Biochemical Genetics, Vol. 45, Nos. 1/2, February 2007 (© 2006)
DOI: 10.1007/s10528-006-9045-y

A Naturally Occurring Variant of Porcine *Mx1* Associated with Increased Susceptibility to Influenza Virus *In Vitro*

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Received 15 September 2005—Final 9 March 2006
Published online: 4 January 2007

Mx1 *has been implicated in resistance to the influenza virus. We have now identified four alleles of the* Mxl *gene in domesticated breeds of pigs. Two of the alleles encode deletion variants (a 3-bp deletion in exon 13 and an 11-bp deletion in exon 14), which might be expected to interfere with Mx activity. The porcine* Mxl *genes corresponding to wild type, the 3-bp deletion mutant, and the 11-bp deletion mutant were cloned and expressed in NIH3T3 cells, and the antiviral activity for influenza virus was assayed. Virus yield was observed to be 10–100-fold greater with the 11-bp deletion allele than that for wild type and the 3-bp deletion alleles. The results suggest that the 11-bp deletion type is lacking antiviral activity able to contribute to the interference of influenza virus replication.*

KEY WORDS: porcine *Mx1*; influenza virus; deletion allele.

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INTRODUCTION

Influenza virus infections are common and occasionally cause epidemic disease as a result of antigenic drift and genetic shift mutations not only in humans but also in a variety of domestic animals, such as the chicken (fowl plague), the horse (equine influenza), and the pig (swine influenza) (Sereda, 1974).

Influenza virus was the first mammalian virus for which replication could be studied biochemically (Hirst, 1941). The genome and transcriptome of this RNA virus have now been well characterized (Enami, 2002). Little is known, however, about the host mechanisms of resistance and susceptibility to influenza virus infection except for the *Mx1* locus in mouse. The mouse influenza virus resistance locus *Mx1* was first identified in 1964 (Lindenmann, 1964), and the Mx protein was subsequently identified (Horisberger *et al*., 1983) in the A2G mouse strain. The mouse *Mx1* protein is induced by interferon (IFN) α/β (Haller *et al*., 1980) as well as by dsRNA or several other viral infections (Pestka *et al*., 1987), and it has an intrinsic antiviral activity for protection against influenza virus infection (Arnheiter *et al*., 1991; Noteborn *et al*., 1987; Staeheli *et al*., 1986).

Mx-related proteins have been described in humans (Aebi *et al*., 1989; Horisberger and Hochkeppel, 1987; Staeheli and Haller, 1985), cattle (Horisberger *et al*., 1988), rats (Meier *et al*., 1990), fish (Staeheli *et al*., 1989), and even in yeast (Rothman *et al*., 1990). The cDNA clone encoding the porcine *Mx1* has been isolated and sequenced (Müller *et al*., 1992). A genetic approach was used to identify the role of *Mx1* protein in protection against virus infection in the mouse, but little is known about the function of Mx proteins in farm animals and humans, as Mx-defective mutants have not been found in these species.

We previously reported polymorphisms (an 11-bp deletion) in exon 14 in the porcine (Landrace) *Mx1* gene (Morozumi *et al*., 2001), and more recently, we have identified an additional mutation of the porcine (Meishan) *Mx1* in exon 13. The percentage of individuals that were homozygous for the 11-bp deletion was relatively low. We suspected therefore that the deletion might have a considerable effect on the susceptibility of pig to some diseases originating from RNA viruses. In order to investigate the antiviral potential of these porcine *Mx1* proteins, we have established stable clones of transfected 3T3 cells that express normal or mutant types of *Mx1*. In this report, we show that the normal porcine *Mx1* confers a degree of resistance to influenza A virus infection *in vitro*, but an 11-bp deletion variant appears to be completely defective in any antiviral activity for influenza A virus infection.

MATERIALS AND METHODS

cDNA Cloning of the Porcine *Mx1*

DNAs were extracted by DNAZol reagent (Invitrogen) from the porcine primary mononuclear cells of Landrace and Meishan breeds according

to the manufacturer's instructions. Exon 13 fragments from the Meishan breed (194 bp) were amplified with 20 pmol of each primer (forward primer 5′-AAGAAGCTGAGACGTCGATCCGGCT-3′ and reverse primer 5′-CTGAAAGATCTCGGCTATGGAGGG-3′). The PCR method consisted of an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C denaturation for 30 s, 64°C annealing for 30 s, and 72°C extension for 1 min, with a final 5 min extension at 72°C. Polymorphism in exon 13 (a 3-bp deletion) was detected by the direct sequencing of these PCR fragments. The 11-bp deletion in exon 14 (Landrace) was detected by restriction fragment length polymorphism (RFLP) as described previously (Morozumi *et al*., 2001).

