A cell-based interferon-tau assay with an interferon-stimulated gene 15 promoter

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ABSTRACT

Interferon-tau (IFNT), a type I IFN expressed in ruminant trophoectoderms, is a known early pregnancy recognition signal (3, 25, 26) and exerts antiviral activity (6, 23). Type I IFNs stimulate the expression of interferon-stimulated genes (ISGs), including interferon-stimulated gene 15 ubiquitin-like modifier (ISG15), MX dynamin-like GTPase 1 (MX1), MX2, and 2′,5′-oligoadenylate synthetase 1 (OAS1), in various cells, such as endometrial, luteal, and peripheral blood cells. Several studies have indicated that the expression of ISGs in bovine leukocytes is increased in early gestation (12, 13, 17, 19). These observations suggest that IFNT is present in the blood stream around the implantation period in cattle, although the concentration is very low.

Promoter assays are regularly used for gene function analysis and the comprehension of gene regulation mechanisms. This system is invaluable for gene analysis, as it can be extremely specific to individual genes. A suitable combination of cells and promoter assay systems may also be applicable to biological functions. The application of promoter assays for the detection of IFN has been examined and suggested to be accurate (4, 5, 11, 18, 20, 28). However, an accurate and convenient detection system is still desired for the diagnosis of gestation status in cows. Furthermore, a cell-based assay system requires the establishment of cells transduced with stable promoter and reporter genes, so no convenient and stable method that complies with the regulations for genetic modification is currently available.

In this study, we first examined the expression and responsiveness of ISGs for IFNT in Madin-Darby...
systems, Foster City, CA, USA) in accordance with the manufacturer’s instructions. The gene expression levels were analyzed by RT-qPCR using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), as described previously (17). The primer pairs used for qRT-PCR are listed in Table 1. The expression levels of ISGs were estimated relative to the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used for the normalization of expression levels between samples.

In silico analysis of transcription factor binding sites. The sequence data of the upstream region of the ISG15 gene were obtained from the NCBI genome database, Bos taurus UMD_3.1.1. The region between 1150 bp before and 50 bp after the ISG15 transcription start site was analyzed to predict the transcription factor binding sites. The IFN-stimulated response elements (ISRE; 5′-CAGTTTCWC TTYYYCC-3′) or nuclear factor kappa B (NF-κB; 5′-GGGAMTTYCC-3′) binding site candidates were computed using the TFBIND program (http://tfbind.hgc.jp) with appropriate thresholds (ISRE: > 0.7; NF-κB binding site: > 0.85). The IFN-gamma (IFNG) activation site (GAS) candidates were directly detected using a consensus sequence (5′-TTNCNNNAA-3′).

Plasmid construction. Genomic DNA was isolated from bovine whole blood using the QIAamp DNA.
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−1150), −550 to +50 (ISG15 −550), −400 to +50 (ISG15 −400), −200 to +50 (ISG15 −200), and −180 to +50 (ISG15 −180), of the ISG15 gene transcription starting point were amplified by PCR using the primers listed in Table 2 and then inserted into the pGL4.10 vector (Promega, Madison, WI, USA) with the In-Fusion HD Cloning Kit (Takara Bio Inc., Kusatsu, Shiga, Japan), which resulted in the construction of ISG15 promoter-reporter vector plasmids (Fig. 1A). These constructs were used in the transient promoter assay for ISG15. The PiggyBac vector (PB-CMV-MCS-EF1-GreenPuro cDNA Cloning and Expression Vector: PB513B-1; System Biosciences, Inc., Palo Alto, CA, USA) was modified as follows. First, the EF1 promoter, green fluorescent protein (GFP), and puromycin-resistance gene sequence were deleted and then the minimum promoter and firefly luciferase (Luc) sequence from pGL4.23 (Promega) were inserted. Then, the sequence of positions −1150 to +50 of ISG15 gene was inserted into the vector to construct PB-ISG-Luc (Fig. 1B). The constructed plasmids were confirmed by restriction enzyme reactions and nucleotide sequencing.

