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Polymorphisms and the Antiviral Property of Porcine Mx1 Protein

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ABSTRACT. We determined the cDNA sequences of the type I interferon-inducible proteins, pig *Mx1* from PK(15) and LLC-PK1 cells, and compared the antiviral activities of both Mx proteins, including *Mx1* polymorphisms against vesicular stomatitis virus (VSV). *Mx1* cDNA derived from PK(15) cells had an 11 bp-deletion in the 3' end of the coding region, and was estimated to encode 8 amino acid substitutions and a 23 amino acid extension compared to that from LLC-PK1 cells. VSV replication was inhibited in the 3T3 cells expressing *Mx1* mRNA after the cDNA was transfected. However, the efficiency of this inhibition was not different between the cells expressing *Mx1* mRNA from both PK and LLC. These results indicate that pig Mx1 protein confers resistance to VSV.
KEY WORDS: antiviral protein, interferon, Mx, swine.

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In the early phase of virus infection *in vivo*, type I interferon (IFN) is produced abundantly in the infected cells. IFN induces a number of proteins that lead to an antiviral state in the stimulated cells [8]. Mx protein is one of those antiviral proteins, and is found in most vertebrates such as mammals, birds and fish [4, 5, 7, 10, 11, 27]. Mx proteins contain a consensus motif seen in GTP binding proteins, and indeed possess GTPase activity [21]. These proteins also contain a leucine zipper motif in the carboxyl (C)-terminus, which is involved in forming a homooligomer [6, 18, 23]. Although human MxA [28], mouse Mx2 [14, 29] and the Mx proteins of various mammals [9] are located in the cytoplasm, some of those in rodents such as mouse Mx1 [24] and rat Mx1 [17] are located in the nucleus. The Mx proteins interfere with replication of various negative strand RNA viruses. Mouse nucleic Mx1 inhibits replication of influenza virus, but not VSV [24]. However, mouse cytoplasmic Mx2 protects the expressing cells against VSV, but not influenza virus [15, 29]. In contrast to mouse Mx proteins, human cytoplasmic MxA inhibits the both influenza virus and VSV, but the human cytoplasmic MxB lacks antiviral activity [22].

It is well-known in laboratory mouse strains that *Mx1* and *Mx2* genes have polymorphism such as deletion or insertion mutations causing frame-shifts, and also nonsense mutations [13, 14, 25, 26]. We recently found a polymorphism resulting in a frame-shift in the pig *Mx1* gene [19]. However, little is known about the antiviral activity of Mx1 protein of the pig. In the present study, we have performed the sequencing of *Mx1* cDNA in pig cell lines to examine the existence of more polymorphisms in these genes, and have estimated an antiviral activity against VSV in *Mx1* mRNA-expressing cells transfected into the mouse fibroblastic cell line BALB/3T3 clone A31 (3T3).

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MATERIALS AND METHODS

Cell culture: Porcine kidney cell lines, PK(15) (JCRB9040) and LLC-PK1 (JCRB0060), were obtained from Human Science Research Resources Bank (Osaka, Japan). 3T3 cells were obtained from RIKEN (Tsukuba, Japan). PK(15) cells were grown in Minimum essential medium (Sigma, Saint Louis, MO, U.S.A.) supplemented with 5% fetal bovine serum (Trace Biosciences, New South Wales, Australia). LLC-PK1 cells were grown in Medium 199 (Sigma) with 3% fetal bovine serum. 3T3 cells were grown in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum. All of the cell lines were seeded in 60 mm dishes at a density of 3×10^5 cells/dish and passaged twice per week.

Northern blot analysis of RNA: Confluent PK(15) and LLC-PK1 cells were treated with or without 50 μg/m*l* of poly(I) poly(C) (Amersham Pharmasia Biotech, Uppsala, Sweden), which was well-known as a chemical to induce IFN-stimulated genes instead of virus RNA molecules, for 6 hr. Total RNA (30 μg) of these cells isolated by TRIzol (Gibco) was electrophoresed, transferred to a nylon membrane (Hybond N+, Amersham Pharmasia Biotech), and subjected to Northern hybridization as described previously [1]. The hybridization cDNA probes for *Mx1* and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained by the RT-PCR method. Total RNA (1 μg) of poly(I) poly(C)-stimulated LLC-PK1 cells was reverse-transcribed with oligo dT(15) primer and 50 U of reverse transcriptase (ReverTra Ace, Toyobo, Tokyo, Japan) in a total volume of 10 μ*l*. PCR was performed with 1 μ*l* of first-strand DNA as described above, using 2.5 U of Taq polymerase (*ExTaq*, Takara, Tokyo, Japan) and a PCR primer set in a total volume of 25 μ*l*. PCR cycles were as follows: 94°C 1 min, 60°C 1 min, 72°C 1 min, 30 cycles. The primer set of 5'-CAC TTC CAA ATG GAG CAG ATC GTG T-3'and 5'-CTC AGC CACT AAC AGC AGG GAC GGT

GG-3' was used for amplification of *Mx1* cDNA of nucleotides 1673–2250 [20]. The primer set for GAPDH cDNA was described previously [2]. PCR products were cloned in the pGEM-T Easy vector (Promega, Madison, WI, U.S.A.) and confirmed by sequencing with an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Autoradiographs were analyzed with a BAS1000 bioimage analyzer (Fuji Photo Film, Tokyo, Japan).