To induce *Mx1* cDNA, the porcine mononuclear cells were treated for 3 h with serum-free RPMI 1640 medium containing 500 U/mL of the recombinant human IFN α (3×10^8 U/mg) (Pepro Tech EC). Total RNAs were prepared using ISOGEN (Nippon Gene), and poly (-A) RNAs were purified using a mRNA Purification Kit (GE Healthcare). Each cDNA of *Mx1* was amplified with the primers of attB1MxF (5′-GGGGACAAGTTTGTACCAAAAAAGCAGGCTGTCACAGCG AAGAAAAGGAAG-3′) and attB2Mx-R (5′-GGGGACCACTTTGTACAAGAA AGCTGGGTCCTTCTATGATGCTATGCGG-3′) or attB2Rdel (5′-GGGGACC ACTTTGTACAAGAAAGCTGGGTCGTCTGTTAGGGAAGGAGG-3′) by Superscript one-step RT-PCR with Platinum *Taq* (Invitrogen). The RT-PCR method consisted of 30 min incubation at 50°C and an initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C denaturation for 15 s, 64°C annealing for 30 s, and 72°C extension for 3 min, with a final 5 min extension at 72°C. These PCR fragments were cloned into pDONR201 (Invitrogen) by the BP reaction according to the instructions for the Gateway cloning procedure (Invitrogen). Recombinant plasmids were purified by alkaline lysis with SDS (Sambrook and Russell, 2001). These plasmids were checked for inserts by colony PCR and direct sequencing of the PCR products (accession no. AB164037). To construct expression clones, recombinant plasmid DNAs were mixed with pDEST26 (Invitrogen) and treated with LR Clonase according to the instructions for the Gateway cloning technology. Each recombinant plasmid DNA was checked by sequencing and purified. The following *Mx1* expression vectors were constructed: for the normal *Mx1*, pExMx1; for the 3-bp deletion variant, pExMx1-3; for the 11-bp deletion variant, pExMx1-11.

Construction of the Transfectants Expressing the Recombinant Porcine *Mx1*

NIH3T3 cells (4×10^6/0.4 mL) were transfected with 5 μg of each expression plasmid by electroporation using an ECM 60 Electroporation system (Btx Inc.) at 13 ohms of resistance, 100 microfarads, and a 200 V setting in cuvettes with 2-mm chamber gaps (1.0 KV/cm). The transfected cells were cultured for 4 days

in DMEM containing 5% FBS and then selected in DMEM (Gibco) supplemented with 500 μg/mL G418 (Geneticin, Invitrogen) and 5% FBS. The G418-resistant cell clones were examined for *Mx1* expression by RT-PCR. Positive clones were subcloned by limiting dilution, and stable cell clones expressing the different *Mx1* variants were established as follows: vector only (pEx), 3T3-vec; by pExMx1, 3T3; by pExMx1-3, 3T3-3; by pExMx1-11, 3T3-11.

Influenza Virus Preparation and Challenge Experiments

Influenza virus A/Aichi (H3N2) was grown in the allantoic cavities of 11-day-old embryonated chicken eggs. The titer of stock virus was $1 \times 10^{7.5}$ the 50% egg-infective dose (EID_{50}) /0.2 m.

Challenge experiments were performed with two different concentrations of the virus, at a multiplicity of infection (MOI) of 1 and MOI of 10, in triplicate. Four cell classes were used for each series; 3T3-vec, 3T3, 3T3-3, and 3T3-11 (5×10^4/well), each grown in 24-well plates for 48 h in DMEM containing 5% FBS and 25 mM HEPES (Invitrogen) (total 108 wells per MOI series). Confluent cell monolayers were infected for 1 h with the diluted virus in DMEM containing 25 mM HEPES. Unadsorbed virus was removed by washing twice with PBS, and the cells were incubated in DMEM containing 0.2% FBS and 25 mM HEPES. Samples of the culture supernatants were harvested (from the triplicate samples) at nine different time points, 0, 6, 12, 18, 24, 30, 36, 48, and 54 h post-infection. The triplicate samples were pooled and centrifuged ($880 \times g$) for 5 min at 4°C. These supernatants were stored at −80°C. The samples were subjected to tenfold serial dilution, and virus titers were determined by the EID_{50} method. The challenge experiment (for both MOI 1 and 10) was conducted three times in total.