**Transfection.** Transient and stable transfections were performed using an electroporator (ECM 2001; BTX Molecular Delivery Systems, Holliston, MA, USA) or Gene Pulser MXcell (Bio-Rad Laboratory, Hercules, CA, USA). In brief, MDBK cells were dispersed by the application of 0.25% trypsin and 50 mM EDTA solution (Sigma-Aldrich), and suspended at 2 × 10^7 cells/mL in HEPES-buffered saline (HBS) solution (21 mM HEPES (pH 7.05), 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM glucose) with constructs. For the electroporation, the following settings were used: 935 V/cm, 50 μF, and 720 Ω for the exponential pulse wave method and 935 V/cm, 2475 μF, and maximum resistance for the square pulse wave method. After electroporation, the cells were placed on ice for 5 min and then plated.

**Promoter assay.** MDBK cells were co-transfected with the ISG15 promoter-reporter vectors (1 μg/cuvette) and pRL-TK (Promega), which constitutively expresses Renilla luciferase as an internal control (1 μg/cuvette), and plated at a density of 1.0 × 10^5 cells/well into 96-well cell culture plates. After incubation for 24 h, the cells were incubated with the medium that contained recombinant bovine IFNT, IFN-alpha (IFNA) (Kingfisher Biotech, Inc., Saint Paul, MN, USA) or IFNG (Kingfisher Biotech Inc.) for 16 h, and measured for luciferase activity using the Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions. The genomic DNA was then used as the template for the amplification of the bovine ISG15 gene. In short, the upstream regions, including positions −1150 to +50 (ISG15 −1150), −550 to +50 (ISG15 −550), −400 to +50 (ISG15 −400), −200 to +50 (ISG15 −200), and −180 to +50 (ISG15 −180), of the ISG15 gene transcription starting point were amplified by PCR using the primers listed in Table 2 and then inserted into the pGL4.10 vector (Promega, Madison, WI, USA) with the In-Fusion HD Cloning Kit (Takara Bio Inc., Kusatsu, Shiga, Japan), which resulted in the construction of ISG15 promoter-reporter vector plasmids (Fig. 1A). These constructs were used in the transient promoter assay for ISG15. The PiggyBac vector (PB-CMV-MCS-EF1-GreenPuro cDNA Cloning and Expression Vector: PB513B-1; System Biosciences, Inc., Palo Alto, CA, USA) was modified as follows. First, the EF1 promoter, green fluorescent protein (GFP), and puromycin-resistance gene sequence were deleted and then the minimum promoter and firefly luciferase (Luc) sequence from pGL4.23 (Promega) were inserted. Then, the sequence of positions −1150 to +50 of ISG15 gene was inserted into the vector to construct PB-ISG-Luc (Fig. 1B). The constructed plasmids were confirmed by restriction enzyme reactions and nucleotide sequencing.

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**Fig. 1** Schematics of transient and stable reporter vector plasmids. The detailed construction of the vector plasmid for transient transfection (A) and PB-ISG15-Luc plasmid for the generation of a stable cell line (B) are illustrated. Each sequence of the ISG15 upstream region (individually positions as indicated in A) was amplified and inserted into pGL4.10 for transient transfection, as described in Materials and Methods. In the DNA sequence represented as thin double lines, bold arrows indicate the protein coding sequence and bold lines indicate other functional sequences. Luc, luciferase 2 gene; MCS, multi-cloning site; miniP, minimum promoter; 5′ or 3′ TR, 5′ or 3′ PiggyBac Terminal Repeat; AmpR, Ampicillin resistance.

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the Dual-Luciferase Reporter Assay System (Promega) in accordance with the manufacturer’s instructions. The luciferase activities were normalized to Renilla luciferase activity for variations in transfection efficiency.

Establishment of ISG15 promoter stable-introduction cell line. MDBK cells were co-transfected with PB-ISG-Luc (1 μg/cuvette), Super PiggyBac Transposase Expression Vector (0.2 μg/cuvette; System Biosciences, Inc.), and PB513B-1 (0.2 μg/cuvette) using an electroporator. Transfected MDBK cells were sorted by fluorescence activated cell sorting (FACS) (MoFlo Astrios Beckman Coulter, Carlsbad, CA, USA) using the fluorescence intensity of GFP to select the stable cell line.

