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# Bovine and water buffalo *Mx2* genes: polymorphism and antiviral activity

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**Abstract** Millennia-long selective pressure of single-strand RNA viruses on the bovine *Mx* locus has increased the advantages of using the bovine Mx protein to evaluate the ultimate significance of the antiviral role of Mx proteins. The conclusions of research based only on the bovine Mx1 protein showed the need for comprehensive studies that demonstrate the role of all isoforms, individually or together, especially in the presence of a second isoform, the bovine *Mx2* gene. This study provides information about bovine and water buffalo *Mx2* genes, as well as their allelic polymorphism and basic antiviral potential. Observation of an *Mx2* cDNA sequence (2,381 bp) obtained from 15 animals from 11 breeds using primers based on a previous sequence (NCBI accession no. AF335147) revealed several nucleotide substitutions, with eight different alleles and two amino acid exchanges: Gly to Ser at position 302 and Ile to Val at position 354, though the latter was found only in the NCBI database. A water buffalo *Mx2* cDNA sequence was identified for the first time, revealing 46 nucleotide substitutions with 12 amino acid variations, in addition to a 9-bp insertion in the 5′ untranslated region UTR, compared with the bovine *Mx2* cDNA. Transfected 3T3 cells expressing bovine *Mx2* mRNAs coding Gly or Ser at position 302, water buffalo *Mx2* mRNA, positive control bovine *Mx1* mRNA-expressing cells, and negative control parental 3T3 were subjected to infection with recombinant vesicular stomatitis virus (VSVΔG*-G), as were empty pCI-neo vector-transfected cells. The positive control and all cells expressing *Mx2* mRNAs displayed significantly higher levels of antiviral activity against VSVΔG*-G ($P<0.01$) than did the negative controls.

**Keywords** Mx2 · Antiviral protein · Type I interferon

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## Introduction

Mx proteins belong to the antiviral proteins that are induced by type I interferon in response to infections caused by a wide range of single-strand RNA viruses (Pavlovic et al. 1993). The Mx proteins are members of the dynamin superfamily, which is characterized by intrinsic GTPase activity (Staeheli et al. 1993). A common feature of this family is the formation of high molecular weight oligomers inside cells (Kochs et al. 2002). Furthermore, conserved properties of Mx proteins are the presence of tripartite GTPase domains in the N-terminal region, a dynamin signature, and a GTP effector domain (GED) containing a leucine zipper motif in the C-terminal region, which plays a key function in the antiviral activity (Melén et al. 1992). The Mx protein shows variable antiviral mechanisms, depending upon the intracellular localization of the individual proteins and the type of pathogen (Lee and Vidal 2002).

Since its first description in the mouse (Lindenmann et al. 1963), the *Mx* gene was reported in various animals, most of which have one to three Mx protein isoforms (Horisberger and Gunst 1991). Ellinwood et al. (1998, 1999) reported the isolation of bovine cDNA encoding *Mx1* and a different splicing isoform, Mx1a, and they mapped the bovine *Mx1* gene to chromosome 1.

It is believed that the bovine *Mx* locus was subjected to intensive selective pressure by single-strand RNA viruses that share many characteristics with those known to be inhibited by Mx proteins. This notion has increased the merits of using the bovine Mx protein to evaluate the real significance of the antiviral role of Mx proteins. Thus, efforts were made to identify corresponding genes and their structures and to implement analyses to determine the polymorphic and functional properties of these genes. Studies on the structural properties have revealed the existence of another splicing isoform of bovine *Mx1*, Mx1B (Kojima et al. 2003), and have identified the fine structure of the bovine *Mx1* gene, which was found to consist of 15 exons and a promoter region approximately 1 kb upstream of the 5′-flanking region (Gerardin et al. 2004).

Using plasmids conditionally expressing bovine Mx1 protein, Baise et al. (2004) demonstrated that bovine Mx1 protein confers antiviral activity against vesicular stomatitis virus (VSV). Moreover, among the different variants of bovine Mx1 proteins, in vitro analysis using recombinant VSV (VSVΔG*-G) revealed positive activities in their antiviral properties (Nakatsu et al. 2004). Recent studies have indicated that bovine Mx1 protein participates in antiviral activity against the rabies virus (Leroy et al. 2006).

Studies have also evaluated the use of upregulated expression of the *Mx* gene as a marker for the disease state in viral infections. Muller-Doblies et al. (2004) have demonstrated that RNA virus infection increases both the expression levels of bovine *Mx* mRNA and Mx protein and have suggested that the bovine Mx1 protein level can be used as a specific marker of acute viral infections for monitoring livestock health (Muller-Doblies et al. 2002).