Inhibition Tests of the IFN α/β Inducible by Influenza Virus Infection

Confluent cell monolayers (3T3-vec, 3T3, 3T3-3, and 3T3-3) in triplicate were incubated with or without anti-mouse IFN α/β (2×10^4 neut. units/0.101 mL) (Biosource) for 14 days. At 14 days, the cells incubated with anti-mouse IFN α/β were infected with the virus diluted at MOI 10 in DMEM containing 25 mM HEPES and anti-mouse IFN α/β for 1 h. The cells incubated without anti-mouse IFN α/β were infected similarly but with virus diluted without anti-mouse IFN α/β. Unadsorbed virus was removed by twice washing with PBS, and the cells were incubated with or without anti-mouse IFN α/β in DMEM containing 0.2% FBS and 25 mM HEPES.

Samples of the culture supernatants were harvested (from the triplicate samples) at 36 h post-infection. The triplicate samples were pooled and centrifuged ($880 \times g$) for 5 min at 4°C. These supernatants were stored at −80°C. The samples were subjected to tenfold serial dilutions and virus infectivity was titrated

by the EID_{50} method. Challenge experiments with or without anti-mouse IFN α/β were conducted three times in total.

Quantitative Analysis of Each Recombinant *Mx1* Expression

For measurement of the *Mx1* mRNA levels in each transfected cell culture, total RNA from 10^6 cells was prepared using ISOGEN (Nippon Gene), and poly (-A) RNA was purified using the mRNA Purification Kit (GE Healthcare) in accordance with the manufacturer's instructions. Each *Mx1* variant was amplified with the primers of attB1MxF and attB2Mx-R or attB2Rdel by Superscript one-step RT-PCR with Platinum *Taq* (Invitrogen). The RT-PCR method consisted of 30 min incubation at 50°C and an initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C denaturation for 15 s, 64°C annealing for 30 s, and 72°C extension for 3 min, with a final 5 min extension at 72°C. The identity of the fragment was confirmed by sequencing as described before (Morozumi *et al*., 2001). Relative quantity of *Mx1* expression was measured by image analyzer (Multi Gauge Fuji Film) as quantum levels of DNA bands emerged in the electrophoresis gel. Expression of mouse *G3PDH* was used as an internal standard.

Amplification of the mouse glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) was performed as an endogenous control with the mouse G3PDH RT-PCR primer set (Toyobo).

Detection of *Mx1* Protein by Western Blot Analysis and Immunofluorescence

For determination of *Mx1* protein expression, the following *Mx1* expression vectors were constructed with pEF5/FRT/V5-DEST (Invitrogen) using the Gateway cloning technology: for normal *Mx1*, pExMx1V5; for the 11-bp deletion variant, pExMx1V5-11. The transfectants expressing *Mx1* recombinant V5-fusion protein were constructed by electroporation using the Flp-In system (Invitrogen) with Flp-In 3T3 cells, each *Mx1* expression plasmid, and pOG44 according to the manufacturer's instructions. Cells were cotransfected with a ratio of 9:1 (w/w) pOG44: *Mx1* expression plasmid. Flp-In 3T3 cells had been cultured in TMEM (Wako) containing 2% FBS, 2 mM glutamine, and 100 mM/mL Zeocin (Invitrogen). The transfected cells had been cultured in TMEM (Wako) containing 2% FBS, 2 mM glutamine, and 100 mM/mL Hygromycin B (Invitrogen). Stable cell clones expressing the normal or mutation *Mx1* were established as pEx-V53T3-vec, pExMx1V5-V53T3, and pExMx1V5-11-V53T3-11.

