Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Available online at www.sciencedirect.com
ScienceDirect
Veterinary Immunology and Immunopathology 124 (2008) 169–176
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Short communication

Induction and sequencing of Rousette bat interferon α and β genes

Tsutomu Omatsu [a,*], Eun-Jung Bak [a], Yoshiyuki Ishii [a], Shigeru Kyuwa [a], Yukinobu Tohya [b], Hiroomi Akashi [b], Yasuhiro Yoshikawa [a]

[a] *Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*
[b] *Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*

Received 29 August 2007; received in revised form 29 February 2008; accepted 14 March 2008

Abstract

Bats are considered to be natural reservoirs for several viruses of clinical importance, including rabies virus, Nipah virus, and Hendra virus. Type I interferons (IFNs) is an important part of the immune system in the defense against viral infection. To investigate the function of type I IFNs upon viral infection in bats, the nucleic acid, and amino acid sequences of Egyptian Rousette (*Rousettus aegyptiacus*) IFN-α and -β were characterized. Sequence data indicated that bat IFN-α consists of 562-bp encoded 187-aa, and IFN-β consisted of 558-bp encoded 186-aa. Phylogenetic analysis of the overall identity of IFN-β shared the highest sequence homology with pig IFN-β in both nucleotide and amino acid level. Stimulation of bat primary kidney cells (BPKCs) and bat lung cell lines, Tb-1 Lu, with polyinosinic–polycytidylic acid (poly(I:C)) or exogenous bat type I IFNs resulted in increased type I IFNs mRNA expression in BPKCs, but not in Tb-1 Lu. Characterization of the bat IFN-α and -β genes allows understanding of the immune responses upon stimulation in different tissues, thus providing practical strategies for control and treatment of clinically important diseases. These results are important especially for the virus infection, and suggest that future molecular studies on virus infection experiment of bats *in vitro* will require careful consideration of the differences of type I IFN expression patterns in different cell types.

Keywords: Bat; Type I interferons; Bat kidney primary cells; Tb-1 Lu

1. Introduction

Bats, Chiropteras, are well-known vectors of rabies and some studies indicate that they may also naturally harbor some emerging viruses such as Nipah virus, Hendra virus, bat-SARS-CoV and Ebola virus (Chua et al., 2002; Halpin et al., 2000; Lau et al., 2005; Leroy et al., 2005; Mayen, 2003; McColl et al., 2000; Normile, 2005). Bat has two suborders, Megachiroptera (flying fox) and Microchiroptera (insectivorous bat). Many emerging or re-emerging viruses, such as rabies, Nipah virus, and Hendra virus, were isolated form Megachiroptera. In particular, European bat lyssavirus type I was also isolated from *Rousettus* sp. (Van der Poel et al., 2000; Wellenberg et al., 2002; Wong et al., 2007). Bats were thought to have an important role for the infection cycle of these emerging and re-emerging viruses. *In vivo* experiment,

* Corresponding author at: Laboratory of Vector-borne Viruses, Department of Virology I, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan.
Tel.: +81 3 5285 1111; fax: +81 3 5285 1208.
E-mail address: t-omatsu@nih.go.jp (T. Omatsu).

0165-2427/$ – see front matter
doi:10.1016/j.vetimm.2008.03.004

Ebola virus inoculation studies showed that both flying foxes and insectivorous bats support viral replication and circulation with high viral titers without becoming ill (Swanepoel et al., 1996). Studies *in vitro* have shown that Ebola virus VP35 protein blocks the activation of interferon regulatory factor 3 (IRF-3) and Ebola virus VP24 protein inhibits interferon (IFN) signaling (Basler et al., 2003; Reid et al., 2006). These data suggested that Ebola virus might evade the anti-viral activity of IFNs in bat cells. Therefore, it is crucial to investigate IFN regulation and function in bats because few immunological studies have been reported for this animal species.