However, in one of the studies to determine the antiviral range of bovine Mx1 protein, transfected cells conditionally expressing bovine Mx1 protein were seen to lack antiviral activity against many important single-strand RNA viruses, such as paramyxoviridae. The conclusion was that the hypothesis of host adaptation through selection of an antiviral Mx isoform is invalidated in the bovine species (Leroy et al. 2005). These findings highlight the need for comprehensive studies that examine the role of all isoforms, individually or together, especially in the presence of a second isoform, the bovine *Mx2* gene.

Although the impression regarding the antiviral potential of *Mx2* genes was adversely affected by the fact that human MxB lacks any antiviral potential (Frese et al. 1995), in two different studies other *Mx2* gene family members such as rat and mouse *Mx2* genes were reported to confer antiviral activity against specific viral infections (Meier et al. 1990; Zürcher et al. 1992). Moreover, in a recent study, transfected cells expressing canine *Mx2* mRNA were shown to resist infection with recombinant VSV (Nakamura et al. 2005). These results encouraged a functional analysis and the search for allelic variation in the bovine *Mx2* gene.

The nucleotide and amino acid sequence of bovine *Mx2* cDNA were reported previously (NCBI accession no. AF355147), but the gene characters remained unknown. Therefore, the goal of this study was to elucidate the gene's polymorphism and antiviral properties. Results revealed the allelic polymorphism among the different bovine *Mx2* cDNA obtained, which were classified into eight genotypes. In addition, transfected cells expressing the two upregulated variants of bovine *Mx2* mRNA and water buffalo *Mx2* mRNA exhibited an antiviral state against recombinant VSV.

## Materials and methods

### Breed and cell culture

Blood samples were collected from 16 animals representing 11 bovine breeds including Holstein, Jersey, Guernsey, Hereford, Angus, Charolais, Limousine, Japanese black, Japanese brown, Japanese short horn, Brahman, and a Swamp buffalo (water buffalo). Leukocytes were separated from the blood samples by centrifugation at 1,800 rpm for 15 min, washed with 8 ml of phosphate-buffered saline, and then transferred to a tube containing 2 ml of HITSOPA QUE-1077 (Sigma, St Louis MO, USA) and centrifuged at 1,800 rpm for 30 min. The obtained pellet of leukocytes was seeded in a 10-cm tissue culture dish (Falcon Labware, Becton Dickinson, Oxnard, CA, USA) containing 10 ml of RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum, 5 μg/ml phytohemagglutin, 1 μg/ml pokeweed mitogen, 100 U/μl penicillin (Nacalai Tesque, Kyoto, Japan), and 100 μg/ml streptomycin (Mejiseika, Tokyo, Japan). The cultured leukocytes were incubated for 42 h at 37°C with 5% $CO_2$.

### RNA extraction and RT-PCR

Cultured leukocytes were treated with 1,000 U/ml of recombinant human interferon-$\alpha_{2b}$ (IFN; Intron-A, Schering, Kenilworth, NJ, USA) for 12 h to stimulate *Mx* mRNA expression. Subsequently, total RNA was extracted by the acid phenol–guanidinium thiocyanate–chloroform extraction method (Chomczynski and Sacchi 1987). The first double-strand cDNA fragments were synthesized from the extracted

**Table 1** Nucleotide substitutions of bovine *Mx2* cDNA in the different breeds

| Breed | Nucleotide position | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 384 | 404 | 515 | 587 | 623 | 629 | 632 | 653 | 662 | 731 | 1179 | 1335 | 1475 | 1484 | 1742 | 2324 | 2418 |
| AF355147 | T | C | G | A | C | T | C | C | T | G | G | A | A | C | T | C | G |
| Hols, Jersey, Guer, JaSH | – | – | – | – | – | – | – | – | – | – | – | G | – | – | C | G | C |
| Brah1-1 | – | – | – | – | – | – | – | – | – | – | – | G | G | T | – | – | – |
| Here, Limo-1, JaBl1, 2, and 3, JaBr-1 | – | – | – | – | – | – | – | – | – | A | A | G | – | – | – | – | – |
| Angus, JaBr-2 | C | T | – | G | T | C | T | G | C | – | – | G | – | – | – | – | – |
| Brah1-2, 2 and 3 | C | T | A | G | T | C | T | A | C | – | – | G | – | – | – | – | – |
| Charolais | C | T | – | G | T | C | T | G | C | – | – | G | G | T | C | G | C |
| Limo-2 | C | T | – | G | T | C | T | G | C | A | A | G | G | T | – | – | C |

