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Molecular characterization of IFN-T expressed in buffalo embryonic trophoblasts and expression of recombinant BuIFN-T1a2 and BuIFN-T8 isoforms in *E. coli*

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ABSTRACT

Interferon tau (IFN-T) acts as a signaling molecule for maternal recognition of pregnancy (MRP) in ruminants. Aim of the present study was to identify various Buffalo Interferon tau (BuIFN-T) transcripts in buffalo trophoblast, phylogenetic comparison of these sequences with known mRNA sequences of buffalo, bovine, caprine and ovine and to express and purify the recombinant BuIFN-T (rBuIFN-T) isoforms. Following RNA extraction from trophectodermal cells, RT-PCR was performed using *Ifn-t* gene specific primers. 13 distinct cDNA variants encoding eight different BuIFN-T proteins were identified. BuIFN-T1a2 and BuIFN-T8 were expressed in prokaryotic expression system at 37 °C, 25 °C and 16 °C with 1 mM IPTG for 12 h and the recombinant proteins expressed at 16 °C were partially purified by Immobilised Metal Affinity Chromatography (IMAC). BuIFN-T isoforms have greater nucleotide and amino acid homology with caprine (98–100%, 96–100%), ovine (94–97%, 90–95%) and bovine (89.6–90.6%, 82–86%). These novel BuIFN-T isoforms contained pronounced nucleotide and amino acid sequence identity with one another (99.1–99.8%, 98–99%) but moderate sequence identity with previously identified buffalo IFN-T (90–92%, 82–86%). Solubility of expressed recombinant isoforms (rBuIFN-T1a2 and rBuIFN-T8) was highest at 16 °C. In conclusion, 13 distinct *Ifn-t* gene variants exist in trophectoderm of *in vitro* developed buffalo blastocysts that encode eight different proteins. rBuIFN-T1a2 and rBuIFN-T8 were successfully expressed in soluble form in *Escherichia coli* expression system at 16 °C with 1 mM IPTG and the resulting recombinant proteins were partially purified by IMAC.

1. Introduction

MRP and subsequent establishment of embryo in uterus are two vital events for fruitful pregnancy. This needs maintenance of corpus luteum (CL) beyond normal estrous cycle which depends on signals received from the developing embryo. Interferon tau (IFN-T) is one such signaling molecule which is synthesized in trophectodermal cells of blastocyst and then secreted to act on uterine epithelial cells for further signaling resulting into diverse physiological actions like implantation, maternal recognition of pregnancy, prevention of immune rejection etc.

IFN-T was initially termed as trophoblastin [1] or trophoblast protein-1 [2,3]. The first report of IFN-T came in ovine [1] then it was discovered in almost all the ungulate species like cattle, goat, buffalo, red deer etc. [4,5]. IFN-T is exclusively expressed in trophectodermal cells of blastocyst and its expression is temporal till the implantation of blastocyst. *Ifn-t* belongs to Type I IFNs [6] and are encoded by multiple genes [7]. Conceptus-derived IFNs are structurally distinct from other Type I IFNs though they possess many activities of Type I IFNs, such as antiviral, immunomodulatory and antiproliferative capabilities. They are collectively referred as IFN-T [8]. Unlike most Type I IFNs, IFN-T expression is not induced by viral or bacterial pathogens [9] and is expressed constitutively by the trophectoderm of blastocyst till the attachment of elongated conceptus to uterine wall [10–13].

IFN-T inhibits regression of CL by suppressing endometrial prostaglandin F2α (PGF2α) release [14,15]. IFN-T prevents oxytocin receptor expression in endometrial epithelium, thereby preventing

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oxytocin from stimulating synthesis and release of PGF2α [16]. It has been estimated that there may be as many as 18 *Ifn-t* genes in cattle, all of them clustered within or in close proximity to the genetic locus of Type I *Ifn* genes [17]. Presently, 18 distinct polymorphic ovine and 18 bovine alleles have been identified [18]. Multiple ovine and bovine *Ifnt* genes are transcribed during early pregnancy which encodes proteins that can possess different biological activities [7,11].

In buffalo, there is report of only one isoform of *Ifn-t* (ACCESSION NO: AY535404). The present work has been done with the objective to identify various *Ifn-t* transcripts in the trophectodermal cells of *in vitro* cultured buffalo blastocyst, to compare these nucleotide sequences phylogenetically with the reported mRNA sequences of buffalo, cattle, sheep and goat, to know the relatively predominant isoform expressed at mRNA level and to clone and express it.

2. Material and methods

2.1. In vitro embryo production

Buffalo ovaries collection, oocyte aspiration, *in vitro* maturation, *in vitro* fertilisation and *in vitro* embryo production were performed as described earlier [19,20].

2.2. Isolation of primary trophectodermal cells from hatched blastocysts

Each of the hatched blastocysts were seeded separately on 4 well plate (Nunc, Denmark) containing standard culture medium (DMEM/F12 supplemented with 10% FBS, 50 μg/ml gentamycin) at 38.5 °C under 5% CO_2. The spent medium was replaced with fresh cultured medium in an interval of 48 h till sufficient outgrowths (10 days) from hatched blastocyst were seen. Inner cell mass was removed mechanically and the outgrowths of the trophectodermal cells were collected by trypsinisation, 0.25% trypsin for 3 min at room temperature.