Cloning and sequencing of the coding region of pig Mx1: The coding region of *Mx1* gene was amplified using the primer set described below, 0.5 μl of Advantage cDNA polymerase mix (Clontech, Palo Alto, CA, U.S.A.) and the first strand DNA synthesized from total RNA of poly(I) poly(C)-stimulated cells of PK(15) or LLC-PK1, in a total volume of 25 μl. PCR cycles was as follows: 94°C 1 min, 63°C 3 min, 25 cycles. The primer set of 5'-CTG CTG ACG GGT CAA CGT CAC AGC GTC-3' and 5'-CTC AGC CAC TAA CAG CAG GGA CGG TGG-3' was used for the coding region of *Mx1* cDNA (nucleotides 29–2250). All of the PCR products were cloned to pGEM-T Easy vector and confirmed by sequencing with an ABI PRISM 377 DNA sequencer.

VSV infection assay with Mx cDNA-transfected cells: The entire open reading frames (ORFs) of pig *Mx1* cDNA were subcloned to mammalian expression vector pCI-neo (Promega). pCI-neo-*Mx1* and intact pCI-neo plasmids were transfected in 3T3 cells with the FuGENE6 transfection reagent (Roche Diagnostics, Indianapolis, IN, U.S.A.), selected with 400 μg/m*l* of G418 (Gibco) according to the manufacturer's protocol and stably cloned *Mx* mRNA-expressing cells. The mRNA levels of *Mx1* in the clonal cells were checked by RT-PCR with the following primers: 5'-AAG AAG CTG AGA CGT CGA TCC GGC T-3' and 5'-TGG AGA CGT GCC CTG GAG ACC C-3' (nucleotides 1647–2136). Infectivity of VSV on transfected 3T3 cells was estimated with recombinant VSV carrying the green fluorescent protein (GFP) gene instead of the G protein gene (VSVΔG*-G) as described previously [14]. Briefly, cells were seeded in collagen type-I-coated 24 well plate (IWAKI, Tokyo, Japan), infected with the recombinant VSV after reaching confluence, and the number of GFP-expressing cells in 10 microscopic fields was counted.

Data analysis: All values are presented as means ± SE. Statistical analysis was performed by analysis of variance with post hoc testing by Fisher's protected least significant difference multiple range test.

RESULTS

Mx1 mRNA expression in pig kidney cell lines: Previous studies indicated that Mx proteins were expressed in pig primary-cultured kidney cells [10]. In the present study, we first investigated the mRNA expression of *Mx1* in two kidney cell lines PK(15) and LLC-PK1, to check whether these cell lines were available to obtain pig *Mx1* cDNA conveniently. The mRNA level of *Mx1* was dramatically increased in both cells treated with poly(I) poly(C), which is well-known as an inducer of IFN-inducible genes (Fig. 1). However, it was almost undetected in untreated cells. The size of *Mx1* mRNA was approximately 2.5 kb.

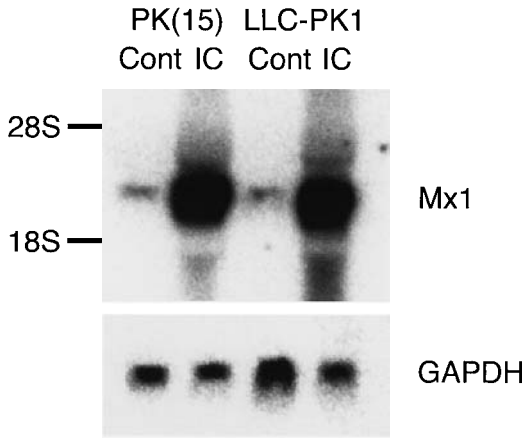

Fig. 1. Northern blot analysis of *Mx1* in pig kidney cell lines. PK(15) and LLC-PK1 cells were treated with 50 μg/m*l* poly(I) poly(C) (IC) for 6 hr. Total RNA (30 μg) was isolated from treated (IC) and non-treated (Cont) cultured cells and analyzed.