The symbol – means the same nucleotide as that in the database of AF355147.
*Hols* Holstein, *Guer* Guernsey, *Brah* Brahman, *Here* Hereford, *Limo* Limousine, *JaSH* Japanese short horn, *JaBl* Japanese black, *JaBr* Japanese brown

total RNA by reverse transcription (RT) using a ReverTra-Ace kit (Toyobo Bio, Osaka, Japan). Bovine *Mx2* cDNA from the fragments was amplified by PCR using specific primers of bMx2 76F (5′-AGCGTAGAGTC CACTTGTCTCC, 76–97) and bMx2 2457R (5′-GATT CATGCACATAAGCAAAACC, 2457–2435) designed from the previously reported nucleotide sequence in the NCBI database (accession no. AF355147). PCR was carried out using *Taq* polymerase (Toyobo Bio) on a DNA Thermal Cycler (PerkinElmer, Norwalk, CO, USA). The cycling protocol consisted of an initial denaturing step at 94°C for 5 min followed by 33 cycles at 94°C for 1 min, 58°C for 1 min, and 68°C for 1 min, and a final extension step of 68°C for 5 min.

## DNA sequencing

Blunt ends of the RT-PCR product of *Mx2* cDNA were treated for the addition of A-tailing with Go*Taq* polymerase (Promega, Madison, WI, USA) and were then ligated to the pGEM-T Easy vector (Promega) using the T4 DNA ligase (Promega). The recombinant plasmids including bovine *Mx2* cDNA were transformed into *Escherichiacoli*-competent cells (J109; Promega) and extracted by the alkaline method after overnight culture and then resuspended in TE (10 mM Tris–HCl and 1 mM EDTA, pH 8.0) and stored at −20°C. The nucleotide sequences of bovine *Mx2* cDNA were determined using an ABI Prism Big Dye terminator chemistry (Applied Biosystems, Foster City, CA, USA) with an ABI-301 Genetic Analyzer (Applied Biosystems). To produce the sequencing samples, PCR was carried out using the primers bMx2 822F (5′-GAGTGGAGATTCGGA GAG, 822–940), bMx2 1250F (5′-CCAGCAGGATATCAC CAACA, 1250–1269), bMx2 1658F (5′-TACCACCAA TATCGAAAAAG, 1658–1677), bMx2 559R (5′-CGCAC CTTCTCCTCATACTT, 559–540), and bMx2 941R (5′-AA GGTCAATGAGGGTCAGAT, 941–922).

## Phylogenetic tree and comparative sequence analysis

Comparative sequence analysis was carried out using BLAST programs at http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi/ (Altschul et al. 1990), while multiple alignments were performed with ClustalW (Higgins and Sharp 1988), available at the DDBJ website http://clustalw.genome.ad.jp/. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987).

**Table 2** Amino acid substitutions of bovine Mx2 protein in the different breeds

| Breed | Amino acid position (nucleotide position) | |
|---|---|---|
| | 302 (1179) | 354 (1335) |
| AF355147 | Gly | Ile |
| Hols, Jersey, Guer, Angus, Charolais, JaSH, JaBr-2, Brah1, 2, and 3 | Gly | Val |
| Here, Limo, JaBl1, 2, and 3, JaBr-1 | Ser | Val |

Abbreviations for names of breeds are the same as those in the Table 1 footnotes.