Cells have many responses to viral infection. One of the responses is the secretion of type I IFNs which are composed of multiple α subtypes and a single β subtype (Sen, 2001). Type I IFNs expression utilize two signal transduction pathways; the Toll-like receptor (TLR)-dependent pathway and TLR-independent pathway. In TLR-dependent pathway, cells recognize viral double-strand RNA, single-strand RNA and CpG DNA via TLR, and subsequently IFN-β is induced. In TLR-independent pathway, intracellular sensors such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene-5 (mda-5) detect viral components in the cytoplasm, and transactivate IFN-β mRNA (Hiscott et al., 2006). Expressed IFN-β binds to the type I IFN receptors and activates numerous IFN-stimulated genes, such as the protein kinase R (PKR) gene, the 2′–5′ oligoa-denylate synthetases (OAS) gene, and the myxovirus resistance (Mx) gene. The product of these genes controls viral infection (Samuel, 2001). Viral double-stranded RNA (dsRNA), a viral intermediate in the proliferation of many RNA viruses, is known as an IFN-inducer through these sensors (Gitlin et al., 2006). Polyinosinic–polycytidylic acid (poly(I:C)) is a synthetic mimetic of viral dsRNA and a strong inducer of type I IFNs *in vivo* and *in vitro* via these sensors (Hertzog et al., 2003).

Type I IFNs stimulate anti-viral activity as mentioned above; however, such studies in bat have not been possible because the bat IFN related genes had not been previously identified. In this study, we determined the sequence of a subtype of IFN-α and IFN-β from *Rousettus aegyptiacus*, including the full open reading frames (ORFs), and analyzed phylogenetically based on IFNs from other mammals. In addition, the up-regulation of these mRNAs in both bat primary kidney cells (BPKCs) and a bat lung cell line, Tb-1 Lu was examined using poly(I:C) or bat type I IFNs derived from BPKCs.

2. Materials and methods

2.1. Preparation of cDNA from bat genomic DNA

Fresh liver sample and whole blood of *R. aegyptiacus* under anesthesia with ketamine (5 mg/ml/kg) and medetomidine (0.2 mg/ml/kg) were collected by heart puncture. Bat liver was fixed with 10% neutral buffer formalin. Bat genomic DNA was isolated from fixed liver with the Wizard Genomic DNA Purification kit (Promega, Madison, WI) and stored at −20 °C until usage.

2.2. Sequencing of bat IFN genes

Bat genomic DNA sample was used as a template of polymerase chain reaction (PCR) using TaKaRa Ex Taq (Takara Bio, Ohtsu, Shiga, Japan). Forward and reverse primers of IFN-α and IFN-β for PCR were designed from the sequence data of human, mouse, cat, pig, and horse IFN-α and IFN-β (Table 1). The accession numbers of these data in GenBank are as follows: IFN-α

Table 1
Sequence of each PCR primers

Primer name		Sequence (5′–3′)
IFN-α	Forward	CTC TCT AGG ATG TGA CCT GCC TCA GA
	Reverse	ACA GGG GCT GTG TTT CTT CTC
IFN-β	Forward	GCT TGG ATT CCA ACT AAG AAG CAG C
	Reverse	ACA GAC GCT GTA CTC CTT GGC CTT CA
GAPDH	F	GAT GGA GCA TCA TAC TGA TCC
	R	GAC CTT CTA CCA CTA CCC AAA
IFN-α	F3	ACA GAG GCA GGT CTT CAC AAC CTA GA
	R2	GAG AAG CAT TTC CAT GTT GAA CCA G
IFN-β	cdsF	TAG GTG ATA GTA GGC ACC ACT GTT CC
	cdsR	CTT TCT CAG AAG TAC AGG CGG AGA GA