Detection of the *Mx1* recombinant V5-fusion proteins was performed using supernatants of cell extracts as described for the Western Breeze Chromogenic Immunodetection Protocol (Invitrogen). Each supernatant of a cell extract was electrophoresed in an SDS-polyacrylamide gradient gel (10–20%). Separated proteins were transferred to a PVDF membrane and detected with the alkaline phosphatase

(AP)-conjugated mouse monoclonal antibody to V5 (Anti-V5-AP, Invitrogen) at 1:2000. Primary antibodies were detected with chromogenic substrate (Western Breeze, Invitrogen).

To detect the *Mx1* V5-fusion proteins by immunofluorescence, each transfectant (8×10^4 cells/well) in triplicate was cultured for 24 h on Lab-Tek chamber slides (Nalge Nunc International). Cultured cells were subjected to immunofluorescence analysis with the fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody to V5 at 1:200 (Anti-V5-FITC, Invitrogen) according to the manufacturer's instructions. The hybridization signals were captured with a cooled CCD camera (Hamamatsu Photonics) and the nuclear images obtained by counterstaining using DAPI solution (Wako) were superimposed on the image of hybridization signals.

RESULTS

Nucleotide Sequence of Exon 13 in the Meishan Breed

The 3-bp deletion at position 1778–1810 is shown in Fig. 1. Comparison of the translated amino acid sequence of exon 13 with the published sequence of the protein (Gene Bank) revealed that exon 13 of *Mx1* in the Meishan breed has lost the serine residue at amino acid 565. The 11-bp deletion found in exon 14 of Landrace breeds (Fig. 1) leads to a frameshift with 8 amino acid substitutions and a 23 amino acid extension in the carboxyl terminal region of the *Mx1* protein (Morozumi *et al.*, 2001).

Influenza Virus Growth in *Mx1* Expressing Cells

MOI 1

The general shape of the growth curve in 3T3-vec and 3T3-11 is similar to that observed in 3T3 and 3T3-3 (Fig. 2). The initial rise in infectivity was observed at 18 h after infection in 3T3-vec and 3T3-11 but 36 h after infection in 3T3 and 3T3-3. Maximal titers were reached by 36 h post-infection in the four transfectants; the infectivity end points were 1.0 $\log_{10}$ EID_{50}/0.2 mL for 3T3, 3T3-3, but 2.8–3.0 $\log_{10}$ EID_{50}/0.2 mL for 3T3-vec, 3T3-11. Moreover, in 3T3-vec and 3T3-11, infectious virus could be detected at 54 h after infection, even though there were no infectious virus in 3T3 or 3T3-3.

MOI 10

In 3T3-vec and 3T3-11, an infectivity of 0.3-0.5 $\log_{10}$ EID_{50}/0.2 mL was observed at 12 h post-infection and the maximum level (4.5–4.7 $\log_{10}$ EID_{50}/0.2 mL) was reached at 36 h after infection (Fig. 2). The infectious virus titer had dropped

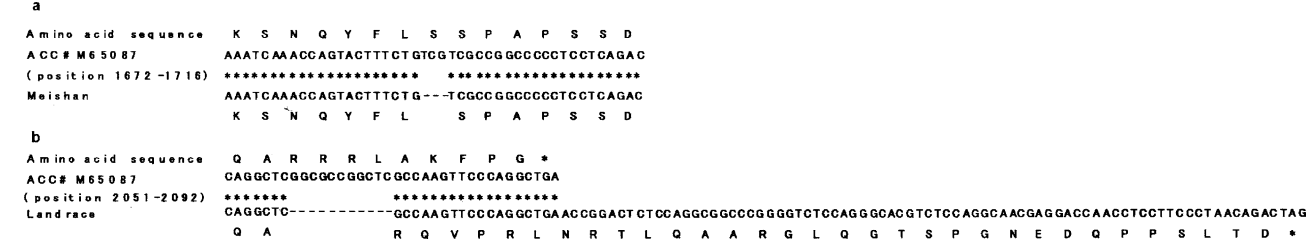

Fig. 1. Comparison of nucleotide and deduced amino acid sequences of exon13 and 14 in porcine *Mx1* in ACC M65087 and conventional pigs. The asterisks mark the coincident sequence. The dashes highlight the 3-bp deletion in exon 13 of Meishan (a) and the 11-bp deletion in exon 14 of Landrace (b)