Validation of stable cell line reactivity to IFNs. MDBK cells with stably introduced ISG15 promoter-reporter were plated at a density of $4.0 \times 10^4$ cells/well into 96-well plates. After incubation for 24 h, the cells were incubated with medium containing recombinant bovine IFNT, IFNA, or IFNG for 16 h, and luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega) in accordance with the manufacturer’s instructions. The limit of detection of IFNT was defined, in accordance with the IUPAC recommendation (7), as the mean value of the untreated control cells plus 3.29 times the standard deviation.

RESULTS

Effect of recombinant IFNT on ISG mRNA expression in MDBK cells
To confirm the responsiveness of MDBK cells to IFNT, we examined the expression of four classical ISGs (ISG15, MX1, MX2, and OAS1) with IFNT (0–100 ng/mL) for 24 h (Fig. 2). IFNT stimulated the mRNA expression of ISG15, MX1, MX2, and OAS1 in a dose-dependent manner in MDBK cells. All four genes were increased by treatment with 1 ng/mL IFNT.

Response of ISG15 gene promoter activity to recombinant IFNs
Next, we searched for the transcription factor binding sites related to IFN signaling in the promoter region of the ISG15 gene and examined their promoter activities, because the ISG15 transcript was highly expressed and responsive in MDBK cells treated with IFNT, as shown in Fig. 2. In silico analysis indicated seven ISRE (start positions −870, −771, −697, −647, −539, −336, and −137), four GAS (start positions −937, −502, −481, and −363), and two NF-κB binding site (start positions −197 and −118) candidates within 1150 bp upstream of the transcription start point on bovine ISG15 promoter region (Fig. 3). When the MDBK cells transfected with various lengths of ISG15 promoter-reporter constructs were treated with IFNT, IFNA, and IFNG (500 ng/mL each) for 16 h, all five different lengths of upstream regions of ISG15 showed approximately two to seven-times responded activities on the expression of the reporter gene compared to those of control (Fig. 3). The promoter activities for IFNT and IFNA were approximately two to three times higher than the activity for IFNG. The deletion of positions −400 to −200 of the ISG15 upstream region significantly decreased the promoter activity for IFNT, whereby the deletion of positions −1150 bp to −400 bp and −200 to −180 did not affect the promoter activity. Although the promoter activity for IFNA was not significantly different to those of IFNT, the activities of IFNG were moderate in comparison, with only a significant difference in the “ISG15 −1150” and “ISG15 −200” promoter regions.

Establishment of an IFNT reactive cell line with PiggyBac vector system
The ISG15 −1150 promoter region, which displayed a sensitive reaction to IFNT, was inserted in the PiggyBac vector plasmid, and then this plasmid, PB-ISG15-Luc, and the GFP-reporter plasmid PB513B-1, were co-transfected into MDBK cells. The cells, termed MDBK-ISG15pro-Luc, were cultured for 7 days and sorted by GFP fluorescence using FACS (Fig. 4A and B). The cells showed a specific increase in reactivity with IFNs for 16 h in a dose-dependent manner (Fig. 5A). At 100 ng/mL, the reactivity to IFNT was the largest (approximately 9.5-time-fold), whereas the reactivity to IFNA was approximately half and IFNG was one sixth compared with that of IFNT. The limit of detection of the assay, which was defined as the value 3.29 times the standard deviation from the untreated control, was 1.20-fold relative luciferase units (equivalent to approximately 0.4 ng/mL IFNT) (Fig. 5B).