of human (BC074029), mouse (BC116872), cat (AY117395), pig (AY526089), and horse (M14540), and IFN-β of human (M25460), mouse (BC119395), cat (AB021707), pig (NM_001003923), and horse (M14546). The PCR products were isolated by electrophoresis in an 1% agarose gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The purified PCR products were cloned into pCR-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and the sequence was determined using the Big Dye™ Terminator kit (ABI, CA, USA) and ABI PRISM™ 377 DNA Sequencer (ABI, CA, USA). We utilized the sequence data to design new primers for PCR amplification of the entire IFN-α and IFN-β cDNAs. Finally, we determined the ORF for the IFN-α and IFN-β cDNA sequences

```
  1  GGTTGAGAAACAGTGCTCTGAACCTATTTAGAAAGTGCATAAAGGAAAGCAAAAGCAGAA
 61  GTAGAAAGTGAGGGGAACATTCAGAAATTGAGAACTCACATGTTCCCTATATATGATACA
121  TGCACAGAGGCAGGTCTTCACAACCTAGAGCCCAAGGTTGGCAGTGTCATCCAGCGCAGC
181  CAGGCCGGCCTCATCTGCAAGATCTCCAATGGCCCTGCCCTGTTCCTTCCTGATGGCCGT
                               M  A  L  P  C  S  F  L  M  A  V   11

241  CGTGGTGCTCAGCTGCCACTCCATCTGCTCTCTGGGCTGTGACCTGCCTCTGACCCACAG
      V  V  L  S  C  H  S  I  C  S  L  G  C  D  L  P  L  T  H  S   31

301  CCTGGTCGACAGGAGGGCGTTGATCCTTCTGGGACAAATGAGGAGAATCTCTCCTTTCTC
      L  V  D  R  R  A  L  I  L  L  G  Q  M  R  R  I  S  P  F  S   51

361  CTGCCTCAAGGACAGAGAAGACTTTGGATTCCCCCAGGGGGTGCTTCATGGCAACCAGTT
      C  L  K  D  R  E  D  F  G  F  P  Q  G  V  L  H  G  N  Q  F   71

421  CCAGGAGGCTCAAGCCATCGCTGTTGCCCACGAGGTGACCCGGCAGACCTTCCTCCTCTT
      Q  E  A  Q  A  I  A  V  A  H  E  V  T  R  Q  T  F  L  L  F   91

481  CTGCACAGAGGCCTCATCCGCAGCTTGGGATGAGACCCTGCGAAGCAGATTCTGCACTGG
      C  T  E  A  S  S  A  A  W  D  E  T  L  R  S  R  F  C  T  G  111

541  ACTCTATCAGCAGCTGATCCACCTGGAAGCCTGTCAGACGCGGGAGGTGGGGGCGGAGGA
      L  Y  Q  Q  L  I  H  L  E  A  C  Q  T  R  E  V  G  A  E  E  131

601  GACTCCCCTGCTGGATGAGGACTCCACACTGGCTGTGAGGAGTTACTTCCAGAGACTCTT
      T  P  L  L  D  E  D  S  T  L  A  V  R  S  Y  F  Q  R  L  F  151

661  CCTCTATCTGCAGGAGAAGAAACACAGCCCTTGTGCCTGGGAGATTATCAGAGCAGAGAT
      L  Y  L  Q  E  K  K  H  S  P  C  A  W  E  I  I  R  A  E  I  171

721  CATGAGGTCCTACTCTTTATCAACACACTTGAAGGAAACCAAGGAGTAAGGATTGACACT
      M  R  S  Y  S  L  S  T  H  L  K  E  T  K  E  *              186

781  GGTTCAACATGGAAATGCTTCTC
```

Fig. 1. The nucleotide and deduced amino acid sequences of bat IFN-α (A) and -β (B). The numbers at left indicate the leftmost nucleotide position. The numbers at right indicate the rightmost amino acid position. Amino acid residues are shown by the one-letter abbreviation code based on the nucleotide sequence. Nucleotides in the 5′ and 3′ non-coding regions are shown preceding the ATG (start methionine codon) and following the TGA or TAA (stop codon, indicated by *), respectively.

```
   1  GTAGATAGTTGAGATTGCCAGACAATGCACTGGTGACTGACAGACAATGCACTGAGTAGA
  61  TAGTTGAGATTGCCAGAGGGGAGGGGGTTGGGGGAAATGGGTGAAAAAGGTGAAAGGGAT
 121  TAAGAAGTTCAAATTACCAGTTATAAAAATAGTCATAGGAATGTGAAGTACAGCATAGGG
 181  AATATAGTCAATAATGATGTAATAACTATGTATGGTGCCAGATGGGTACTAGATTTTTTG
 241  GGGTGATCACTTCATATGGTATATAAATATCTAACCACTATGTCGTACATCTAAAACTGA
 301  TATGATTTTCATTCCATTGTAATTGAAAAATATAAATGACAAAAGAAAACTGAAAAGGAG
 361  AACTGAAAATGGGAAATTCCTCTGAAATAGAAAGGGTTGATGACCGTATAAATAGCCCAG
 421  GCTCATGGAGAAAAGACATTCACACTGCCAACACTTGAAGCCTTCCCTTCAGTGCCTAGG
 481  TGATAGTAGGCACCACTGTTCCCGTTTTCACCATGACCAACAGGTGCATCCTCCAAATTG
                                   M  T  N  R  C  I  L  Q  I  A  10

 541  CTCTCCTGTTGTGTTTCTCCACCACAGCTCTTTCCATGAGCTACGACTTGCTTCGATTTC
        L  L  L  C  F  S  T  T  A  L  S  M  S  Y  D  L  L  R  F  Q  30

 601  AACAAAGAAGCAGCAATTTAGCCTGCCTGAAGCTCCTGTGGCGGTTAAATGGAACCCCTC
        Q  R  S  S  N  L  A  C  L  K  L  L  W  R  L  N  G  T  P  Q  50

 661  AATATTGCCTCAAGGACAGGATGGACTTCAAGATCCCTGCGGAGATTAAACAACCACAGC
        Y  C  L  K  D  R  M  D  F  K  I  P  A  E  I  K  Q  P  Q  Q  70

 721  AACTCCAGAAGGAGGACGCAGTATTGATCATCCATGAGATGCTCCAGCAGATCTTTGGTA
       L  Q  K  E  D  A  V  L  I  I  H  E  M  L  Q  Q  I  F  G  I   90

 781  TTCTCCAAAGAAATTTCTCTAGCACTGGCTGGAATGAGACCATCATTGAGACTCTCTTTG
        L  Q  R  N  F  S  S  T  G  W  N  E  T  I  I  E  T  L  F  V 110

 841  TGAAACTTGATAAGCAGATAGACCTTCTGGATACAGCCCTGGAGAAACTGGAGAAGGAAA
        K  L  D  K  Q  I  D  L  L  D  T  A  L  E  K  L  E  K  E  N 130

 901  ACTTCACCTGGGAAAGCATGACAGTGCTGCACCTAAAGAATTACTACTTTAGGATCATGA
        F  T  W  E  S  M  T  V  L  H  L  K  N  Y  Y  F  R  I  M  R 150

 961  GGTACCTGAAGATCAGGTTGTACAGCAGATGTGCCTGGACAGTAGTCCGAGCGGAAATTC
        Y  L  K  I  R  L  Y  S  R  C  A  W  T  V  V  R  A  E  I  L 170

1021  TCAGGAACTTTTCCTTCCTTATTGGACTTACAGAATACCTCCGAAACTGAAGATCTCTCC
        R  N  F  S  F  L  I  G  L  T  E  Y  L  R  N  *              185

1081  GCCTGTACTTCTGAGAAAGAACAGTGCTTG
```

