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Molecular and Functional Characterization of Canine Interferon-Epsilon

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In this study, we provide the first comprehensive annotation of the entire family of canine interferons (IFNs). Canine IFN-ε (IFNE), IFN-κ (IFNK), and IFN-λ (IFNL) were discovered for the first time. Ten functional and 2 truncated IFN-α (IFNA) pseudogenes were found in the genome, which also enriched the existing knowledge about canine IFNA. The canine type I IFN genes are clustered on chromosome 11, and their relative arrangements are illustrated. To further investigate the biological activity of canine IFNE, it was expressed and purified in *Escherichia coli*. Recombinant canine IFNE (rCaIFN-ε) displayed potent antiviral activity on both homologous and heterologous animal cells *in vitro*, indicating that rCaIFN-ε has more broad cross-species activity than recombinant canine IFNA (rCaIFN-α). The antiviral activities of rCaIFN-ε and rCaIFN-α7 against different viruses on MDCK cells were also evaluated. The antiviral activities of recombinant canine IFNK and IFNL were demonstrated using a VSV-MDCK virus-target cell system. rCaIFN-ε exhibited a significant anti-proliferative response against A72 canine tumor cells and MDCK canine epithelial cells in a dose-dependent manner. rCaIFN-α7 was approximately 16-fold more potent than rCaIFN-ε in promoting natural killer cell cytotoxicity activity. Further, rCaIFN-ε can activate the JAK-STAT signaling pathway.

Introduction

Interferons (IFNs) are a heterogeneous group of pleiotropic cytokines consisting of 3 structurally unrelated types (I, II, and III). There is no significant sequence homology between the IFN types, and each family has a specific receptor. Type I IFNs include IFN-α (IFNA), IFN-β (IFNB), IFN-ω (IFNW), IFN-ε (IFNE), IFN-κ (IFNK), IFN-δ (IFND), IFN-ζ (IFNZ), and IFN-τ (IFNT). IFN Types II and III only include one member each, IFN-γ (IFNG) IFN-λ (IFNL), respectively. IFNL is also known as interleukin (IL)28 and IL29. IFNs evoke antiviral, anti-proliferative, antitumor, and immunomodulatory cellular responses. Although type I IFNs are central to prime innate immunity, some subfamilies, including IFND and IFNT, on the other hand, are not induced by viruses but instead are produced by the trophectoderm and are involved in maternal recognition during pregnancy, they appear to trigger responses in maternal uterine endometrium that allow the pregnancy to become established (Roberts and others 1999; Demmers and others 2001; Walker and Roberts 2009). IFND has been found in pig, sheep, and horse; while IFNT was only described in ruminants (Lefevre and others 1998; Cochet and others 2009). In addition, type I IFNs have diverse expression profiles, and their antiviral activities are virus- and cell dependent (Cheng and others 2006; Sang and others 2010b). Type I and type III IFNs have similar mechanisms of induction, activate the same signaling pathways, and trigger the same biological actions in target cells (Ank and others 2008). Type III IFNs have been retained in some species, including humans, mice, chickens, pigs, and cattle, but have been lost in others (Kotenko and others 2003; Walker and Roberts 2009; Sang and others 2010a; Diaz-San Segundo and others 2011). A functional type I receptor consists of 2 subunits, IFNAR1 and IFNAR2, both of which are in the class II cytokinereceptor family. Within a species, type I IFNs appear capable of binding to the receptor and competing with other type I IFNs for occupancy. Binding of IFN to receptor is the first step initiating the signal transduction cascade that activates the IFN biological function in cells (Walter 2004).

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Human IFNE was first identified in 1999 (Chen and others 1999), which has found to be constitutively expressed in the lung, brain, small intestine, and reproductive tissues (Hardy and others 2004; Peng and others 2007). Since then, mice, pigs, and cattle IFNE have been reported (Hardy and others 2004; Yulei and others 2008; Walker and Roberts 2009). Moreover, seminal plasma up-regulates IFNE expression in cervicovaginal tissues (Sharkey and others 2007). Therefore, IFNE is considered as playing a role in reproductive function, in either viral protection or early placental development in placental mammals (Hardy and others 2004; Xi and others 2012).

The research on canine IFNs is not systematic. To date, only 3 subtypes of canine IFN have been identified: IFNA, IFNB, and IFNG (belonging to types I and II) (Himmler and others 1987; Devos and others 1992; Zucker and others 1992; Iwata and others 1996). No member of the type III IFN family has previously been described in canines. A detailed annotation of the genomic loci containing the IFN gene cluster in canines is an important step toward elucidating the full spectrum of the type I IFNs and will provide further insights into their biological functions and pathological significance.