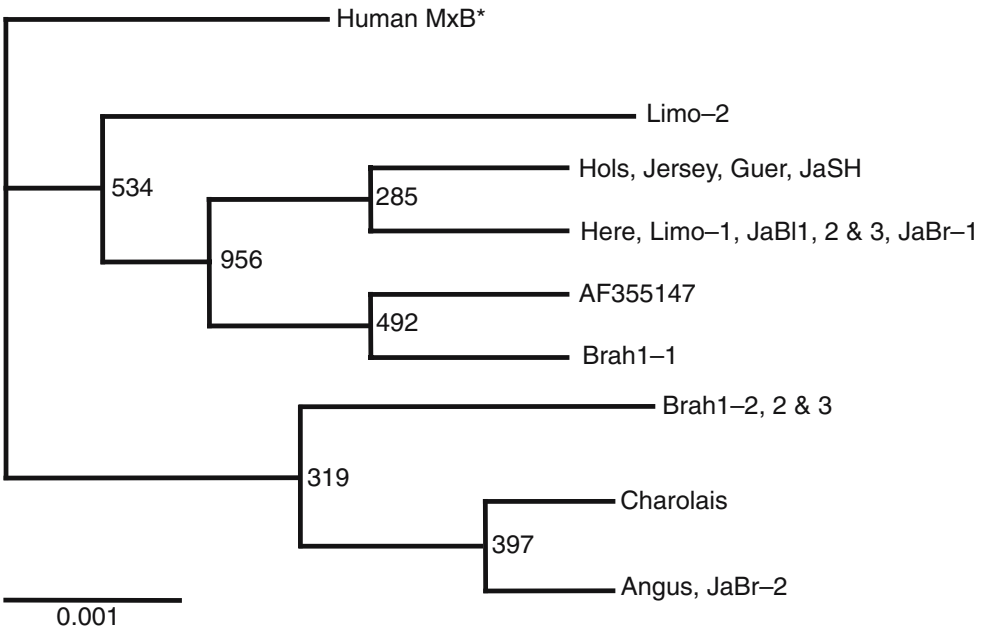



**Fig. 1** Phylogenetic tree of the eight bovine *Mx2* cDNA haplotypes and human *MxB* cDNA as out-group candidate constructed using the neighbor-joining method (Saitou and Nei 1987). Abbreviations for names of breeds are the same as those in the Table 1 footnotes. *Numbers* indicate bootstrap values, while an *asterisk* indicates the out-group candidate

## Establishment of an expression vector and permanently transfected 3T3 cells

A 2,381- and 2,390-bp *Not*I-digested fragments of bovine and water buffalo *Mx2* cDNA, respectively, from the recombinant pGEM-T Easy vector was religated to the *Not*I site of the pCI-neo expression vector (Promega), which contains the human cytomegalovirus immediate–early enhancer/promoter and the neomycin phosphotransferase gene. Embryonic 3T3 fibroblast cells from a Swiss mouse (Riken Cell Bank, Tsukuba, Japan) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. A construct of the pCI-neo expression vector including *Mx2* cDNA was transfected into 3T3 cells using FuGene transfection reagent (Roche Diagnostics, Indianapolis, ID, USA) in accordance with the manufacturer's instructions. Selection of the transfected cultured clones was carried out in a medium containing 500 μg/ml of geneticine (G418; Gibco BRL, Gaithersburg, MD, USA) for 2 weeks. The expression of *Mx2* mRNA in individual clones of stably transfected cells was ascertained using the gene-specific primers bMx2 76F; the expression of *Mx1* mRNA was confirmed using bMx1 44F (5′-GC CAAC TAGTCAGCACTACATTGTC, 44–68) and bMx1 2343R (5′-ATGGGGTGTGAAAGGCTATT, 2343–2324); and the expression of mouse β-actin mRNA was ascertained using mbActinF (5′-TGGAATCCTGTGGCATC CATGAAAC, 886–/910) and mbActinR (5-TAAAACG CAGCTCAG TAACAGTCCG, 1234–1210) by RT-PCR against total RNA, which was extracted and treated with a DNA-free kit

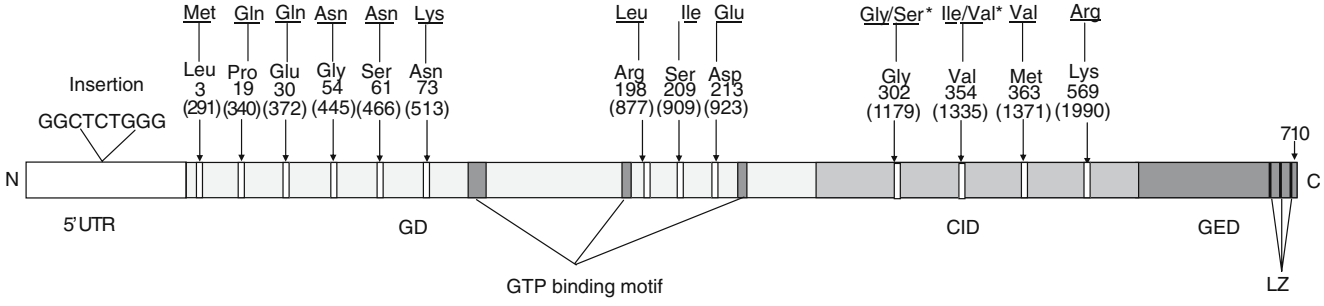



**Fig. 2** A schematic diagram showing the amino acid exchanges detected in water buffalo Mx2 protein in comparison with bovine Mx2 protein (NCBI accession no. AF355147). The *underlined* amino acids indicate those of bovine corresponding to same position; an *asterisk* indicates nonsynonymous mutations of bovine *Mx2* cDNA; *vertical arrows* indicate the genetic variations. *Numbers* denote the amino acid exchanged positions, and *numbers* in *parentheses* are the nucleotide substitution positions. *GD* GTP-binding domain, *CID* central interactive domain, *GED* GTP effector domain