Fig. 1. (*Continued*).

using IFN-α F3, R2 and IFN-β cdsF, cdsR (Table 1). The ORFs and the deduced amino acid sequences were analyzed using the genetic information processing software GENETYX-WIN Version 4.0.2 (Software Development, Tokyo, Japan).

2.3. Phylogenetic analysis of IFNs

Mammalian IFN nucleotide sequence information was obtained from GenBank. Species in the phylogenetic tree were limited because only several species

have enough numbers of subtypes of known INFs sequences. Sequences were aligned using Clustal W (Version 1.83; http://www.cf.ac.uk/biosi/research/biosoft/downloads/clustalw.html), checked by eye, and all positions with gaps or ambiguous alignments were excluded from the analysis. A phylogenetic tree was constructed using Phylip (Version 3.6.5; http://evolution.genetics.washington.edu/phylip.html) with the following full-length IFN ORFs referred to GenBank: human (IFN-α1 (NP_076918), IFN-α2 (NP_000596), IFN-α5 (NP_002160), IFN-α14 (NP_002163), and IFN-β (AAC41702)), horse (IFN-α1 (P05003), IFN-α2 (P05004), IFN-α3 (P05005), IFN-α4 (P05006), and IFN-β (P05012)), pig (IFN-α1 (NP_999558), IFN-α3 (ABI26095), IFN-α10 (ABB51634), IFN-α14 (ABB51627), and IFN-β (NP_001003923)), dog (IFN-α1 (P81255), IFN-α5 (BAD18111), IFN-α7 (NP_001006655), IFN-α8 (NP_00100713) and IFN-β (XP_538679)), cat (IFN-α1 (AAM78030), IFN-α7 (BAC75983), IFN-α10 (NP_001027000), IFN-α14 (NP_001027002), and IFN-β (BAA93629)), mouse (IFN-α1 (AAO63592), IFN-α5 (AAI20911), IFN-α7 (NP_032360), IFN-α14 (NP_996858)), chicken (IFN-α (BAA83090) and IFN-β (NP_001020007)).

2.4. Preparation of bat primary kidney cells and bat cell line

Fresh kidney of *Rousettus leschenaulti* under anesthesia with ketamine (5 mg/ml/kg) and medetomidine (0.2 mg/ml/kg) were removed, sliced and treated with 0.25% Trypsin–EDTA in phosphate-buffered saline (PBS). Whole blood of bat was collected by heart puncture under anesthesia. Collected bat primary kidney cells were seeded on 10-cm^2 plate in Dulbecco's modified eagle's medium (DMEM) (Invitrogen) with 5% heat-inactivated fetal calf serum (FCS). Bat lung epithelial cell line, Tb-1 Lu, was maintained in incubation of 5% CO_2, at 37 °C in DMEM containing 10% FCS.