Here, based on a screen of the canine genome, we present a detailed description and annotation of the entire canine IFN repertoire, consisting of 15 functional genes and 2 IFNA pseudogenes, distributed on chromosomes 11, 10, and 24. We identified canine IFNE, IFNK (type I IFNs), and IFNL (type III IFN), which have never been reported. Several IFNA sequences, which were not described earlier, were also found. In addition, canine IFNE was cloned and expressed in *Escherichia coli*, and the recombinant protein demonstrated high antiviral activity, anti-proliferation activity, and Natural Killer (NK) cell stimulatory activity. Meanwhile, it was demonstrated to have the capability of activating the JAK-STAT pathway. These results indicate that canine IFNE behaves in a similar manner to orthologs in other species and has the potential to be developed as a novel protein pharmaceutical.

Materials and Methods

Bioinformatics and phylogenetic analysis of canine IFNs

To gain insight into the canine IFNs, the sequences of canine IFNA, IFNB, and IFNG from GenBank (Table 1) were used to perform a megaBLAST search of the canine genome database (Build 3.1) in the National Center of Biotechnology Information (http://blast.ncbi.nlm.nih.gov/). Human IFNE, human IFNW, human IFNK, human IFNL, porcine IFND, bovine IFNT, and murine IFNZ sequences (Table 1) were queried using a cross-species megaBLAST of the canine genome database, because no canine homologues for these genes have been reported. Subsequently, the newly discovered canine IFN (IFNE, IFNK, and IFNL) nucleotide sequences and the deduced amino-acid sequences were aligned with the corresponding GenBank IFN sequences from canines and other animals using ClustalW (version 1.83). A phylogenetic tree was constructed of the newly discovered canine IFN sequences and other publicly available representative IFN sequences using the MAGA program (version 5.0) and neighbor-joining analysis (Tamura and others 2011).

IFN gene isolation and construction of expression constructs

Canine epithelial cells (MDCK) stimulated with polyI:C were collected, and total RNA was isolated using TRIzol reagent (Invitrogen). Then, total RNA was used as a template for reverse transcription (RT) and PCR amplification using a one-step RT-PCR kit (Newpep). The canine IFNE primers were designed based on the obtained IFNE nucleotide sequence. The sense primer was 5′-ATGATTAACAAGCATTG CTTTG-3′, and the anti-sense primer was 5′-TTATTTGCTTA GATTTCTTATCAGT-3′. The amplification conditions were as follows: incubation at 42°C for 30 min and initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. A final extension at 72°C for 7 min was then performed. Amplified product (~560 bp) was purified from an agarose gel and sequenced using an ABI 310 Prism automated sequencer (PE Applied Biosystems). The IFNK, IFNL, and IFN-α7 cDNAs were also cloned according to our screened sequences and a previous report (Taira and others 2005). The DNA fragments encoding mature canine IFNE, IFN-α7, IFNK, and IFNL protein were subcloned into the prokaryotic expression vector pET-21a (Merck Millipore) and verified by sequencing.

Expression and purification of canine IFNs

The 4 resulting plasmids were transformed into Origami B(DE3) cells (Merck Millipore). Freshly isolated colonies were chosen and incubated overnight at 37°C in LB medium containing 100 μg/mL ampicillin. The overnight cultures were diluted 1:100 in fresh medium and incubated at 37°C to an OD_{600} of 0.6. Then, 0.2 mM isopropyl-b-d-thiogalactopyranoside (IPTG) was added to the culture, and the cells were incubated at 25°C for 8 h. Cells were collected and disrupted by sonication. The purification of the recombinant protein was performed using nickel-chelating Sepharose (GE Healthcare) according to the supplier's instructions. Then, the purified protein was fractionated by gel filtration on a Superdex-200 column (GE Healthcare) to exclude misfolded protein and exchange the buffer. The purity of the proteins was assessed by 12% SDS–PAGE and N-terminal sequencing. The protein concentration was estimated with standard BCA assays (Yang and others 2007).

Table 1. Query Sequences Used for the Genomic Searches

Species	*Gene*	*Accession No.*
Canine	IFNA	AB125934.1
Canine	IFNA	AB125936.1
Canine	IFNA	AB125937.1
Canine	IFNB	NM__001135787.1
Canine	IFNG	NM_001003174.1
Human	IFNE	AY190045.1
Human	IFNW	X58822.1
Human	IFNK	AF315688.1
Human	IFNL	AY184372
Porcine	IFND	GQ415074.1
Bovine	IFNT	AF238611.1
Murine	IFNZ	NM_197889

In vitro *viral inhibition assay*

The antiviral activities of rCaIFN-ε and rCaIFN-α7 were determined by the cytopathic effect inhibition assay using Vesicular stomatitis New Jersey virus (VSV) on MDCK (canine), MDBK (bovine), CRFK (feline), and WISH (human) cells according to previously described protocols (Armstrong 1971). In addition, the antiviral activities against canine parvovirus (CPV), canine distemper virus (CDV), and influenza virus A/WSN/33 (H1N1) were measured in MDCK cells via the same method (Taira and others 2005). According to a previous report (Taira and others 2005), rCaIFN-α7 was more potent than other alpha subtypes of CaIFN in antiviral activity; so, we selected it as a positive control to compare it with rCaIFN-ε. To confirm that canine IFNK and IFNL are functional proteins, their antiviral activities in the VSV-MDCK system were also assessed. All antiviral assays were repeated thrice. Antiviral activity was calculated by the Reed–Muench method (Reed and Muench 1938), with 1 U as the highest dilution that reduced cell number by 50%.

