conceptus. Coexpression of IFN-alpha and IFN-omega subtype mRNA in ovine and bovine conceptus has been demonstrated (Farin et al., 1991; Stewart et al., 1990; Cross et al., 1991).

It could be biologically advantageous to activate bIFNT genes, perhaps at different times during early pregnancy, in order to achieve the magnitude of protein production necessary for maternal recognition of pregnancy. Transcripts for bIFNT1 predominated, compared to those for bIFNTc1, during the period of maternal recognition of pregnancy (day 17-22). Coexpression of the bIFNT1 with c1 mRNA was evident (day 20), bIFNT1 was highly expressed on day 17 and transcripts were gradually weakly detectable on day 20 and 22. However, the transcrips of other bIFNTc1 gene was highly expressed on day 20 and transcripts were weakly detectable on day 17 and 21 bovine conceptuses. Collectively, perhaps more interestingly, a conceptus specific and temporal pattern of gene expression was observed in this study. More specifically, there appears to be a transition from trophoblastic and bIFNT1 and c1 genes activation.

The observed patterns of transcripts further suggested that control of activation of the bIFNTc1 gene is a few difference from that of bIFNT1 or c2 or c3 (Fig. 2, 3), but the functional significance of bIFNT1 and c1 has yet to be identified. Differential expression of other types of bIFNTc1 has been reported by these data and could be attributed to subtle differences in the promoter regions. There are good evidences that show the molecular basis for differential transcription of bIFNT1 and c1 genes in the utero. In this study, the luciferase activity assay resulted from the upstream region of wild type bIFNT1 and c1 gene transfected to JEG3 cell reflected the expression levels of these genes in the utero. These results indicated that the expressions of bIFNT1 and c1 genes are regulated at transcriptional level and transient transfection analyses using JEG3 cell and also demonstrated that high and low expression levels from bIFNT1 and c1 genes, respectively, are probably due to a short, contiguous region of the promoter that is unique to the bIFNT1 and c1 genes.

In summary, we report the patterns of expression of mRNA corresponding to distinct bIFNT1 and related c1 genes. The temporal change in abundance of mRNA, coupled with the somewhat conceptus specific pattern of expression, suggests that bIFNT1 and c1 genes expression in bovine are developmentally regulated.

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