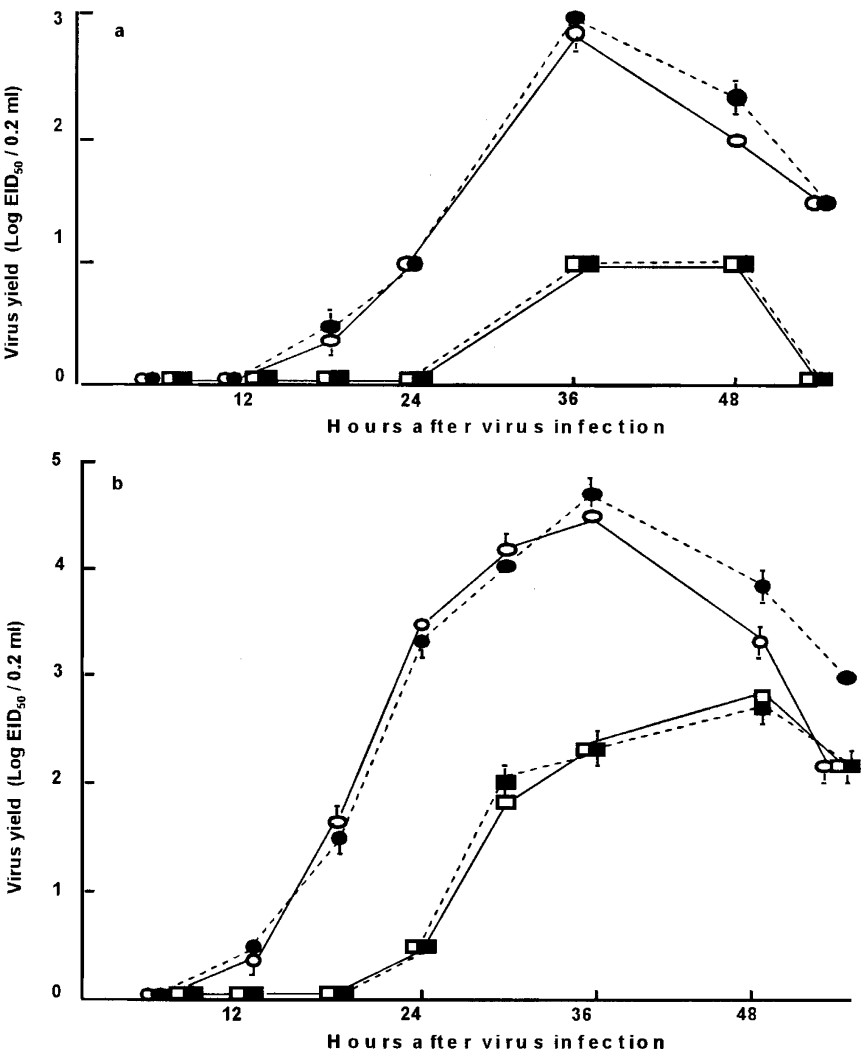

Fig. 2. Inhibition of virus multiplication by *Mx1*. The 3T3-vec (*open circle*), 3T3 (*open box*), 3T3-3 (*solid box*), and 3T3-11 (*solid circle*) cells were infected with influenza A virus at MOI 1 (a) and MOI 10 (b). The data, derived from three independent experiments, are shown as mean ± standard deviation. Bars show standard deviations.

at 48–54 h after infection. While the general course of viral increase in 3T3-vec and 3T3-11 was similar to that observed in 3T3 and 3T3-3, infectious virus was detected 12 h earlier, and the maximum level of 4.5–4.6 $\log_{10}$ EID_{50}/0.2 mL was