DNA sequences of Mx1 cDNA in two pig cell lines: We isolated *Mx1* cDNA from total RNA of poly(I) poly(C)-treated PK(15) and LLC-PK1 cells by RT-PCR. When the nucleotide sequences of pig *Mx1* cDNA were determined, *Mx1* cDNA derived from PK(15) cells had a deletion of 11 bp in the 3' end of the coding region, and was estimated to encode an 8 amino acid substitution and 23 amino acid extension compared to that from LLC-PK1 cells and reported data [20]. This frame-shift might influence the leucine zipper motif in the C-terminus (Fig. 2). Moreover, in the other region ten nucleotide substitutions, which caused four amino acid exchanges, were seen in *Mx1* cDNA derived from both LLC-PK1 and PK(15) cells in comparison with previous data (Table 1). These amino acid substitutions did not occur in the consensus sequences of the GTP binding region or the leucine zipper motif in the C-terminus.

Infectivity of VSV in pig Mx1 mRNA-expressing cells: To investigate the antiviral effect of pig Mx1 protein, we examined the infectivity of recombinant VSV in the *Mx1* mRNA-expressing 3T3 cells. As shown in Fig. 3, the numbers of VSV-infected cells in individual *Mx1*-expressing clones were approximately 25% lower than empty vector-transfected or non-treated cells. However, the degree of the infectivity against VSV was not changed significantly among the cells that were transfected *Mx1* cDNA from PK(15) or LLC-PK1.

DISCUSSION

In the present study, we sequenced *Mx* cDNAs from two pig kidney cell lines, PK(15) and LLC-PK1 cells. They showed some nucleotide differences, with changes of amino

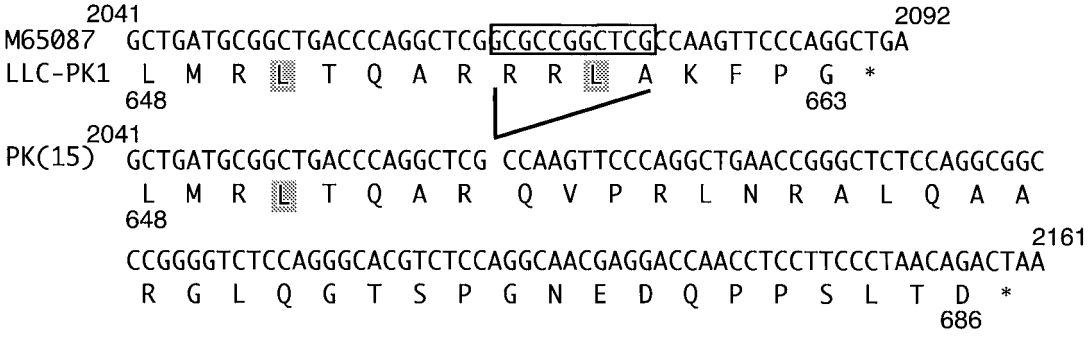

Fig. 2. Nucleotide and deduced amino acid sequences of C-terminal region in pig *Mx1*. The sequences and the positions derived from a previous report [20] (accession number: M65087), LLC-PK1, and PK(15) are shown and compared with the results of this study. Open box indicates a deletion site in PK(15). Leucine residues in the leucine zipper motifs of *Mx1* are shown in gray boxes. Asterisks indicate the positions of stop codons.

Table 1. Nucleotide and amino acid substitutions in the coding region of pig *Mx1*

Nucleotide Position	Previous data	This study	Amino acid position and deduced amino acid substitution
96	G	A	Silent
114	G	A	5 Ser - Asn
373	G	A	Silent
613	T	C	Silent
760	T	C	LLC only, Silent
967	A	C	289 Glu - Asp
1023	C	A	308 Ala - Glu
1341	G	A	414 Arg - Lys
1424	A	C	Silent, PK only
1444	G	A	Silent, LLC only

Nucleotides of previous data are derived from the report [19] (accession number M65087).

acid residues in the coding region of *Mx1* cDNA compared to that of reported previously [20]. In particular, an 11 bp-deletion was seen in the 3' end of *Mx1* cDNA from PK(15). The position of this deletion was identical to that observed in the genomic DNA from several breeds of pig such as Landrace and Yucatan miniature pig [19]. This caused a frame-shift mutation with 8 amino acid substitutions at the C-terminal end and the extension of 23 amino acids at the protein. It is known that nucleotide differences of the *Mx* gene exist in various species such as the mouse [13, 14, 25, 26], duck [3] and chicken [16]. For example, most mouse laboratory inbred strains have polymorphisms in both *Mx1* and *Mx2* genes such as deletion or insertion mutations with frame-shifts resulting in the loss of antiviral function, but a few laboratory and feral strains have functional gene(s) for antiviral activity. Furthermore, the amino acid substitution in *Mx1* of PK(15) involved a leucine residue, which is located in the leucine zipper motif (Fig. 2). The leucine zipper motifs are involved in oligomerization of the Mx protein [18]. Considering our observation and the previous reports, it is conceivable that the *Mx1* gene of the pig has variety in not only the sequence but also in its potential for antiviral activity.