Assessment of anti-proliferation activity in canine cell lines

The anti-proliferation activity of rCaIFN-ε and rCaIFN-α7, which were produced in Origami B(DE3) cells, in MDCK cells and the A72 canine tumor cell line was determined as previously described (Loveland and others 1992; Tsang and others 2007). MDCK and A72 cells were cultured in 96-well plates (5×10^3/well) with DMEM containing 10% bovine serum. A dilution series of rCaIFN-ε or rCaIFN-α7 was then added. After a 72-h treatment, 20 μL MTT solution (5 mg/mL) was added to each well and mixed. The cells were further incubated for 4 h. Then, the media was removed by aspiration, dimethyl sulfoxide was added to each well to elute the dye, and the color intensity at 490 nm was measured using an ELISA plate reader. The data are reported as the percentage of growth, which was calculated as (OD of sample/OD of mock)×100%.

Ability to promote NK cytotoxicity activity

Heparinized peripheral blood was collected from clinically healthy adult beagle dogs, and peripheral blood lymphocytes (PBLs) were isolated by Ficoll–Hypaque gradient separation according to a previously reported method (Timonen and Saksela 1980; Vissers and others 1988). The isolated PBL concentration was adjusted to 10^6/mL using RPMI 1640 medium supplemented with 10% fetal calf serum. The ability of IFNs to promote NK cytotoxicity was evaluated using a reported protocol but with some modifications (Krakowka 1983; Knapp and others 1993; Gondolf and others 1996; Peng and others 2007). Lymphocytes (effector cells) were treated with 4-fold serially diluted rCaIFN-α7 or rCaIFN-ε, which were produced in Origami B(DE3) cells, for 6 h and then mixed with NK-susceptible canine thyroid adenocarcinoma (CTAC) cells at a ratio of 80:1. All tests were performed in triplicate. After incubation for 15 h at 37°C, supernatants were obtained by centrifugation at 400 *g* for 6 min. The level of lactate dehydrogenase in the supernatants was measured via the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega), which represents the activity of NK cytotoxicity. Spontaneous release from the target cells was determined in wells without effector cells, whereas maximal release was achieved by lysis of target cells with 1 M HCl. The percentage of NK cell cytotoxicity was calculated as (Experimental release - Spontaneous release)/(Maximal release - Spontaneous release)×100%.

Analysis of activate JAK-STAT pathway ability

To analyze the ability of rCaIFN-ε to activate the JAK-STAT pathway, an IFN-stimulated response element (ISRE)-luciferase assay was performed as previously described but with some modifications (Schindler and Darnell 1995; Zhao and others 2012). MDCK cell was cultured in 24-well plates for 12 h and then transfected with 250 ng of ISRE-luc plasmids containing 50 ng of the reference Renilla luciferase reporter vector pRL-TK (Promega) using Lipofectamine 2000 (Invitrogen). The cells were further incubated for 24 h, and then, 10U rCaIFN-ε or rCaIFN-α7 (titrated with VSV-MDCK system), which were produced in Origami B(DE3) cells, were added to each well and mixed. After incubation for 6 h, the cells were lysed in Reporter Lysis Buffer (Promega), and luciferase activity was measured. All tests were performed in triplicate. The results are indicated as the relative luciferase activity presented as the ratio of the reporter plasmid and the reference Renilla luciferase reporter plasmid.

Results

Identification of IFN genes in the canine genome

The canine IFNE and IFNK subtype nucleic acid sequences are located on chromosome 11 of the canine genome assembly 3.1 (GenBank: NW_003726080.1). Similar to other vertebrate type I IFNs, IFNE and IFNK are intronless. The canine IFNL sequence was discovered on chromosome 24 of the canine genome (GenBank: NW_003726101.1), specifically located on a scaffold from 36,440,345 to 36,440,917 bp. The canine IFNG sequence was identified as only a single copy on chromosome 10 of the canine genome (GenBank: NW_003726077.1), specifically located on a scaffold from 10,407,391 to 10,411,501 bp. IFNE, IFNK, and IFNL are present as single genes with intact ORFs and are assumed to be functional, providing the first evidence that they are present in canines. The IFNE, IFNK, and IFNL sequences are available at GenBank™ as accessions KC527684, KC754971, and KC754970. Ten functional IFNAs and 2 truncated IFNAs were also found in the canine genome, which form an IFNA gene cluster on chromosome 11 (from 31,671,841 to 31,808,293 bp). The number of functional IFNAs is more than previously reported for canines (Himmler and others 1987; Taira and others 2005). IFNW, IFND, IFNT, and IFNZ were absent in the canine genome database, consistent with previous reports (Lefevre and others 1998; Oritani and others 2000; Hardy and others 2004; Walker and Roberts 2009).