2.5. Expression of type I IFNs mRNA using poly(I:C) treatment

BPKCs and Tb-1 Lu cells were treated with 5% FCS-DMEM including 10 μg/ml poly(I:C) (Sigma, St. Louis, MO) and 150 μg/ml diethylaminoethyl dextran (DEAE-Dextran) (Sigma) in 5% CO_2 at 37 °C for 3 h. Cells were then washed twice with PBS and total RNA was isolated using ISOGEN solution (NIPPON GENE, Toyama, Japan).

2.6. Preparation of bat type I IFNs-containing medium

BPKCs were treated with 5% FCS-DMEM including 10 μg/ml poly(I:C) and 150 μg/ml DEAE-Dextran in 5% CO_2 at 37 °C for 3 h. After treatment, the cells were washed twice and cultured in fresh 5% FCS-DMEM for 24 h. The whole supernatant, bat type I IFNs-containing medium, was collected and stored at 4 °C until usage.

2.7. Expressions of bat IFNs mRNA under bat type I IFNs treatment

BPKCs and Tb-1 Lu cells were prepared at 5×10^5 ml^{-1} in 6-well culture plate containing 2 ml per well for 2 days, and then treated with 200 μl of bat type I IFNs-containing medium for 1 h. After that, cells were washed three times and then incubated in 5% CO_2 at 37 °C in DMEM containing 5% FCS (primary kidney cells) or 10% FCS (Tb-1 Lu cells) for additional 0, 4, and 8 h. After incubation, total RNA was isolated from these cells with ISOGEN solution (NIPPON GENE).

2.8. Reverse-transcription PCR analysis of type I IFNs mRNA expression

Total RNAs were treated with DNase I (Takara Bio) according to the manufacturer's instructions. RNA samples were then reverse-transcribed using the $Oligo(dT)_{12–18}$ primer and SuperScript™ II (Invitrogen) for synthetic first-strand cDNA. cDNAs were used as a template for semi-quantitative PCR with TaKaRa Ex Taq using GAPDH F and R, IFN-α F3 and R2 and IFN-β cdsF and cdsR (Table 1) as primers. The PCR products were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide.

In this study, the experiment was performed in accordance with the Animal Experimentation Guideline, the University of Tokyo, and was approved by the Institutional Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

3. Results and discussions

3.1. Cloning of full-length bat type I IFN ORFs and phylogenetic analysis of IFNs

The nucleic acid and amino acid sequences of one of the IFN-α genes and the IFN-β gene from *R. aegyptiacus* were determined. The full-length bat IFN-α ORF was 562 bp and encoded 187-aa polypep-

tides. Bat IFN-β ORF was 558 bp and encoded 186-aa polypeptides (Fig. 1(A and B)). Direct comparison of bat IFN-α genes simultaneously against various animal species was complicated by the fact that there are various IFN-α subtypes and difficulties in the identification of subtype of the bat IFN-α. Therefore, sequence comparisons were performed using IFN-β between bat and human, pig, cat, horse, and mouse. The identity of bat IFN-β with human, pig, cat, horse, and mouse IFN-β were 77.5, 82.0, 78.3, 77.5, and 66.5% at the nucleotide level and 64.2, 72.0, 61.8, 61.3, and 49.5% at the amino acid level, respectively. Phylogenetic analysis using the amino acid sequences from several representative eutherian type I IFNs and chicken type I IFN found that both bat IFN-α and bat IFN-β are homologous to the mammalian IFN group. Further analysis showed that both bat IFN-α and IFN-β were most closely related to those of pig, and followed by horse (Fig. 2). Although phylogenetic relationship between bat and other animals remains inconclusive, molecular phylogenetics using mitochondrial DNA or retro-transposon insertions indicated that Chiroptera is included in Fereuungrate or Pegasoferae (Perissodactyla, Carnivora, Pholidota, and Chiroptera) (Nikaido et al., 2000; Nishihara et al., 2006). Comparison of the amino acid sequences of the cell surface molecule CD4 showed that bat is more closely related to cat and dog (Omatsu et al., 2006). Our findings and these molecular phylogenetic analyses suggested that bat might have anti-viral mechanism similar to these animals. Some investigators indicated that Nipah virus spread from Megachiroptera to pig and then from pig to human (Tan and Wong, 2003), and pig might be more susceptible to the virus than other animals. In contrast, relatively low