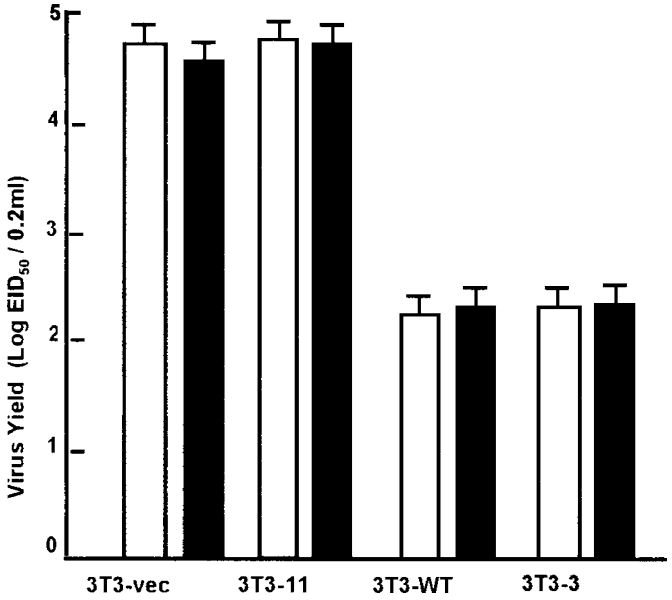

Fig. 3. Influenza virus growth in the *Mx1* expressing cells pretreated with anti-IFN α/β. The *Mx1* expressing cells had been treated with (*open bars*) or without anti-mouse IFN α/β (*solid bars*) for 14 days before and during influenza virus infection (MOI 10). This experiment was repeated in triplicate, and data are shown as mean $\pm$ standard deviation. Bars show standard deviations.

reached at 36 h, or 12 h earlier than for 3T3 and 3T3-3. The maximum virus titers in 3T3-vec and 3T3-11 were 100 times higher than those in 3T3 and 3T3-3.

Influenza Virus Growth in *Mx1* Expressing Cells Pretreated with Anti-IFN α/β

As the influenza virus is an IFN-inducing virus, the influenza virus-infected cell is capable of releasing IFN α/β, which may induce an antiviral state in neighboring cells. To eliminate this possibility, we treated 3T3-vec and three transfectants with anti-mouse IFN α/β (Biosource) for 14 days before and during virus infection (MOI 10). The results were essentially the same as for untreated cells irrespective of the porcine *Mx1* genotype (Fig. 3).

Expression of Recombinant *Mx1*

To standardize the amount of sample RNA from each transfected cell, RT-PCR was performed with 10^6 cells having the same passage history as that of the cells

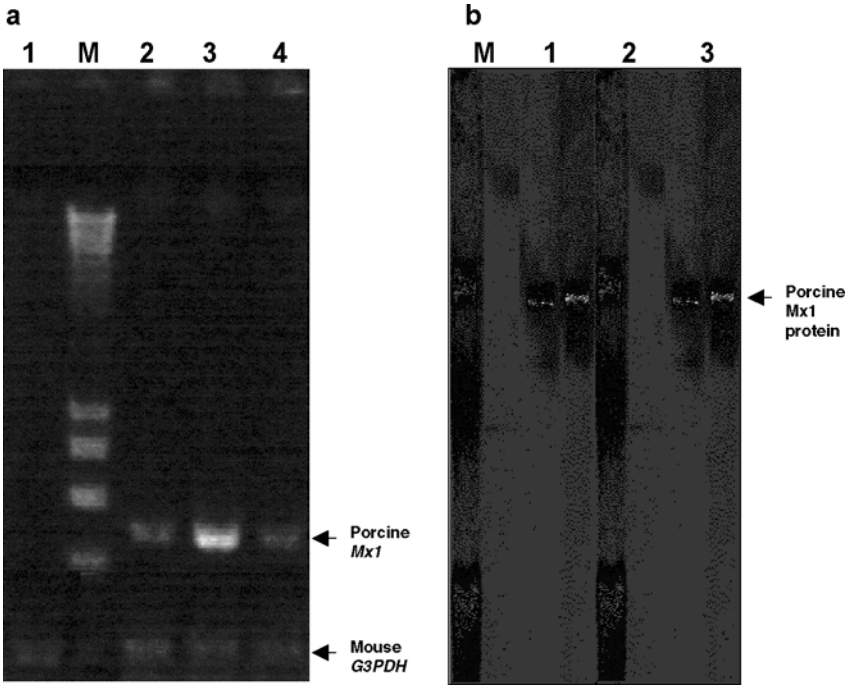

Fig. 4. Expression of recombinant *Mx1* and Western blot analysis of *Mx1* protein. (a) For measurement of *Mx1* mRNA level in each transfected cell (3T3-vec, 3T3-WT, 3T3-11, and 3T3-3), RT-PCR was performed with mRNA purified from 10^6 cells. Amplification of *G3PDH* was performed as an endogenous control. M, Markers; lane 1, 3T3-Vec; lane 2, 3T3-WT; lane 3, 3T3-11; lane 4, 3T3-3. (b) Western blot analysis of the Flp-In 3T3 cells transfected with the porcine *Mx1*. Each cell (V53T3-vec, V53T3-WT, and V53T3-11) extract was electrophoresed in an SDS-polyacrylamide gradient gel (10–20%). Separated proteins were transferred to PVDF membrane and detected with the Anti-V5-AP. M, Markers; lane 1, V53T3-vec; lane 2, V53T3-WT; lane 3, V53T3-11.