Canine IFNE shares more than 70% identity with human and porcine IFNE at the nucleic-acid and amino-acid levels but only 56.7% nucleotide identity with mouse IFNE. A comparison of IFNE to the previously reported canine IFN-α7 (AY117391), IFN-β1 (AB021707), and IFN-γ (D30619) sequences revealed homologies of approximately 29.2%, 33.5%, and 10.8%, respectively (Table. 2). A phylogenetic tree was constructed, showing that canine IFNE, IFNK, and IFNL belong to their respective IFN clusters (Fig. 1).

Table 2. Homology (%) of Amino Acids (Top Line) and Nucleotides (Bottom Line) Among Each Canine Interferon Subtype and Other Reported Mammalian IFNEs

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	
1	***	99.5	100	93.6	99.5	27.9	29.2	25.7	10.9	10.8	29.4	24.6	29.4	CaIFN-α4
2	99.8	***	99.5	93	98.9	27.9	29.2	25.7	10.9	10.8	29.4	24.6	29.4	CaIFN-α5
3	99.8	99.6	***	93.6	99.5	27.9	29.2	25.7	10.9	10.8	29.4	24.6	29.4	CaIFN-α6
4	96.5	96.3	96.3	***	93	26.8	29.2	26.7	10.9	10.2	29.4	24.6	29.9	CaIFN-α7
5	99.8	99.6	99.6	96.3	***	27.3	28.6	25.7	10.9	11.4	28.9	24.1	28.9	CaIFN-α8
6	52.5	52.3	52.3	51.9	52.3	***	33.5	26.9	12.2	9.8	36	31.4	36	CaIFN-β1
7	49.7	49.5	49.5	49	49.5	49.8	***	31.6	13.3	10.8	71.7	56.7	75.4	CaIFN-ε
8	47.5	47.3	47.7	48.8	47.3	45.9	51.2	***	9.8	10.8	31.6	25.4	30	CaIFN-κ
9	37.2	37.2	37.4	38	37.1	36.2	37.8	34.6	***	4.9	13.3	10.2	11.2	CaIFN-λ
10	34	34	34.2	32.7	33.8	38.6	38.2	33.9	33.2	***	11.4	10.8	10.8	CaIFN-γ
11	49.6	49.4	49.4	48.7	49.4	51.2	83	50.8	37.6	39.8	***	58.3	76.2	HuIFN-ε
12	44.8	44.6	45	44.8	44.6	48.2	70.7	46.1	35.4	36.9	72.1	***	55.7	MuIFN-ε
13	49.2	49.4	49.4	48.7	49	52.4	84.9	50.8	36.8	38.6	84.5	70.3	***	PoIFN-ε

***Same sequence.

In most species, the IFNE and IFNB genes are located on the 2 extremities of type I IFN gene clusters (with the exception of IFNK), while the other type I IFN genes are randomly located relative to one another. Similar to most species, the canine IFNB and IFNE genes define the outer limits of the locus, with all of the IFNA genes distributed between these 2 markers. The IFNK gene is located 4.76 Mb from this gene cluster, similar to other mammals (LaFleur and others 2001; Walker and Roberts 2009). The IFNB and IFNE genes are transcribed in the same direction, while IFNK is transcribed in the opposite direction. The relative arrangement of the canine IFN locus is illustrated in Fig. 2.

Cloning and expression of canine IFNs in E. coli

The canine IFNE, IFN-α7, IFNK, and IFNL genes were obtained by reverse transcription and PCR from RNA isolated from canine cells, and prokaryotic expression plasmids were constructed. The plasmids were transformed into *E. coli* Origami B(DE3) cells, and the transformants were induced to express IFNs in a highly soluble form. The