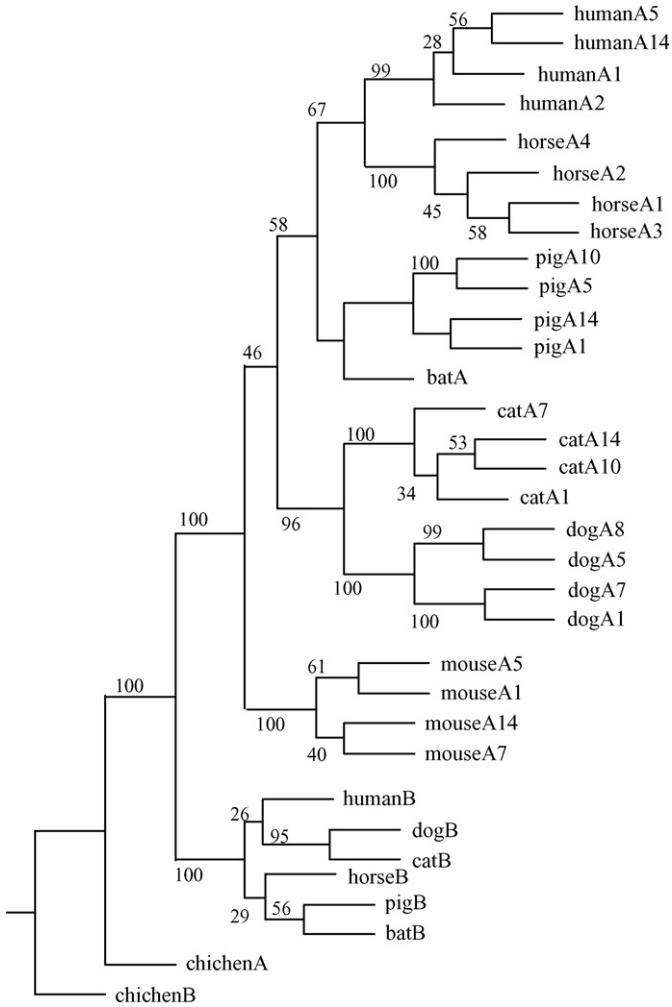

Fig. 2. Maximum likelihood phylogenetic tree constructed by the Phylip 3.65 program using amino acid sequences from human, horse, pig, cat, dog, mouse, chicken, and bat type I IFNs. The numbers at the nodes indicate bootstrap values. 'A' and 'B' reflect IFN-α and -β, respectively.

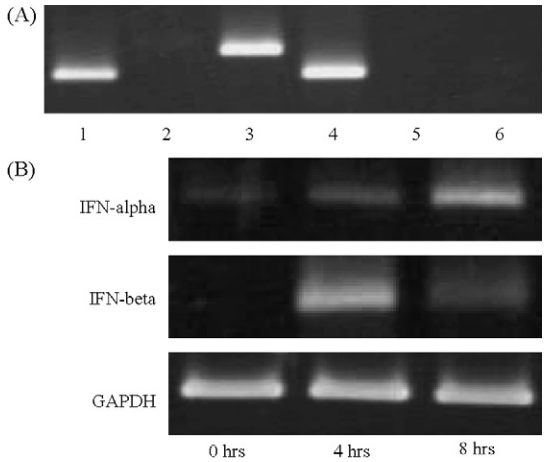

Fig. 3. Expression of type I IFNs in BPKCs and Tb-1 Lu. (A) Expression of type I IFN mRNAs in response to poly(I:C) treatment. IFN-α mRNA (lanes 2 and 5), IFN-β mRNA (lanes 3 and 6), and GAPDH control mRNA (lanes 1 and 4) from BPKCs (lanes 1–3) and Tb-1 Lu cells (lanes 4–6) were analyzed using semi-quantitative reverse-transcription PCR of total RNA followed by 1% agarose gel electrophoresis and ethidium bromide staining. (B) Temporal change of type I IFN mRNAs in BPKCs after treatment with bat IFNs-containing medium. IFN-α mRNA (upper panel), IFN-β mRNA (middle panel) and GAPDH mRNA (control; lower panel) from BPKCs were analyzed following treatment with bat IFNs-containing medium for the indicated period of time.

homology of immune factors between bat and human suggested the presence of different anti-viral activity against some viral infections. These factors might be one of the key factors to control zoonoses from bat.