DISCUSSION

ISG15 is a representative ISG that has been shown to respond to both IFNT and other type I IFNs in cattle (1, 14–16, 24). It has been reported that the IFNT-stimulated increased gene expression of ISG15 may occur in endometrial epithelial cells and mater-
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We performed an in silico analysis of the transcription factor binding sites in the bovine ISG15 upstream region, and found seven ISRE, four GAS, and two NF-κB binding site candidates up to 1150 bp upstream from the transcriptional start point. These facts and the present results indicated that ISG15 is a highly sensitive gene to IFNT, in addition to MX1, MX2, or OAS1; therefore, we chose the ISG15 promoter as the reporter gene promoter candidate for IFNT. We performed an in silico analysis of the transcription factor binding sites in the bovine ISG15 upstream region, and found seven ISRE, four GAS, and two NF-κB binding site candidates up to 1150 bp upstream from the transcriptional start point.
more, we also examined the effect of IFNG on transcription of ISG15. IFNG is a type II IFN and activates the transcription of mRNA by GAS via the STAT1 homodimer by binding to IFNG receptor (8). The transcription activity of ISG15 stimulated by IFNG was slightly altered by the deletion of three GAS candidates, even though GAS is a cis-element of IFNG signaling. The transcription activity of ISG15 stimulated by IFNG was additionally explored because it has been suggested that NF-κB increases JAK-STAT signaling pathway-independent gene expression.

**Fig. 4** Generation of a stable cell line with an integrated ISG15 upstream-luciferase gene. (A) FACS profiles for isolation of a stable cell line. MDBK cells were stably transfected with PB-ISG15-Luc and PB513B-1 for the selection using the electroporator. The cells with strong GFP fluorescence (inside the gate; 0.25%) were collected and defined as a stable cell line (MDBK-ISG15pro-Luc). (B) GFP-positive cells collected using FACS.

**Fig. 5** Validation of stable cell line for IFN responsiveness. (A) Responsiveness of MDBK-ISG15pro-Luc cells to IFNs. The cells were treated with 0–100 ng/mL IFNT, IFNA, or IFNG for 16 h and analyzed for luciferase activity. The promoter activities are indicated relative to the activities of the untreated controls. The values are shown as the mean ± SE (n = 3). (B) The determination of the limit of detection of MDBK-ISG15pro-Luc cells. The cells were treated with 0–1 ng/mL IFNT and the luciferase activity was measured. The values are shown as the mean ± SE (n = 4). The dashed line indicates the limit of detection.
pression of ISG15 (9, 22). However, the deletion of a potential candidate NF-κB binding site caused no significant variation in the transcriptional activity of ISG15. These complex results implied the absence of a true transcription binding site that binds to the trans-element within the analyzed range; that is, the sites are located in a region further upstream.

Although cytopathic antiviral assays have been widely used for the quantification of the biological activity of IFNs, there are disadvantages; for example, the viruses used for antiviral assays have high virulence and infectivity and, consequently, the assay requires advanced biological containment and safety measures (2, 21, 27, 30). These complexities have limited the widespread use of antiviral assays. Although reporter gene assay systems to measure IFNs have been proposed over the past 20 years as alternative methods to antiviral assays (4, 5, 11, 18, 20, 28), these are not without complications, such as the use of viral vectors and the regulations of genetic modification. In the present study, we developed a novel cell-based IFN-τ bioassay system with ISG15 promoter-luciferase reporter genes. This assay system resolves the problems of the quantification of the biological activity of IFNs described above; in other words, no viruses are required to perform the assay. The limit of detection for IFNT in the present study was 0.4 ng/mL, which is equal to approximately 8 IU/mL. The value was comparable with that in a previous report (20). These observations indicate that, after further investigation, the assay may be suitable to replace the traditional plaque assay for IFNT. Moreover, the cell line was integrated with the reporter gene using the PiggyBac transposon system (10), which uses PiggyBac transposase for gene integration into the genome of the host cells; thus, even the production and maintenance of this cell line only utilized virus-free processes. This will allow laboratories to perform simple quantification of the biological activity of IFNT; potentially, the process may also be applicable for other IFNs, but further studies are required.

In conclusion, the present study describes the selection of the ISG15 promoter for a cell-based assay system. Our results also showed that the promoter assay of the upstream region of ISG15 indicated that an ISRE between −400 and −200 bp upstream of transcription start site of ISG15 may positively participate in the transcription of ISG15 gene. A novel cell-based IFN bioassay system, with the ISG15 promoter-luciferase gene in MDBK cells, was used to detect and quantify type I IFNs in a dose-dependent manner. Because of its convenience, this assay represents a novel alternative to the biological plaque assay for the quantification of IFNT and other type I IFNs.

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