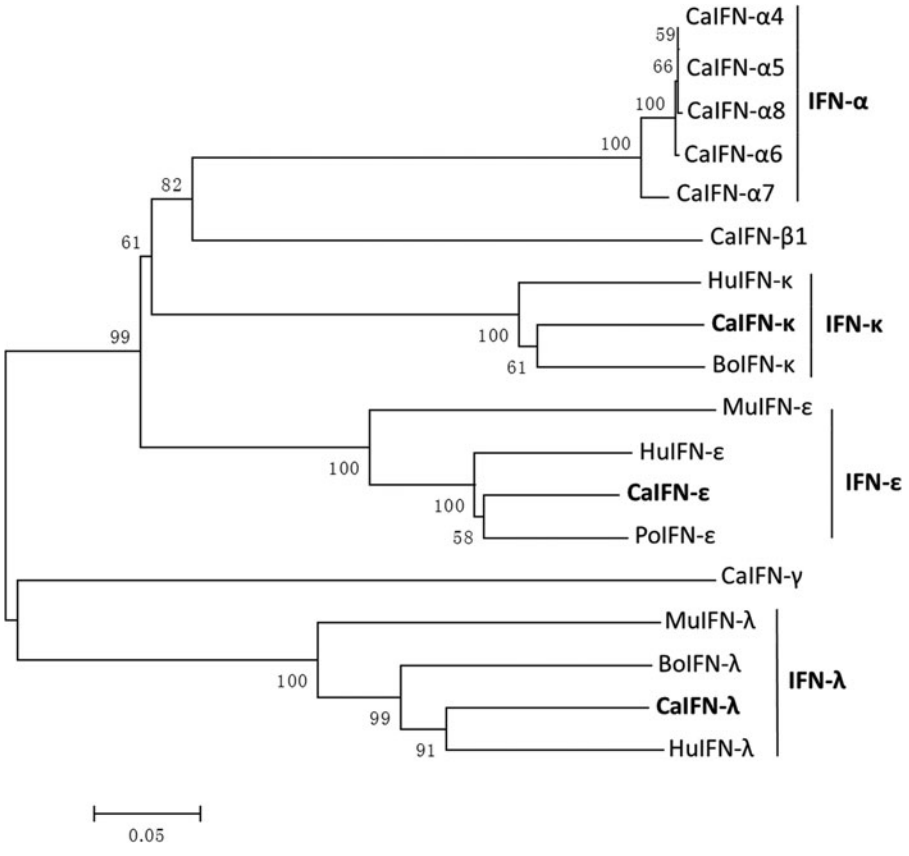

FIG. 1. Phylogenetic analysis of interferons (IFNs) in canines and other species. Groups of sequences are indicated to demonstrate the divergent pattern of IFNs. The newly identified CaIFN-ε, CaIFN-κ, and CaIFN-λ belong to their respective IFNs clusters, as the tree shows. The phylogenetic tree was generated with the MAGA program (version 5.0) using the neighbor-joining method.

FIG. 2. Canine type I IFN locus schematic. This schematic is drawn to scale and shows that IFN-β (IFNB) and IFN-ε (IFNE) are located on the 2 extremities of the IFN-α (IFNA) clusters. Each IFN gene is represented by its abbreviated name, and the arrows denote the direction of transcription. The 2 truncated IFNA are indicated by gray arrows.

recombinant IFNs were purified in a soluble form by 2-step purification, that is, Ni^{2+} affinity chromatography followed by gel filtration. Nonreducing SDS-PAGE analysis of the 4 His-tagged purified proteins showed bands of approximately 21, 20, 23, and 20 kDa for IFNE, IFN-α7, IFNK, and IFNL (respectively) with at least 98% purity (Fig. 3). N-terminal sequencing of these IFNs by Edman degradation confirmed their correct expression (Table 3).

We also attempted to express these IFNs in the *E. coli* BL21(DE3) strain, but they formed inclusion bodies, which were subsequently denatured and renatured. When the denatured rCaIFN-ε inclusion bodies were directly diluted 20-fold in refolding solution, all of the protein precipitated. Thus, we performed multi-step renaturation by gradually reducing the concentration of guanidine hydrochloride to a 1 M final concentration. In this manner, rCaIFN-ε was successfully renatured. All the renatured IFNs were purified using gel-filtration chromatography, and the purity of the final products was ~98%.

Antiviral activities of canine IFNs

The purified canine IFN proteins produced in *E. coli* were tested by viral inhibition assays, and all of them demonstrated high biological activity; the antiviral activity of proteins produced in Origami B(DE3) vs. in BL21(DE3) was not significantly different. rCaIFN-ε and rCaIFN-α7 displayed high antiviral activities in canine cells (MDCK) and heterologous animal cells (MDBK), but very low activity in CRFK and WISH cell lines, which is slightly different than previously reported (Taira and others 2005). rCaIFN-α7 was 3 to 4 fold more active than rCaIFN-ε in the canine cells, while rCaIFN-α7 demonstrated significantly lower activity than rCaIFN-ε in heterologous cells, indicating that rCaIFN-ε has relatively broad cross-species activity (Table 4). rCaIFN-ε and rCaIFN-α7 were tested for antiviral activities against VSV, CPV, CDV, and H1N1 in the MDCK cells and displayed activity against these viruses. The activity of rCaIFN-α7 was higher than rCaIFN-ε against VSV and CPV, while rCaIFN-ε was more active against CDV and H1N1. rCaIFN-α7 was most active against VSV and least active against H1N1, while rCaIFN-ε was most and least active against VSV and CPV, respectively (Table 5).

To determine whether the identified canine IFNK and IFNL sequences encoded for biologically active IFN products, we tested their antiviral activities using the VSV-MDCK virus-target cell system. The antiviral activities of recombinant canine IFNK and IFNL were $9.1\times10^6\pm0.7\times10^6$ U/mg and $5.5\times10^5\pm0.6\times10^5$ U/mg, respectively, proving that these IFNs are not pseudogenes.