**Table 3** Nucleotide substitutions between bovine and water buffalo *Mx2* cDNA

| Nucleotide position | Bovine | Water buffalo |
|---|---|---|
| 9 | Del | Ins |
| 111 | T | C |
| 127 | T | C |
| 151 | G | T |
| 152 | T | C |
| 191 | A | C |
| 214 | C | T |
| 219 | G | A |
| 230 | A | G |
| 241 | A | G |
| 247 | G | A |
| 282 | A | C |
| 299 | G | A |
| 331 | A | C |
| 363 | C | G |
| 436 | A | G |
| 457 | A | G |
| 494 | A | C |
| 497 | G | A |
| 508 | T | G |
| 578 | C | T |
| 599 | C | T |
| 809 | T | C |
| 812 | C | T |
| 815 | C | A |
| 818 | A | G |
| 836 | G | T |
| 868 | T | G |
| 900 | A | G |
| 914 | A | C |
| 986 | T | C |
| 1055 | C | T |
| 1064 | C | T |
| 1088 | G | A |
| 1115 | C | A |
| 1133 | C | A |
| 1335 | A | G |
| 1362 | G | A |
| 1418 | G | A |
| 1730 | C | T |
| 1981 | G | A |
| 2081 | G | A |
| 2090 | G | A |
| 2162 | T | C |
| 2237 | C | T |
| 2428 | A | C |

The compared nucleotide sequence of bovine *Mx2* cDNA is based on the NCBI database (accession no. AF355147).
*Dl* Deletion, *Ins* insertion

(Invitrogen, Carlsbad, CA, USA). Mouse β-actin mRNA expression was examined as an internal control (Suzuki et al. 2000).

### Functional analysis

Permanently transfected clones of 3T3 cells were subjected to experimental infection with VSVΔG*-G carrying the green fluorescent protein (GFP) gene instead of the viral G protein gene (Ko et al. 2002). The antiviral activity against VSVΔG*-G was determined by counting the number of GFP-expressing cells in 10–20 microscopic fields. At least three independent experiments were carried out for each clone.

### Statistical analysis

All results are expressed as mean values±standard errors of the mean (SE). Statistical significance was analyzed using Fisher's protected least significant difference test. $P<0.01$ or 0.05 was considered statistically significant.

## Results

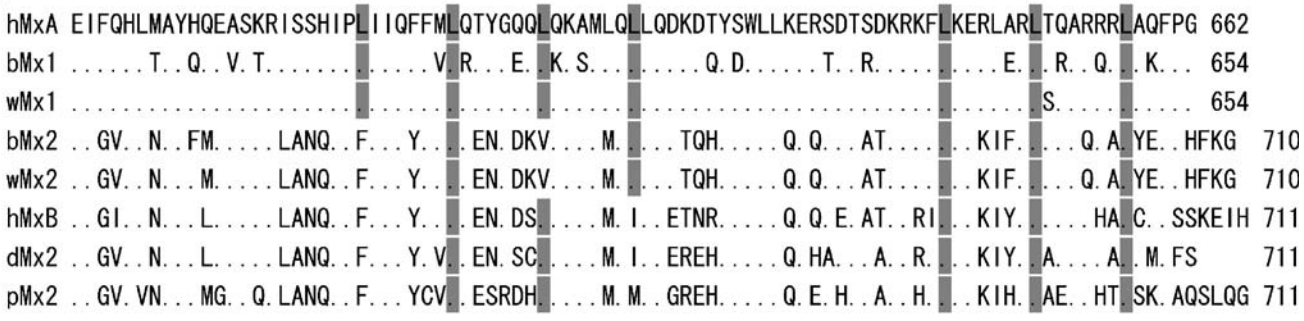
### Genetic variation of bovine Mx2 cDNA

Bovine *Mx2* mRNA expression was clearly detected in the IFN-treated cultured leukocytes by RT-PCR using the bMx2 76F and bMx2 2457R primers. The 2,381-bp products of *Mx2* cDNA from 15 animals comprising 11 bovine breeds were sequenced and estimated to encode a protein of 710 amino acids. The obtained nucleotide sequences were compared with each other and also with the sequence of the bovine *Mx2* gene in the NCBI database (accession no. AF355147). As shown in Table 1, 17 nucleotide substitutions were detected; of these, 14 in the coding region (276–2408) were synonymous mutations and only two led to amino acid exchanges, while one was detected at the 3′ untranslated region (UTR). Nucleotide substitutions at positions 1179 and 1335 resulted in amino acid exchanges from Gly to Ser at position 302 and from Ile to Val at position 354, respectively (Table 2). However, nucleotide A at position 1335 corresponding to Ile at amino acid position 354 was observed only in the database. The loci coding the *Mx2* gene from three individuals of Limousine, Japanese brown, and Brahman1 were heterozygous, but those of the other 12 animals possessed a single homozygous *Mx2* allele.

### Phylogenetic tree and comparative sequence analysis

Based on the nucleotide substitutions detected in this study, the genotypes corresponding to bovine *Mx2* cDNAs could be classified into eight alleles. A phylogenetic tree was constructed using the nucleotide sequences among the eight alleles as shown in Fig. 1. The eight alleles were divided into two major groups that showed a considerable genetic distance between them.