used for virus challenge experiments. There was no expression of porcine *Mx1* in control 3T3 cells. All transfectants expressed the porcine *Mx1* gene. It appears, however, that the amount of *Mx1* expressed in 3T3-11 cells was greater than that expressed in 3T3 and 3T3-3 cells (Fig. 4 and Table I) based on the similar expression levels observed for mouse *G3PDH* (used as an internal control).

Detection of *Mx1* Protein

Expression of the porcine *Mx1* protein in transfected cells was analyzed by Western blotting with a mouse monoclonal antibody, which allows detection of recombinant fusion proteins containing a V5 epitope tag. Figure 4 shows that both

Table I. Relative Expression of the Pig *Mx1* Genes Transfected into Mouse 3T3 Cells

	Quantum level		
Type of cell	Porcine *Mx*	Mouse *G3PDH*	*Mx*/*G3PDH*
3T3-vec	0	16372	0
3T3-WT	42877	18965	2.2
3T3-11	124574	18580	6.7
3T3-3	42794	15920	2.7

Note. Relative expression of *Mx* was measured by quantum level of electrophoresis picture of DNA obtained by RT-PCR of mRNA. Expression of mouse *G3PDH* was used as internal standard. 3T3-vec: Only vector was transfected. 3T3-WT: Wild-type *Mx1* was transfected. 3T3-11: *Mx1* of 11-bp deletion was transfected. 3T3-3: *Mx1* of 3-bp deletion was transfected.

V53T3 (normal *Mx1*) and V53T3-11 (11 bp deletion) cells synthesized proteins of approximately 77 kDa recognized by anti-V5-AP antibody. Untransfected V53T3-vec cells did not synthesize a protein recognized by anti-V5-AP antibody.

Cytoplasmic Distribution of the Porcine *Mx1* Protein

To determine the intracellular localization of the porcine *Mx1* protein, immunofluorescence experiments were performed. The FITC-conjugated mouse monoclonal antibody to the V5-porcine *Mx1* fusion protein stained cytoplasmic structures similarly in both V53T3 and V53T3-11 (Fig. 5). The V53T3-vec cells that had been transfected with pEF5/FRT/V5 and pOG44 did not contain the porcine *Mx1* protein.

DISCUSSION

It is well established that the mouse *Mx1* gene is necessary and sufficient to establish resistance to influenza virus infection in this species. We demonstrated that the 3T3 cells transfected with the porcine full-length *Mx1* cDNA were more resistant to influenza virus infection than untransfected cells. *Mx1* is normally not expressed in these cells, and it appears that reduced influenza virus replication takes place in 3T3 cells expressing the porcine *Mx1*. Expression of *Mx1* has been shown to be induced by IFN α/β treatment or in viral infection through the action of IFN α/β (Haller *et al*., 1980). However, 3T3 cells expressing the porcine *Mx1* retained antiviral activity even when the cells were treated with anti-IFN α/β antibody. This suggests that the inhibition of replication of influenza virus by the porcine *Mx1* expressing cells was dependent on the function of the *Mx1* protein and was not dependent on induction of *Mx1* by IFN α/β. Moreover, it seems likely that the other proteins specifically induced by IFN are not essential for the inhibition observed here. It would be interesting to test whether increasing