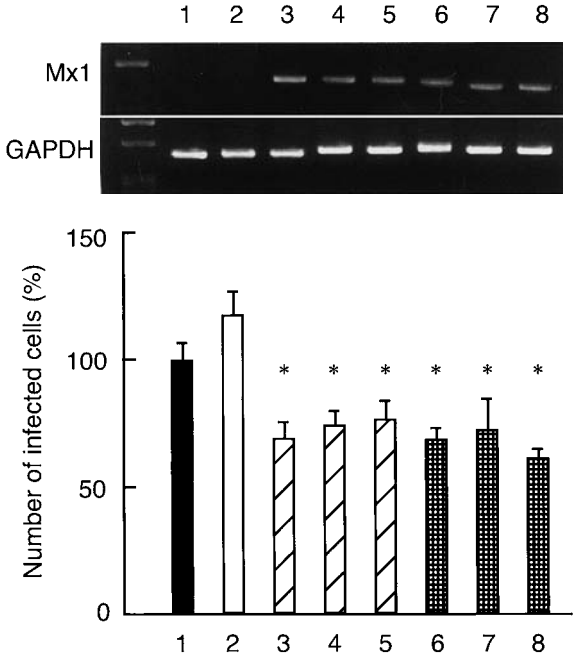

Fig. 3. The infectivity of *Mx1* cDNA-transfected clonal lines against recombinant VSV. The expression of *Mx1* mRNA and the control *GAPDH* mRNA in each clonal cell line was detected by RT-PCR (upper panels). The values of infectivity are expressed as relative to those of non-treated 3T3 cells (lower graph). 1, non-treated parental 3T3 cells; 2, 3T3 clonal cells transfected with empty plasmid; 3–5, *Mx1* mRNA-expressing clonal cells transfected with the LLC-PK1 cDNA; and 6–8, *Mx1* mRNA-expressing clonal cells transfected with PK(15) cDNA * $P<0.05$ vs. non-treated cells.

Horisberger [10] has reported that type I IFN causes pig primary kidney cells and PK(15) cells to inhibit the replication of VSV. Therefore, we compared the antiviral effect against VSV in the stably *Mx1* mRNA-expressing cell clones. As shown in Fig. 3, all clones expressing *Mx1*

mRNA from both PK(15) and LLC-PK1 cell lines were resistant against VSV. However, the inhibition of VSV replication in these clones was weak (approximately 25% inhibition) compared to that in human MxA-expressing cells, which was more than 90% inhibition [4, 22]. Our previous study demonstrated that mouse Mx2-expressing cells show about 50% inhibition of VSV replication under the same experimental conditions as the present study [14]. Besides, both rat Mx3 and human MxB are not able to inhibit VSV replication [17, 22]. It is suggested that the characteristic of pig Mx1 on VSV inhibition is close to that of mouse Mx2, rat Mx3 and human MxB rather than human MxA.

There was no difference in the effect on VSV infectivity between PK(15)-type *Mx1* and LLC-PK1-type *Mx1* (Fig. 3). It is considered that the difference of structure at the C-terminal end of pig Mx1 protein did not influence the antiviral effect against VSV. In fact, a mutant human MxA with a substitution from Leu to Lys in the leucine zipper motif lacks the activities of GTPase and oligomerization, but maintains antiviral activity against Thogoto virus [12]. In the present study, we also found four amino acid substitutions of the *Mx1* protein in both LLC-PK1 and PK(15) cells compared with the reported sequence previously. Since a cell line LLC-PK1 is derived from Hampshire pig, these substitutions may exist in this breed. A single amino acid substitution from Glu to Arg near the C-terminus is known to change the antiviral properties of human MxA protein [28]. This mutant MxA protein blocks the replication of influenza virus but not VSV, in contrast to the wild type MxA, which blocks both influenza virus and VSV replication. Furthermore, we reported recently that amino acid variations of chicken Mx protein exist in various breeds and a specific amino acid substitution at position 631 (Ser to Asn) is considered to affect the antiviral activity against VSV [16]. Amino acid substitutions of Mx1 protein of the pig may be possible to influence the antiviral activity against VSV and other viruses. Therefore, it is necessary to identify the variation of this protein in the respective breeds of the pig, and to examine whether those substitutions affect the antiviral properties against VSV and other viruses.

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