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 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).

**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/ml 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 µl. PCR was performed with 1 µl 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 µl. 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 CAC GTG T-3’ and 5’-CTC AGC CACT AAC 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 AGC GGT CAA CGT CAC AGC GTC-3′ and 5′-CTC AAG AAG CTG AGA CGT CGA TCC GGC T-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/ml 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 (VSV-G*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.

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 acid sequences.
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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 frameshift 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.

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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