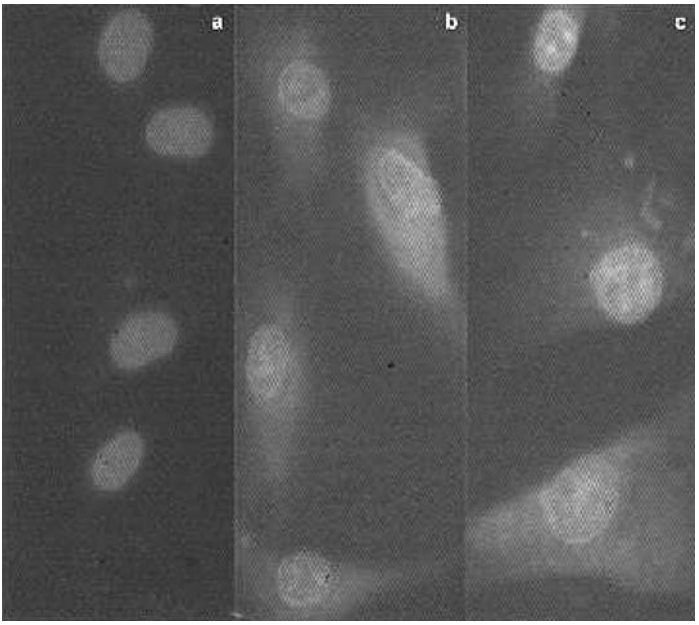

Fig. 5. Cytoplasmic distribution of the porcine Mx protein. Each transfectant (8×10^4 cells/well) in triplicate was cultured for 24 h and subjected to immunofluorescence analysis with Anti-V5-FITC at 1:200. The nuclear images were obtained by counterstaining using DAPI solution. (a) V53T3-vec. (b) V53T3. (c) V3T3-11.

the level of expression of the *Mx1* protein in 3T3 cells would result in complete inhibition of influenza virus replication.

The 3T3 cells transfected with *Mx1* cDNA of the 3-bp deletion variant expressed *Mx1* protein at a level similar to that of normal *Mx1* cDNA transformed cells and also demonstrated reduced replication of influenza virus. It seems likely that the 3-bp deletion of the porcine *Mx1* cDNA does not affect the function of *Mx1* protein.

On the other hand, the cells transfected with *Mx1* cDNA of the 11-bp deletion variant appear to lack the capability to suppress influenza virus multiplication. Similar levels of *Mx1* transcript were found in 3T3, 3T3-3, and 3T3-11 (Fig. 4 and Table I, with similar levels of *G3PDH* expression), although expression of the 11-bp deletion variant may actually be increased, suggesting that normal expression may be self-regulated. Western blotting analysis suggests that the molecular weight (approximately 77 kDa) and amount of expressed *Mx1* protein were similar in cells transfected with the normal or the 11-bp deletion variant of the porcine *Mx1* gene. The size of the product, which is similar to human *Mx1*

protein, suggests that a full-length copy of *Mx1* is expressed in these cells. Asano *et al*. (2002) reported that VSV replication was inhibited not only in the 3T3 cells expressing wild-type porcine *Mx1* mRNA but also in the 3T3 cells expressing the 11-bp deletion variant *Mx1* mRNA. The evidence that the 11-bp deletion variant *Mx1* lost antiviral activity to influenza virus but not to VSV will be defined in experiments based on the viewpoint of which domain or domains of *Mx1* protein is correlated with each virus multiplication.

The 3-bp deletion in exon 13 has been observed only in the Meishan breed to date, where the frequency is approximately 21% with the genotype classes being in line with Hardy–Weinberg. However, although an allele frequency of around 30% was observed for the 11-bp deletion in several herds of Landrace and other breeds of pigs, the frequency of individuals homozygous for the 11-bp deletion was relatively low (3.7%). We suspect therefore that pigs homozygous for the 11-bp deletion might be susceptible to some RNA virus infectious diseases.

Evidence of interspecies influenza virus transmission has been reported, for example, the finding of avian H1N1 viruses in swine, human H3N2 viruses in swine, swine H1N1 viruses in humans, and other avian viruses in seals, whales, and mink (Gorman *et al*, 1990, 1991; Kida *et al*., 1988; Scholtissek *et al*., 1983). This indicates that pigs may serve as significant reservoirs of H1N1 and H3N2 influenza viruses and are frequently involved in interspecies transmission of influenza viruses. Influenza virus maintenance in pigs and the frequent introduction of new viruses from other species may be important in the generation of pandemic strains of human influenza virus. Early production of or increased expression of an active *Mx1* protein may yield pigs with increased resistance to influenza virus. The existence of variation in the porcine *Mx1* gene suggests that it may be possible to select pigs less susceptible to influenza virus, which may also have a positive impact on the frequency of the generation of pandemic strains of the virus.

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