**a**




**b**

| | bMx2 | wMx2 | pMx2 | hMxB | dMx2 | mMx2 | bMx1 |
|---|---|---|---|---|---|---|---|
| bMx2 | | 98 | 67 | 70 | 71 | 60 | 58 |
| wMx2 | (97) | | 66 | 70 | 70 | 60 | 58 |
| pMx2 | (73) | (72) | | 69 | 70 | 57 | 56 |
| hMxB | (71) | (72) | (71) | | 74 | 60 | 57 |
| dMx2 | (69) | (68) | (70) | (74) | | 59 | 57 |
| mMx2 | (53) | (53) | (52) | (53) | (53) | | 69 |
| bMx1 | (54) | (54) | (53) | (53) | (54) | (62) | |

**Fig. 3** Comparative sequence analysis based on the nucleotide and amino acid sequences of *Mx2* genes of several mammalian species, with human MxA and bovine Mx1 as references. **a** Variations in the GED regions of Mx proteins from different mammalian species, with human MxA as reference. A *gray highlight* indicates conserved leucine residues participating in leucine zipper domains. **b** Similarity percentage based on the nucleotide and amino acid sequences of *Mx2* genes of several mammalian species. *hMxA* human MxA (accession no. M30817), *bMx2* bovine Mx2 (Jersey), *wMx2* water buffalo Mx2, *pMx2* porcine Mx2 (P. Tungtrakoolsub et al., unpublished data), *hMxB* human MxB (accession no. M30818), *dMx2* canine Mx2 (accession no. AF239824), *mMx2* murine Mx2 (accession no. NM_013606), *bMx1* bovine Mx1 (accession no. AF047692), *wMx1* water buffalo Mx1 (Y. Nakatsu et al., unpublished data). *Numbers* indicate the similarity percentage between amino acids, and *numbers* in *parentheses* show the similarity percentage between nucleotides

Results of a comparative sequence analysis (Fig. 3b) suggested that the nucleotide sequence of bovine *Mx2* cDNA is close to that of porcine *Mx2* cDNA (73%) and that the amino acid sequence of bovine Mx2 is close to the sequences of canine Mx2 (71%) and human MxB (70%). However, the nucleotide and amino acid sequences of bovine *Mx2* showed very low homologies (54 and 58%, respectively) to those of bovine *Mx1*.

## Water buffalo Mx2 cDNA

In this study, water buffalo *Mx2* cDNA was originally cloned and sequenced. The 2,400-bp nucleotide sequence was determined, and the coding region (285–2427) was deduced to contain 710 amino acids. The sequence data is available in the GenBank through accession no. EF052266 for *Mx2*. In a comparison of water buffalo *Mx2* cDNA and bovine *Mx2* cDNA (NCBI accession no. AF355147), 46 nucleotide substitutions, of which 34 were synonymous mutations and 12 led to amino acid exchanges, were observed as shown in Table 3 and Fig. 2; in addition, a 9-bp insertion was detected in the 5′ UTR. However, in the most important GED region, such as that containing the leucine zipper motifs, there was no amino acid substitution between bovine and water buffalo Mx2 proteins (Fig. 3a).

## Antiviral activity examination of bovine Mx2-transfected cells

To determine the antiviral potential, an experimental infection using VSVΔG*-G was carried out on 3T3 cell lines permanently expressing bovine and water buffalo *Mx2* mRNAs. Four kinds of *Mx* cDNAs were transfected into 3T3 cells, including Jersey *Mx2* cDNA coding Gly at amino acid position 302, Hereford *Mx2* cDNA coding Ser at the same position, water buffalo *Mx2* cDNA, and Holstein *Mx1* cDNA as a positive control (Nakatsu et al. 2004). Parental 3T3 and empty pCI-neo vector-transfected 3T3 cells were used as negative controls. *Mx* mRNA expression in the transfected cell lines was checked by RT-PCR using specific