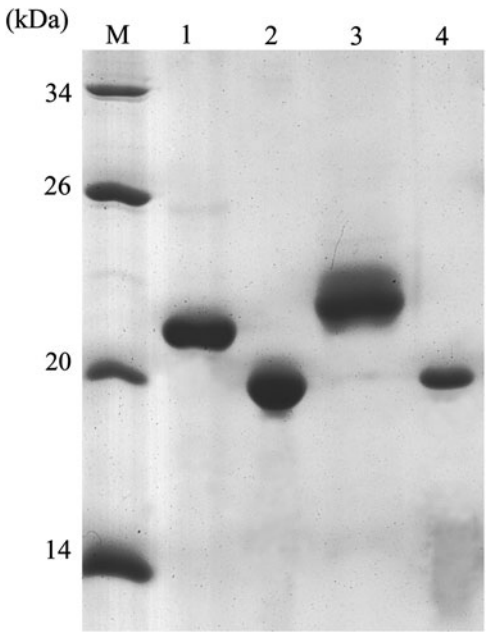

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified rCaIFNs expressed in *Escherichia coli*. Lane M, molecular-weight markers in kDa; lane 1–4, represent purified recombinant canine IFNE, IFN-α7, IFN-κ (IFNK), and IFN-λ (IFNL), respectively.

Proliferation inhibition activities of rCaIFN-ε and rCaIFN-α7 on various cells in vitro

We evaluated the anti-proliferation effect of rCaIFN-ε and rCaIFN-α7 against 2 canine cell lines. As shown in Fig. 4, rCaIFN-ε and rCaIFN-α7 exhibited significant anti-proliferative responses in a dose-dependent manner for approximately 90% for the lowest dilution tested. Fifty percent cell growth inhibition occurred when approximately 470 pg/mL rCaIFN-ε or 130 pg/mL rCaIFN-α7 was mixed with A72 malignant canine tumor cells. For MDCK cells, derived from the canine kidney, approximately 35,600 pg/mL rCaIFN-ε or 3,300 pg/mL rCaIFN-α7 resulted in 50% cell growth inhibition, respectively. A comparison of the anti-proliferation activities of rCaIFN-ε and rCaIFN-α7 indicated that rCaIFN-α7 is approximately 3.6 and 10.8 times more potent than rCaIFN-ε against A72 and MDCK cells, respectively, in MTT assays.

rCaIFN-ε and rCaIFN-α7 promote NK cytotoxicity

The effects of rCaIFN-ε and rCaIFN-α7 on canine NK responses to NK-susceptible cells were determined by performing

Table 3. n-Terminal Sequencing Results of 4 Recombinant Canine Interferons

Recombinant CaIFN	*N-terminal sequence*
IFNE	LELKLAFFQQ
IFN-α7	CHLPDTHGLR
IFNK	LDCNLLHFHL
IFNL	PTSKPTTTRR

The n-terminal methionine residue has been omitted.

Table 4. Antiviral Activities of Rcaifn-ε and Rcaifn-α7 Produced in Origami b(de3) Against Vesicular Stomatitis New Jersey Virus on Various Animal Cell Lines

	Antiviral activity ($\times 10^6$ U/mg) on the cells			
CaIFN	*MDCK*	*MDBK*	*CRFK*	*Wish*
IFN-ε	60.6	52.2	0.001	0.0003
IFN-α7	198.10	10.1	0.0001	<0.00006

Data are shown as the mean of 3 independent experiments.

NK assays, and both IFNs augmented cytotoxicity when assayed in CTAC target cells. rCaIFN-ε enhanced cytotoxicity to 40% at a concentration of 40 ng/mL, while rCaIFN-α7 exhibited the same activity at a concentration of 2.5 ng/mL. Thus, rCaIFN-α7 was approximately 16-fold more potent than rCaIFN-ε in this assay (Fig. 5).

rCaIFN-ε can stimulate JAK-STAT pathway

To determine whether canine IFNE could activate the JAK-STAT pathway, rCaIFN-ε or rCaIFN-α7 were incubated with ISRE-luc plasmid transfected MDCK cells. If the STAT1 and STAT2 were activated, ISGF3 will form and bind to the ISRE of the reporter plasmid, inducing luciferase expression. Luciferase fluorescence was measured using a luminometer. As shown in Fig. 6, compared with the mock-treated group, rCaIFN-ε- or rCaIFN-α7-treated groups significantly induced luciferase expression, which indicated that these 2 IFNs can activate the IFN JAK-STAT signal pathway *in vitro*.