3.2. Expression of bat type I IFN

To investigate whether BPKCs and Tb-1 Lu cells have the capacity of IFN production, we first examined the up-regulation of type I IFNs in response to poly(I:C) treatment. In BPKCs, there is an increase in the expression of IFN-β mRNA, but not IFN-α mRNA, at 3 h after poly(I:C) treatment. However, in Tb-1 Lu cells, poly(I:C) treatment induced IFN-α mRNA production but production of INF-β mRNA was not observed (Fig. 3(A)). To examine whether BPKCs or Tb-1 Lu expresses type I IFN mRNA in response to the bat IFN-containing medium (exogenous IFN), the expression of type I IFNs mRNA was examined at 0, 4, and 8 h after the exogenous IFN-treatment. In the case of BPKCs, IFN-α mRNA was detected at each time point with a gradual increase, and IFN-β mRNA which was not initially detected, peaked at 4 h in response to exogenous IFN (Fig. 3(B)). In Tb-1 Lu, however, type I IFNs mRNA expression was not detected at all (data not shown). Although BPKCs could induce type I IFNs mRNA in response to poly(I:C) via TLR3, RIG-I, and mda-5, type I IFNs-inducing signal was not sufficient for the stimulation of IFN-α mRNA synthesis. In contrast, when type I IFNs were supplied to BPKCs, IFN-β mRNA was induced more rapidly than IFN-α. This indicated that IFN-β is involved in immediate response to invasion of viruses or microbes and IFN-α, which is responsible for anti-viral activity via stimulation of PKR, OAS, and Mx synthesis, is induced by IFN-β and has more prolonged response in BPKCs (Fig. 3(B)). When Tb-1 Lu were treated with either poly(I:C) or bat IFNs-containing medium, these cells did not express any IFN-α or IFN-β mRNA. This suggests that in Tb-1 Lu the mechanism of dsRNA recognition or the signaling pathway reacted to exogenous IFNs is not utilized for up-regulation of type I IFNs mRNA. Thus, the IFN signal responding to both poly(I:C) and exogenous type I IFNs was different between BPKCs and Tb-1 Lu. Bat is diversified into about a thousand species in the world. These results further indicated that extra considerations should be taken in the interpretation of experimental data of anti-viral dynamics among various bat cell types and species.

The bat immune system is of particular interest because of its ability to act as a reservoir for a variety of pathogens that pose serious health threats to humans. However, these studies are complicated because few studies on anti-viral mechanism of bat are available. Thus, the nucleotide sequences of type I IFNs of Rousette bat were characterized for the first time. To investigate whether and how bats harbor clinically important pathogens, some basic information from inoculation studies performed *in vivo* and *in vitro* is very important. To determine how wild animals remain asymptomatic to pathogens, it will be necessary to understand their viral control mechanisms, such as IFN signaling. Using bats as representative pathogenic carriers, this study provides some basic and important immunological information about bat. It is necessary for understanding zoonoses from bat, especially for Megachiroptera.

Acknowledgements

R. aegyptiacus and *R. leschenaulti* were kindly supplied by Ueno Zoo, Tokyo, Japan and Asa Zoo, Hiroshima, Japan. This work was supported by Research on Emerging and Re-Emerging Infectious Diseases grant (H18-emerging disease-ippan-008) from The Ministry of Health, Labor and Welfare of Japan.

References

Basler, C.F., Mikulasova, A., Martinez-Sobrido, L., Paragas, J., Muhlberger, E., Bray, M., Klenk, H.D., Palese, P., Garcia-Sastre, A., 2003. The Ebola virus VP35 protein inhibits activation of interferon regulatory factor 3. J. Virol. 77, 7945–7956.

Chua, K.B., Koh, C.L., Hooi, P.S., Wee, K.F., Khong, J.H., Chua, B.H., Chan, Y.P., Lim, M.E., Lam, S.K., 2002. Isolation of Nipah virus from Malaysian island flying-foxes. Microbes Infect. 4, 145–151.

Gitlin, L., Barchet, W., Gilfillan, S., Cella, M., Beutler, B., Flavell, R.A., Diamond, M.S., Colonna, M., 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. Proc. Natl. Acad. Sci. U.S.A. 103, 8459–8464.