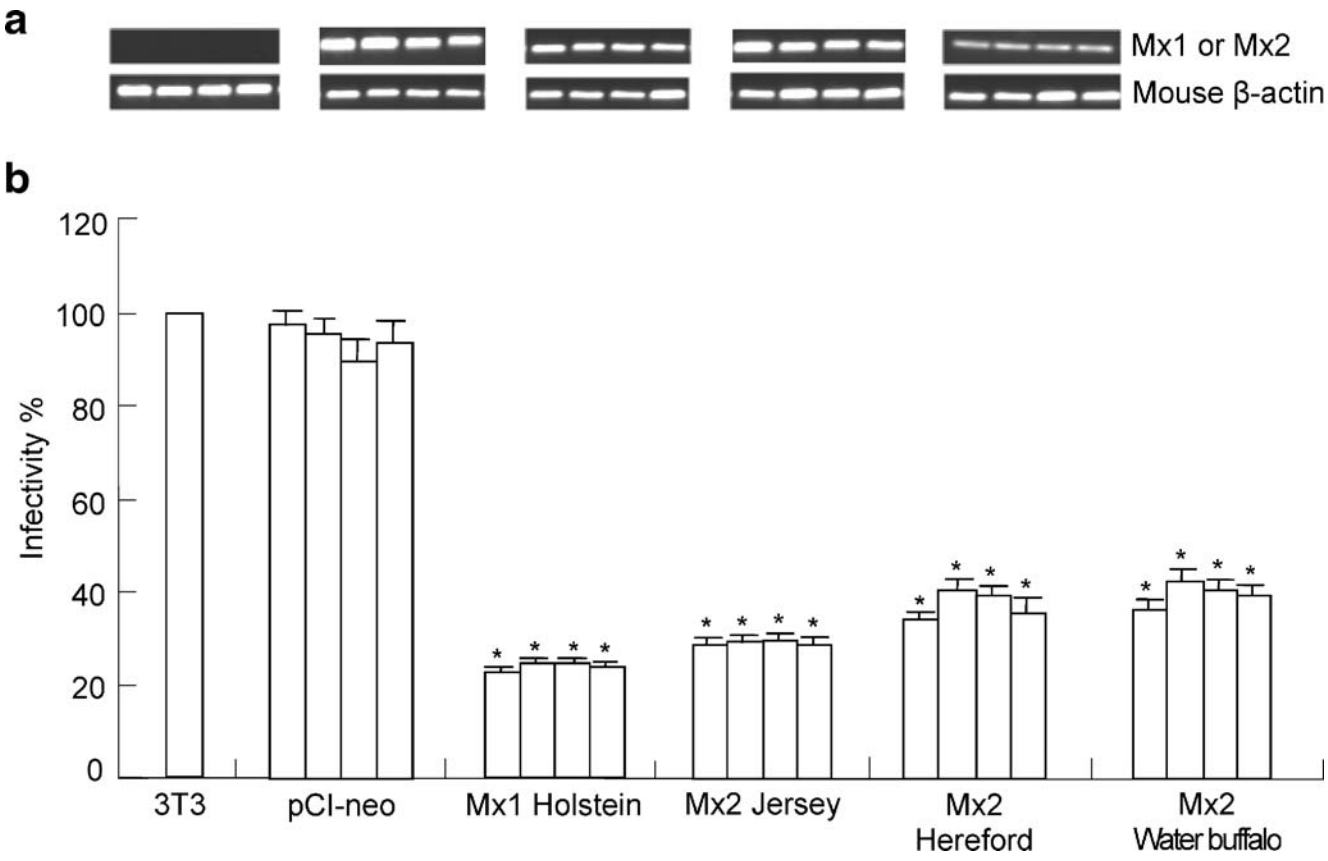



**Fig. 4** Experimental infection using VSVΔG*-G in bovine and water buffalo *Mx* mRNA-expressing 3T3 cell lines. **a** Expression of *Mx1* or *Mx2* mRNA and mouse β-actin mRNA as an internal control. Total RNA was extracted and subjected to RT-PCR using specific primers for bMx1 44F and bMx1 2343R for *Mx1* mRNA, bMx2 76F and bMx2 2457R for *Mx2* mRNA (*top*), and mbActinF and mbActinR for mouse β-actin cDNA (*bottom*). **b** Infectivity to VSVΔG*-G of parental 3T3, Holstein Mx1, Jersey Mx2, Hereford Mx2, or water buffalo Mx2 mRNA-expressing cells. Infectivity of parental 3T3 cells was expressed as 100%. Data are shown as mean values±SE. An *asterisk* indicates statistical significance at $P<0.01$ compared with the infectivity of parental 3T3 cells

primers of bMx2 76F and bMx2 2457R or of bMx1 44F and bMx1 2343R. Results are depicted in Fig. 4a. As shown in Fig. 4b, all of the transfected clones expressing *Mx1* or *Mx2* mRNAs showed significantly higher levels of antiviral activity against VSVΔG*-G than did the parental 3T3 and the empty pCI-neo vector-transfected control ($P<0.01$). Cells expressing Hereford *Mx2* mRNA with the two nonsynonymous mutations at position 302 and 354 revealed a relative drop in the protection level against VSVΔG*-G compared with those expressing Jersey *Mx2* mRNA, which disclosed only one nonsynonymous mutation at position 354 ($P<0.05$). The same was found in cells expressing water buffalo *Mx2* mRNA with the 12 nonsynonymous mutations, which revealed lower antiviral activity than did those expressing the Jersey *Mx2* mRNA ($P<0.05$).