Discussion

Since IFNE was first discovered in 1999, it has only been found in 4 species to date: humans, mice, pigs, and cattle. Moreover, our understanding of the canine IFNs is still very scarce, because only IFNA, IFNB, and IFNG have previously been identified, so, the canine type I IFN gene cluster is not well characterized. Thus, to better elucidate the canine IFN family, we systematically analyzed the IFNs in the canine genome. Two previously unreported type I IFN subclasses (IFNE and IFNK) were discovered using genomic database screening. Further, 10 functional IFNAs and 2 truncated IFNA pseudogenes were found, increasing the number of described canine IFNAs. IFNB, represented in humans and mice by one copy, is also present as a single gene in canines. IFNW is present in humans, mice, felines, and cattle (Leaman and Roberts 1992; Hardy and others 2004; Yang and others 2007), but was not found in canines. IFND, IFNT, and IFNZ, which have been identified in pigs, ruminants, and mice, respectively, are also absent in canines.

Slightly different from humans and mice, all type I IFN in canines, except IFNK, are clustered in an approximately 212-kb length of DNA located on chromosome 11. The canine type I IFN gene cluster consists (to date) of 15 genes: 12 IFNAs (include 2 pseudogenes) and single IFNB, IFNE, and IFNK genes. The arrangement of type I IFN genes within this locus likely reflects the origins and subsequent evolution of individual family members. IFNE and IFNB, 2 genes of ancient origin, define the outer limits of the locus. All of the IFNAs are distributed between these 2 IFNs. The IFNK gene is located 4.76 Mb from this gene cluster, which is similar to other mammals. Given the high nucleotide and amino-acid similarity between the different IFNAs, it is likely that they

Table 5. Antiviral Activities of Rcaifn-ε and Rcaifn-α7 Produced in Origami b(de3) Against Various Viruses on MDCK Cells

	Antiviral activity ($\times 10^6$ U/mg) against the viruses		
CaIFN	*CPV*	*CDV*	*H1N1*
IFN-ε	0.001	0.09	0.003
IFN-α7	0.012	0.07	0.0006

Data is shown as the mean of 3 independent experiments.

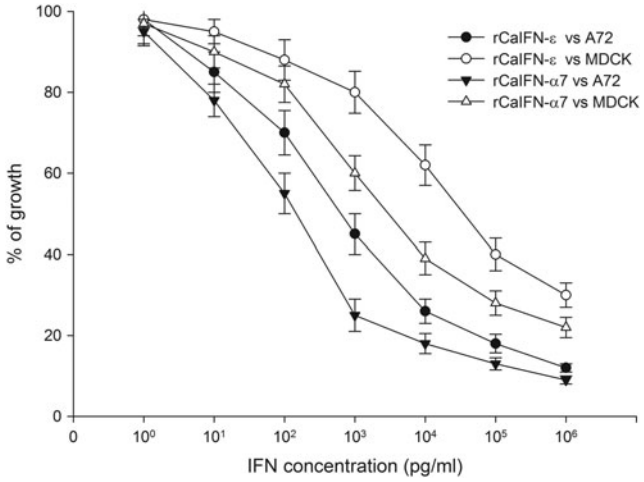

FIG. 4. Comparison of the anti-proliferation activity in 2 canine cell lines between rCaIFN-ε and rCaIFN-α7 produced in Origami B(DE3). Data are means ($n = 3$) ± SEM.

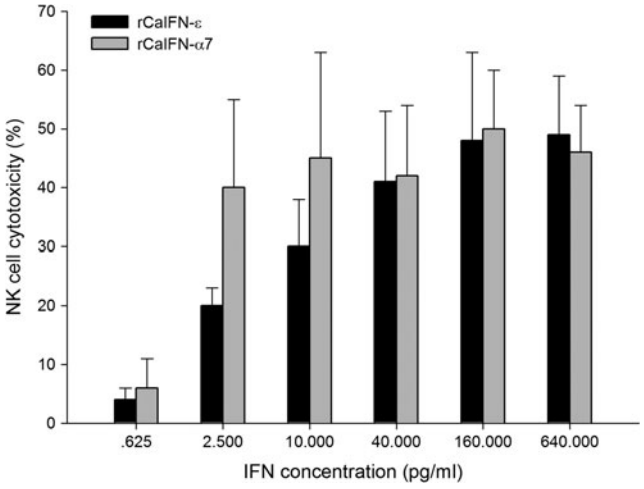

FIG. 5. Comparison of the NK cytotoxicity-promoting activity between rCaIFN-ε and rCaIFN-α7 produced in Origami B(DE3). Data are means ($n = 3$) ± SEM.

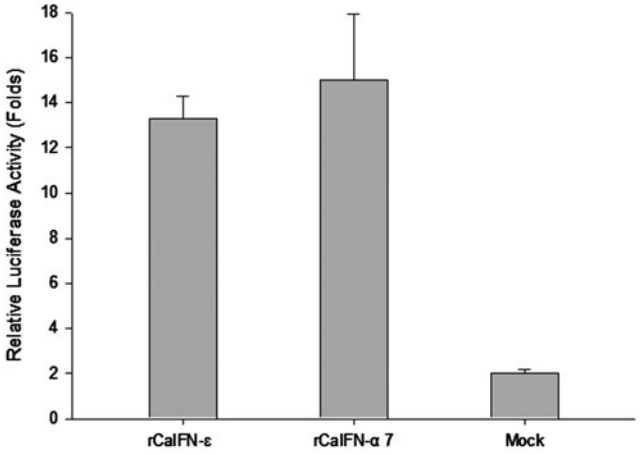

FIG. 6. Luciferase reporter assay. Relative luciferase activity is presented as the ratio of the reporter plasmid and the reference Renilla luciferase reporter plasmid. Data are means $(n=3)\pm$SEM.

arose by gene duplication via unequal recombination and then evolved into their own family. Different from the traditional IFNs in phylogeny, type III IFNs are only found in humans, mice, pigs, and cattle. The canine type III IFN was first localized to chromosome 24 and identified as biological activity.