Halpin, K., Young, P.L., Field, H.E., Mackenzie, J.S., 2000. Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. J. Gen. Virol. 81, 1927–1932.

Hertzog, P.J., O'Neill, L.A., Hamilton, J.A., 2003. The interferon in TLR signaling: more than just antiviral. Trends Immunol. 24, 534–539.

Hiscott, J., Nguyen, T.L., Arguello, M., Nakhaei, P., Paz, S., 2006. Manipulation of the nuclear factor—kappaB pathway and the innate immune response by viruses. Oncogene 25, 6844–6867.

Lau, S.K., Woo, P.C., Li, K.S., Huang, Y., Tsoi, H.W., Wong, B.H., Wong, S.S., Leung, S.Y., Chan, K.H., Yuen, K.Y., 2005. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proc. Natl. Acad. Sci. U.S.A. 102, 14040–14045.

Leroy, E.M., Kumulungui, B., Pourrut, X., Rouquet, P., Hassanin, A., Yaba, P., Delicat, A., Paweska, J.T., Gonzalez, J.P., Swanepoel, R., 2005. Fruit bats as reservoirs of Ebola virus. Nature 438, 575–576.

Mayen, F., 2003. Haematophagous bats in Brazil, their role in rabies transmission, impact on public health, livestock industry and alternatives to an indiscriminate reduction of bat population. J. Vet. Med. B: Infect. Dis. Vet. Public Health 50, 469–472.

McColl, K.A., Tordo, N., Aguilar Setien, A.A., 2000. Bat lyssavirus infections. Rev. Sci. Tech. 19, 177–196.

Nikaido, M., Harada, M., Cao, Y., Hasegawa, M., Okada, N., 2000. Monophyletic origin of the order Chiroptera and its phylogenetic position among mammalia, as inferred from the complete sequence of the mitochondrial DNA of a Japanese megabat, the Ryukyu flying fox (*Pteropus dasymallus*). J. Mol. Evol. 51, 318–328.

Nishihara, H., Hasegawa, M., Okada, N., 2006. Pegasoferae, an unexpected mammalian clade revealed by tracking ancient retroposon insertions. Proc. Natl. Acad. Sci. U.S.A. 103, 9929–9934.

Normile, D., 2005. Virology. Researchers tie deadly SARS virus to bats. Science 309, 2154–2155.

Omatsu, T., Nishimura, Y., Bak, E.J., Ishii, Y., Tohya, Y., Kyuwa, S., Akashi, H., Yoshikawa, Y., 2006. Molecular cloning and sequencing of the cDNA encoding the bat CD4. Vet. Immunol. Immunopathol. 111, 309–313.

Reid, S.P., Leung, L.W., Hartman, A.L., Martinez, O., Shaw, M.L., Carbonnelle, C., Volchkov, V.E., Nichol, S.T., Basler, C.F., 2006. Ebola virus VP24 binds karyopherin alpha1 and blocks STAT1 nuclear accumulation. J. Virol. 80, 5156–5167.

Samuel, C.E., 2001. Antiviral actions of interferons. Clin. Microbiol. Rev. 14, 778–809 (table of contents).

Sen, G.C., 2001. Viruses and interferons. Annu. Rev. Microbiol. 55, 255–281.

Swanepoel, R., Leman, P.A., Burt, F.J., Zachariades, N.A., Braack, L.E., Ksiazek, T.G., Rollin, P.E., Zaki, S.R., Peters, C.J., 1996. Experimental inoculation of plants and animals with Ebola virus. Emerg. Infect. Dis. 2, 321–325.

Tan, C.T., Wong, K.T., 2003. Nipah encephalitis outbreak in Malaysia. Ann. Acad. Med. Singapore 32, 112–117.

Van der Poel, W.H., Van der Heide, R., Van Amerongen, G., Van Keulen, L.J., Wellenberg, G.J., Bourhy, H., Schaftenaar, W., Groen, J., Osterhaus, A.D., 2000. Characterisation of a recently isolated lyssavirus in frugivorous zoo bats. Arch. Virol. 145, 1919–1931.

Wellenberg, G.J., Audry, L., Ronsholt, L., van der Poel, W.H., Bruschke, C.J., Bourhy, H., 2002. Presence of European bat lyssavirus RNAs in apparently healthy *Rousettus aegyptiacus* bats. Arch. Virol. 147, 349–361.

Wong, S., Lau, S., Woo, P., Yuen, K.Y., 2007. Bats as a continuing source of emerging infections in humans. Rev. Med. Virol. 17, 67–91.