## Discussion

Knowledge about the bovine Mx2 protein is slight compared with what is known about the bovine Mx1 protein. This report presents for the first time data on the polymorphism and antiviral activity of bovine and water buffalo Mx2 proteins. The 17 nucleotide substitutions detected divided the bovine *Mx2* cDNA into eight different alleles (Table 1). However, only one amino acid exchange, Gly to Ser at position 302, seems highly likely to occur; the Ile to Val exchange at position 354 is unlikely to happen because it was found only in the database (NCBI accession no. AF355147), while all of the breeds examined carried Val at the same position (Table 2). This finding, which indicated the conservativeness of the amino acid sequence in the bovine *Mx2* gene, is in accordance with that reported previously in the bovine *Mx1* gene, which also showed only a single amino acid exchange in the breeds examined (Nakatsu et al. 2004).

The phylogenetic tree constructed from the sequence of the different bovine *Mx2* alleles divided them into two major groups. This finding is entirely different from the previously reported result based on the phylogenetic tree constructed using the different bovine *Mx1* alleles (Nakatsu et al. 2004).

A water buffalo *Mx2* cDNA nucleotide sequence was identified for the first time and showed 12 nonsynonymous mutations compared with that of bovine Mx2 protein (Fig. 2). None of these nonsynonymous mutations, however, were detected in the GED, which is considered to be a structurally important region affecting the potential of Mx protein (Fig. 3a).

In vitro analysis using VSVΔG*-G revealed that cell lines expressing both bovine and water buffalo *Mx2* mRNAs clearly conferred antiviral activity (Fig. 4b). It was clear there was a relative drop in the antiviral activity in cells transfected with bovine *Mx2* cDNA and with a nonsynonymous mutation at position 302 (Fig 4b). This drop led to a protection level lower than was seen in cells lacking this nonsynonymous mutation at position 302. At the same time, cells expressing water buffalo *Mx2* mRNA with 12 nonsynonymous mutations also revealed a level of antiviral activity lower than with those cells expressing the Jersey *Mx2* mRNA, though the nonsynonymous mutation at position 302 was not detected in the water buffalo *Mx2* cDNA. The apparent reason for these variations in the antiviral potential is the presence of the nonsynonymous mutations. However, in the absence of more specific research, the lack of a recognized uniform mechanism governing how the Mx protein confers an antiviral state makes it difficult to confirm that these nonsynonymous mutations led to the variations. Moreover, information about the expression in the protein level of bovine Mx2 remains unknown because no specific antibodies for bovine Mx proteins are available. Future work will focus on determining this level in a comparative study including the bovine Mx1 protein. Alternatively, an RT-PCR analysis [which was widely used in similar studies (Nakatsu et al. 2004; Nakamura et al. 2005)] of the total RNA extracted from the transfected cells was used to confirm the upregulation of the transfected plasmids.

Despite the presence of two variants of the bovine Mx2 protein, the antiviral activity level of bovine Mx1 and Mx2 is relatively close. This similarity is also consistent with the antiviral properties of water buffalo Mx1 and Mx2 because cells expressing water buffalo *Mx1* mRNA showed the same levels of antiviral activity as cells expressing water buffalo *Mx2* mRNA (K. Yamada et al., unpublished data). However, a specific study is required to compare the antiviral potential of the two bovine *Mx1* and *Mx2* isoforms.

According to results obtained by comparing the bovine Mx2 sequence with those of other members of the Mx2 family of proteins, the probability that bovine and water buffalo Mx2 could exhibit antiviral activity was poor; both bovine and water buffalo Mx2 proteins exhibited a similarity to human MxB (Fig. 3b), and the latter was devoid of any antiviral activity (Frese et al. 1995). The result was the same with canine *Mx2* cDNA, which showed a similarity to the human MxB cDNA and conferred antiviral activity against VSV (Nakamura et al. 2005). Unfortunately, in both cases no specific reason for the variation in antiviral activity is currently evident, and a comparison analysis in the GED failed to reveal any significant structural differences (Fig. 3a). Hence, an analysis using chimeric construction is required in the near future to explain the factor responsible for this variation in the antiviral activity of human MxB and bovine Mx2 and of other Mx protein family members. The finding that both isoforms of bovine and water buffalo Mx proteins, Mx1 and Mx2, confer antiviral activity is of interest because it is known that in most of the domestic mammals possessing more than one Mx protein isoform only one isoform confers antiviral activity. And, with the exception of canine, it was the first isoform of Mx1 that demonstrated this feature.

Although the present report deals only with in vitro analysis, this type of evidence is of considerable value with regard to the antiviral properties of the bovine Mx protein.

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