To explore the biological activities of canine IFNE, we cloned it and expressed soluble protein in *E. coli*. The antiviral, anti-proliferative, and NK cytotoxicity-promoting activities of the recombinant protein were then examined. The canine IFNE gene encodes an 187 amino-acid (aa) precursor with a putative 21 signal peptide in the N-terminal. The native IFNE sequence includes cysteine residues at positions 52, 162, and 174, which is very similar to human IFNE. A pair of Cys residues (52 and 162) that are conserved in all human type I IFNs and are known to form a disulfide linkage crucial for activity are also conserved in IFNE (Chen and others 1999; Hardy and others 2004). Without this disulfide bond, the protein is degraded or accumulates as inclusion bodies. As expected, rCaIFN-ε expressed in BL21 cells formed inclusion bodies. A limitation of the production of properly folded proteins in *E. coli* is the relatively high reducing potential of the cytoplasmic compartment. The Origami B strain, which carries thioredoxin reductase (trxB) and glutathione reductase (gor) mutations, enhances the formation of disulfide bonds in the *E. coli* cytoplasm. As anticipated, rCaIFN-ε expressed in Origami B was highly soluble and biologically active. Unlike rCaIFN-α7, the renaturation of rCaIFN-ε inclusion bodies was slightly difficult. The refolded rCaIFN-ε was very unstable, needing to be stored in a solution containing 1 M guanidine hydrochloride. The properly and improperly folded IFN proteins were separated by gel filtration, thereby increasing the specific activities of the IFN products by orders of magnitude.

To elucidate the antiviral potency of canine IFNE, it was essential to systematically analyze its antiviral activity in cells from different species and against various viruses. We found that compared with IFNA, canine IFNE displayed relatively broad cross-species antiviral activity. There were some differences in antiviral activity against different viruses between canine IFNE and IFNA. For instance, IFNE was most active against VSV and least active against CPV, but IFNA was most active against VSV and least active against H1N1. These results suggest the different physiological functions of these IFNs in the body.

IFNs are believed to cause anti-proliferative effects via cell-cycle arrest and/or by inducing apoptosis. To test for anti-proliferative activity of canine IFNE, we chose an approach similar to one previously described (Loveland and others 1992; Tsang and others 2007), which involved canine tumor cells and nontumor cells. rCaIFN-ε exhibited dose-dependent inhibitory effects against cell proliferation in these 2 cells. In addition, the suppressive activity in canine tumor cells was more potent than in nontumor cells, indicating that canine IFNE has an antitumor function.

NK cells spontaneously lyse a variety of tumor cells *in vitro* and are believed to play an important role in host resistance to tumor growth and metastasis *in vivo*. IFNs are known to enhance NK-mediated cellular cytotoxicity. Our study demonstrated that canine IFNE enhances NK cytotoxicity against NK-susceptible target cells. These observations suggest that canine IFNE may be useful for the treatment of canine neoplasms. A comparison of the abilities to suppress the proliferation of cells and promote NK cytotoxicity between canine IFNE and IFNA indicated that the activity of IFNE is less than that of IFNA. These data are similar to human IFNE, which implicates that canine IFNE may not be functional in blood circulation (Peng and others 2007).

IFNs are cytokines playing an important role in the defence against viruses, which achieve this function through activation of the JAK/STAT signaling pathway. Type I IFNs signal through IFNAR1-IFNAR2 heterodimeric complex, which activates JAK1 and TYK2 kinases and causes dimerization of STAT1 and STAT2 factors that bind to ISRE promoter. Together, these responses initiate the antiviral immune responses. The rCaIFN-ε induces JAK-STAT signaling pathway, which, in turn, triggers the expression of IFN-stimulated genes (ISGs) and then establishes an antiviral response in target cells.

In summary, we explored the canine IFNs by genomic database screening. Canine type I and III IFNs, including IFNE, IFNK, and IFNL, were discovered and identified for the first time. We have provided the first comprehensive annotation of the Type I IFN locus in canines, thereby providing insights into the functional evolution of the canine type I IFN family. The biological functions of canine IFNE, including antiviral, anti-proliferative, and NK cytotoxicity-promoting functions, were further assessed. These results indicate that IFNE is a likely candidate for a novel, effective therapeutic agent.

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Author Disclosure Statement

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