2.3. RNA extraction, RT-PCR, cDNA cloning and sequence analysis

In order to avoid the variation in the gene in population, the trophoblast outgrowths of single hatched blastocysts were used in the whole experiment. Total cellular (tc) RNA was extracted from trophectodermal tissue using the RNeasy mini kit (Qiagen Corp., Carlsbad, CA) according to the manufacturer's instructions. RNA preparations were treated with RNase-free DNase enzyme (Qiagen Corp., Madison, WI) for 1–2 min at room temp to remove genomic DNA contamination.

2.4. Primer designing

Ifn-t sequences of different isoforms in cattle were retrieved from NCBI Genbank (Accession No: AF238611.1, AF238612.1, AF238613.1) and a consensus sequence was determined using clustalW multiple alignment program of DNAstar. The consensus sequence was used to design primers by Primer3 software (primer3.ut.ee/) to amplify full length *Ifn-t* in buffalo.

2.5. Synthesis of cDNA, PCR and sequence analysis

Two micrograms of tcRNA was incubated at 65 °C for 5 min, then reverse transcribed with M-MuLV reverse transcriptase (#K1621, Fermentas Corp, USA), oligo(dT) primer, and 10 mM each of dNTP mix at 42 °C for 60 min. PCR amplification of *Ifn-t* genes were performed with high fidelity dream taq DNA Polymerase (Fermentas Corp, USA) and *Ifn-t* specific primers in 20 μl reaction volume. Briefly, reaction mix contained 10 μl dream taq™ green PCR master mix (Fermentas Corp, USA), 8 μl NFW, 0.5 μl of each primer and 1 μl cDNA. The cyclic conditions used for PCR were: initial denaturation at 94 °C for 3 min; 35 cycles of - 94 °C for 30 s; 62 °C for 30s and 72 °C for 1 min; and final extension at 72 °C for 10 min. The sequence of primers used in this study was- forward primer: 5′-AACCTACCTGAAGGTTCACCCAGA-3′ and reverse primer: 5′-TGAGTGTACGAAGGTGATGTGGCA-3′. PCR product was purified on 1.5% agarose and ligated into the pJET1.2/blunt cloning vector (Fermentas Corp. USA) and transformed in TOP10 competent *E. coli* cells according to the manufacturer's instructions (Invitrogen Inc., Carlsbad, CA, USA) and plated on Luria Bertani Agar (LB) plates containing 50 μg/ml ampicillin. Bacterial colonies were picked and propagated in 5 ml Luria Broth containing ampicillin at 37 °C overnight. Plasmid was isolated using the Nucleospin Plasmid Miniprep Kit (Genetix Biotech Asia, New Delhi). PCR amplification using IFN-T specific primers verified presence of Buffalo IFN-T gene. Total 30 clones were sequenced twice from two different labs (Eurofins MWG Operon, India; Xceleris genomics, Ahmedabad), in both directions using vector primers and sequences were compiled. The identified novel buffalo *Ifn-t* variants and the coding sequences of bovine, ovine and caprine *Ifn-t* variants listed in Genbank, were compared through multiple alignment using ClustalW, BioEdit version 7.09. For phylogenetic analysis, a consensus tree was constructed using MEGA version 4 (http://www.megasoftware.net/) through the Maximum Parsimony method [21].

2.6. Construction of prokaryotic expression vector of BuIFN-T

TOP10 cells harbouring isoforms BuIFNT1a2 and BuIFNT8 in pJET vector were verified, and PCR amplified, without signal sequence, using 5′-end primers having NcoI restriction sites (5′-atcgCCATGGTGTTACCTATCTCGGAGACTCATG-3′) and 3′-end primer having XhoI restriction site (5′-atcgCTCGAGAGGTGAGTTCA-GATTTCCACCCAT-3′). The cyclic conditions used for PCR amplification were same as described earlier. Amplicons were cloned in pET-28b vector after double restriction digestion and transformed in TOP10 cells. Recombinant colonies having correct ORF were used to transform BL21 (DE3) *E. coli* cells.

2.7. Expression of recombinant buffalo IFN-T (rBuIFN-T) isoforms

The colonies containing recombinant plasmid pET-28b-BuIFN-T (the positive clones) were grown to 0.6 OD at 600 nm and were initially induced with 1 mM IPTG at 37 °C for 4 h. To improve solubility, expression was tried at lower temperature- 25 °C and 16 °C for overnight and 22 h, respectively with 0.5 mM IPTG. Soluble proteins from respective cell lysates were extracted using Q proteome Bacterial Protein prep kit (Qiagen, USA) according to manufacturer's instruction. The soluble protein and insoluble fraction from pellet were subjected to SDS-PAGE and western blot to analyze expression of recombinant proteins.

2.8. Purification of rBuIFN-T isoforms using His-tag affinity column

For purification of soluble rBuIFN-T1a2 and rBuIFN-T8, single colony of BL-21 DE3 cells harbouring BuIFN-T1-pET28b + & BuIFN-T8-pET28b + expression constructs were cultured in 2 L LB broth for 12 h at 16 °C with 50 μg/ml ampicillin. Cells were harvested at 6000 g for 20 min and resuspended in His binding buffer (0.3 M NaCl, 10 mM imidazole in 50 mM phosphate buffer) and sonicated (conditions- 40 amplitude, 5 s pulse, 5 s on/off for 30 min). Soluble and insoluble fractions were then separated at 15,000 g for 30 min at 4 °C and the cleared lysates were used for purification. The supernatant of each recombinant protein, was loaded into 1 ml